3.
Techniques and Application of Crop Engineering

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TRANSORMATION OF PLANTS FOR CROP PROTECTION PURPOSES

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No paper submitted

PROGRESS WITH TRANSGENIC PLANTS AND INSECT RESISTANCE

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PROGRESS WITH TRANSGENIC TREES

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ABSTRACT

Current progress in the transfer of genes of scientific and economic importance to both fruit and forest trees is reviewed. Limitations to progress in gene transfer, the need for long lived stable and tissue specific gene expression and future goals are identified. The apple has been used as a model to discuss some of the opportunties presented since most information is available with this species. More importantly flowering and fruiting tissue is now available for detailed molecular, genetic, physiological and biochemical examination. In this tree species it is now possible to report the stable expression and Mendelian segregation of transgenes with evidence for a 1:1 segregation of one of the genes, nopaline synthase (*nos*) in the R1 of transgenic apple progeny. In addition stable gene expression of both *nos* and the co-transferred gene *nptII* was observed in the fruit flesh of the apple fruit some 7 years after the initial transformations.

INTRODUCTION

The limitations of attempting to use conventional breeding strategies for long-lived species tree species have been pointed out previously (James, 1987). Long life cycles where seedlings have an extended juvenile phase and high levels of heterozygosity are two of the most formidable problems facing genetic improvement programmes for tree breeders. It is not surprising therefore that in the past few years other so-called 'non-conventional or biotechnological strategies' have been invoked. These invariably involve somatic methods of gene transfer either by using disarmed strains of the soil organisms *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* or by the use of naked DNA which is delivered directly to the cells by electroporation or by free DNA uptake or by the use of DNA-coated microprojectiles. A detailed review of these techniques and how they have been used to transform woody species has very recently been published (Dandekar *et al.*,1993) and no attempt will be made to summarise this work here. The intention here is to discuss the special considerations and demands made by trees when genetic transformation is attempted. I should like to consider four main areas.

1) the identification of the major constraints to successful regeneration and transformation of trees species
2) the need for long-lived stability of gene expression and for inheritance of the transgenes
3) the special requirements for tissue-specific expression in trees
4) future prospects for the insertion of scientifically, economically and commercially important genes in trees.

The apple tree will be used as a suitable example since not only does considerable information on its transformation already exist (James *et al.*, 1989, 1992; James and Dandekar 1991; Lambert and Tepfer, 1991; Maheswaran *et al.*, 1992) but transgenic apple trees have recently flowered and fruited at East Malling making this species particularly suitable for consideration of topics 1-4 listed above.

1) MAJOR CONSTRAINTS TO TRANSFORMATION OF TREES

Low frequency of transformation

There are many possible causes of this and many of them are common to non-tree species as well.

i) Tissue culture

Although the list of tree species where transgenic plants have been obtained now numbers a dozen or so (see Table 7.2 in Dandekar *et al.*, 1993) the frequency of transformation (i.e. number of transgenic plants

produced per explant) is often extremely low (< 1% and often < 0.1%) even where good tissue culture and regeneration systems are available. Low frequency of transformation is often a reflection of lack of competence for regeneration even without the confounding effects of transformation. An even worse scenario is where the species in question is difficult to propagate in culture making germplasm for regeneration and transformation experiments very scarce. Many commercially important tree species fall into one or both of these categories and where trees produce copious amounts of secondary products mere survival of organs in culture is an achievement in itself. These problems in tree species have been reviewed by Haissig (1989).

Although not peculiar to tree species their long lived nature means they may have established special relationships with other organisms which are difficult or impossible to replicate in *in vitro* conditions e.g the need for mycorrhizae for tree root systems. Failure to replicate these unique biochemical and physiological relationships often leads to low or no *de novo* regeneration from cells and tissues. Obviously until such problems can be overcome there is little prospect for genetic transformation. These failings underscore the paucity of knowledge of the behaviour of cells and tissues *in vitro* and of the role of the different classes of plant growth regulator and how tissue and organ growth is integrated.

Even when excellent propagation and regeneration systems are available, as in the case of apple, the regeneration of transgenic plants as a routine procedure is far from secured. The reasons for this are many, not least of which is the complexity of plant - microbe interactions when *Agrobacterium* is the gene transfer method of choice as it most often is.

ii) Vector design - selectable markers and reporter genes

The binary vector system of Agrobacterium has been successfully used to transform a wide range of plants (van Wordragen and Dons, 1992) but this system may still yield to improvements since the broad host range plasmids used are relatively large and have low copy number. Mozo and Hookyaas, (1992) have recently started to design new co-integrate vectors using the *loxP*-Cre site -specific recombination system of phage P1 that will lack the disadvantages of both the binary vector and co-integrate vector systems and hopefuly produce transgenic plants having increased stability of T-DNA and lacking large direct repeats.

Other aspects of design relate to the marker and reporter genes used in transformation experiments. It is now clear that in early work a mutant form of the commonly used selectable marker gene *nptII*, which encodes the enzyme neomycin phosphotransferase and permits transformed cells to grow in the presence of kanamycin, was unknowingly being used by many laboratories world-wide (Yenofsky *et al.*, 1990). The mutation reduces phosphotransferase activity of the encoded NPTII protein such that cells transformed with the mutant form of the gene are less able to withstand elevated levels of kanamycin in the media. Recent experiments on apple using the wild type gene have indeed led to superior rates of transformation but at the moment we have been unable to compare the two forms of the gene in a common *Agrobacterium* background. The introduction of an intron into the coding region of the widely used reporter gene ß-glucuronidase (GUS) (Jefferson *et al.*, 1987) which permits both histochemical visualisation of transformed cells and quantitation by fluorimetry has also been improved by the introduction of an intron into the coding region of the *uid*A gene to prevent its expression in contaminating *Agrobacterium* cells (Vancanneyt *et al.*, 1990).

Variations in the virulence of *Agrobacterium* strains carrying different Ti-plasmids on many plant species including apple, has been reported (Dandekar *et al.*, 1990). These authors reported that the presence of additional copies of the *vir*G gene in these strains carrying the plasmid pTiBo542 increased tumour growth on apple explants.

iii) Plant -microbe interactions and the complexity of the interaction between *Agrobacterium* and plant cells

The complexity of the mechanism of T-DNA transfer from *Agrobacterium* to host plant cell has been the subject of many excellent reviews (e.g. Zambryski, 1988) to which the reader is referred. Recently we have attempted to identify some of the limiting factors and possible causes of the low frequency of apple transformation (James and Dandekar, 1991) by exploiting some of the findings concerning the physiological and biochemical factors that are known to affect *Agrobacterium* virulence (Zambryski 1988). The most important of these is the plant phenolic compound acetosyringone (AS) (Stachel *et al.*, 1985). Incubating bacteria with this compound at pH 5.2 prior to infection induces the transcription of virulence genes of *Agrobacterium tumefaciens* (At) through a two component regulatory system involving the virulence genes *vir*A and *vir*G (Rogowsky *et al.*, 1987). Although the growth of *Agrobacterium* is inhibited at low pH the addition of the osmoprotectant betaine

phosphate (BP) has been shown to improve growth and virulence induction at pH 5.2 (Vernade *et al.*, 1988). In a series of experiments we have included AS and BP in the induction medium for At prior to inoculation of apple leaf discs (James *et al.*, 1993). Disarmed strains of At harbouring plasmids carrying the reporter gene GUS between the T-DNA borders have been used to monitor the effects of manipulating the induction medium on subsequent leaf disc transformation. Stable leaf disc transformation was assessed by measuring GUS activity fluorimetrically in individiual leaf discs six weeks after infection. Results showed that there was a synergistic effect of AS and BP in the virulence induction media that led to significantly increased expression of the GUS gene. Other experiments showed that the efficiency of stable GUS gene expression in apple cells may depend on the length of the induction phase, and the temperature at which this is carried out.

A number of parameters that affect virulence induction have now been assessed and when these optimised variables are used in experiments where leaf discs are not destructively assayed but permitted to regenerate plants striking increases in transformation efficiency have been observed i.e from $<1\%$ to 5% (James *et al.*, unpublished). This encouraging result emphasises the need to pay the utmost attention to already available basic information on the control of virulence induction in *Agrobacterium*.

2) STABILITY OF EXPRESSION AND INHERITANCE OF TRANSGENES IN APPLE

The presence of flowering and fruiting transgenic apple trees offers unique opportunities to study both long term stability and the inheritance and viability of these genes in the progeny of trees. We first produced transgenic apple clones (cv Greensleeves) in 1986 (James *et al.*, 1989) using the disarmed Ti-binary vector pBIN6 (Bevan, 1984). The plants were transgenic for a reporter gene nopaline synthase (*nos*) and the selectable marker neomycin phosphotransferase (*nptII*). Annual tests, since 1987, for expression of *nos* in leaves have always been positive but in 1992 and 1993 these clones flowered under contained growth room conditions. Controlled pollinations were performed after emasculation of the transgenic plants. Fruit set and subsequent growth was normal on all the trees pollinated with Malus cv Baskatong.

Gene expression assays for *nos* (Otten *et al.*,1978) on both the fruit 'flesh' and excised immature embryos from the segregating R1 progeny were carried out using paper electrophoresis and a double anti-body sandwich ELISA method for the NPTII protein (Nagel *et al.*, 1992). Freshly excised 'flesh' tissue always gave a positive response for *nos* from several different apple fruits. To test for expression of the co-transferred *nptII*, sterile 'flesh' tissue was introduced into culture and induced to form callus. During a 5 week growth period tissue from transgenic fruit flesh was able to form callus on induction media containing either 0 or 50μg/μl of kanamycin whereas control flesh from untransformed fruit of the same cultivar and grown under the same conditions failed to grow in the presence of the antibiotic. In addition the NPTII protein was detectable in both fruit 'flesh' callus (8-11 ng/mg protein) and leaf material (1-1.5 ng/mg protein) from transgenic plants but not in equivalent material from control untransformed plants.

Segregation and inheritance of transgenes in R1 apple progeny

Micropropagated immature embryos from two independent transgenic apple clones, B and E, were assessed for segregation and expression of the *nos* gene. Data from electrophoresis assays showed that the gene segregated 1:1 according to a normal Mendelian ratio where clone E showed 27 'seedlings' positive for expression of nopaline synthase and 25 negative from a population of 52 whilst clone B showed 7 positive and 5 negative from a population of 12 'seedlings'. If these results can be reproduced in other transgenic apple clones and cultivars the spectre of genetic engineering of fruit quality via ripening manipulation and of increased pest and disease resistance is brought that much closer.

3) TISSUE SPECIFIC EXPRESSION

Where the tree produces a valuable product such as timber or consumables such as fruit or nuts there will often be requirements for tissue-specific expression such that the transgene is either expressed or not depending on the nature of gene and the desired effect. The spatial and temporal control of gene expression using tissue specific and inducible promoters is now a reality for many non-woody species (Edwards and Coruzzi, 1990) and in the future we should expect to see this extended to tree species.

Obviously the requirements for fruit and forest trees can be both similar and very different depending on the gene of interest. All trees would benefit from increased disease and pest resistance although the nature

of the pest or pathogen will determine which promoters should be used. A good example are the genes coding for modified endotoxins produced as intracrystalline proteins (ICP's) by *Bacillus thuringiensis* (B.t.). Preparations of these ICP's have been used for many years as insecticidal sprays (Dulmage, 1981) and have been shown to have no mammalian toxicity. Upon ingestion by insects the protoxin (ca. 130kD) is proteolytically cleaved to the mature toxin peptide (ca. 66kD) which binds to the epithelial cells of the midgut, eventually causing their death and leaking of electrolytes into the haemocoel. Fatal changes in pH and ion balance result. Transformed apples with the CryIA(c) gene from B.t. encoding endo-toxins that are specifically targetted at Lepidopteran pests have already been produced with expression controlled by the constitutive CaMV35S promoter (Dandekar *et al.*,1992). Since there is no mammalian toxcity associated with this gene presumably there would be no need to target the fruit specifically. On the other hand it is not known to what extent levels of transgene expression are affected by the type of promoter driving the Bt gene.

Very different situations between fruit and forest trees might be expected in other cases. Where for example the goal is to protect trees from mammals that damage bark, genes coding for lectins with known 'unpalatiblity properties' may be used (Kaku *et al.*, 1990). To give protection the genes should ideally be expressed only in the bark if the subject is a fruit tree otherwise the unpalatibility may well extend to other tissues required for consumption by a different group of mammals i.e. the consumer! This consideration of course does not apply to forest trees where a constitutive promoter driving the lectin gene may be all that is necessary. In a similar vein the need for specifity of gene expression in fruit trees compared to forest trees is further highlighted in the case of the vascular tissues. The use of vascular-specific promoters such as sucrose synthase to drive genes encoding glycoproteins that deter phloem or xylem-feeding insects (Hilder et al., 1992) may be necessary to avoid any spurious carry over of gene expression in the fruit.

4) FUTURE PROSPECTS FOR THE INSERTION OF SCIENTIFICALLY, ECONOMICALLY AND COMMERCIALLY IMPORTANT GENES IN TREES.

The first tree species to be transformed was a hybrid poplar (*Populus alba x Grandidentata)* by Fillatti *et al.*, (1987) using an *Agrobacterium*-mediated leaf disc procedure. These plants were transformed with the *aroA* gene that encodes the bacterial enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, conferring resistance to the overall herbicide glyphosate. Although the use of this gene for either forestry or fruit and nut trees has not been developed there are undoubtedly situations and circumstances where foresters and fruit and nut growers would benefit from its more widespread use (Atkinson, 1985; Lund-Hoie, 1985). However , environmental and political barriers may first have to be removed before we see this happening (Sullivan, 1985).

Some reference has already been made to the importance of genes for pest and disease resistance and transgenic apple trees carrying genes coding for Bt toxins that may control codling moth (*Cydia pomonella*) damage have already been released for field trialling in the USA (Dandekar *et al.*, 1992). Progress in this area and with another gene that is active against some insects , the cow pea trypsin inhibitor (CpTI) (Hilder *et al.*, 1987) has recently been reviewed (James *et al.*, 1992). Progress in the control of plant diseases caused by fungal pathogens via transgenic plants may accelerate if some of the findings of Broglie *et al.*, (1991) concerning the use of chitinase genes to reduce damage caused by the pathogen *Rhizoctonia solani* can be extended more widely to other crops including tree species.

Although molecular evidence is still being sought to confirm the inheritance of the transgenes in our fruiting apples the data presented here suggest that long-lived stable gene expression in both vegetative and reproductive parts of the apple plant should in the future permit the introduction of novel genes that control the process of fruit ripening. The two key genes responsible for this, ACC synthase and ACC oxidase have already been isolated, cloned and sequenced from apple fruit tissues (Dong *et al.*, 1991; 1992). Making the anti-sense form of the genes and then transforming suitable cultivars with these genes is the next step. If the spectacular results achieved for the molecular control of ripening in tomatoes (Picton *et al.*, 1993) can be reproduced with climacteric fruit trees there is no reason why the characteristics of ripening in potentially important cultivars cannot be altered in a commercially and environmentally safe way that is acceptable to the consumer. To be able to control ripening in this way will inevitably affect such processes as fruit damage and spoilage in store with consequential beneficial effects on control of pests and diseases.

Finally the control of tree form and architecture is of great importance in both fruit and forest trees. In recent years some spectacular effects on plant form have been observed in transgenic plants transformed with the so-called *rol* genes that are present in Ri plasmids of *Agrobacterium rhizogenes* (White *et al.*, 1985). When

transferred as a group to recipient plant cells that are then regenerated into transgenic plants they cause the 'hairy root phenotype' where pronounced effects on adventitious root formation, root growth rate and habit, apical dominance, leaf and flower morphology and fertility may be observed. Thus far 4 *rol* genes have been identified as A,B,C, and D and these sequenced (Slightom, 1986). Transferring these genes singly or in combination induces distinct developemntal abnormalities for each *rol* gene (Schmulling *et al.*, 1988). The modification of root growth and architecture in tree species in order to modify important characters such as propagation potential, nutrient uptake, and achorage are obvious uses of these genes if they do not adversely affect equally important aspects of performance such as fertility, shoot branching and tree size. Genetic chimeras of apple have now been produced (James, 1987; Lambert and Tepfer, 1991) whereby transgenic root systems were induced to develop on normal shoots. These plants displayed more extensive and faster growing root systems than control plants, a type of plant that may well find uses in some fruit and forestry management systems. However as with all the facets of transgenic plant production ultimately the technology will only be implemented if it can be shown to be environmentally acceptable and commercially justifiable.

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DISPERSAL OF GENES BY POLLEN - THE PROSAMO PROJECT

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ABSTRACT

This study involved using a transgene conferring resistance to the herbicide, glufosinate-ammonium, to determine the frequency of pollination with non-transgenic oilseed rape over distances from 1-70 m. The field plot consisted of a 9m diameter central plot containing transgenic plants, surrounded by a 105m x 105m plot of non-transgenic oilseed rape. All plants were from the spring cultivar Westar. Six honey bee hives were included to aid pollen movement. Pollination fell sharply up to a distance of 12m (0.016%, sample size >110,000) and at a distance of 47 m was 0.00034% (sample size >295,000). No evidence of pollination was observed at 70 m, but the sample size was smaller (>12,000) because it was taken from the corners of the plot. Observations were also made on patterns of pollination and bee behaviour. Data from this study and subsequent experiments in the PROSAMO Programme will contribute to defining isolation distances for the early evaluation of novel transgenic plants.

INTRODUCTION

By the turn of the century, it is likely that the first transgenic plant varieties will be contributing to agricultural production. The comprehensive evaluation of transgenic plants under standard field conditions is an essential step towards achieving this goal. In order to have approval from the Regulatory Authorities to evaluate novel transgenic plants in field trials, it is necessary to assess the likelihood and consequences of those transgenes being passed through pollen to other plant populations. This transfer might be to plants of the same or related species, to crop plants or to wild populations.

The PROSAMO Programme (Planned Release of Selected and Modified Organisms) has, over the past 3 years, investigated some important issues associated with the release of transgenic plants and microbes (Amin-Hanjani *et al*, 1993; Dale *et al*, 1992a; Gray *et al*, 1992). Studies in crop plants have included investigating the survival and growth characteristics of a range of transgenic plant species (Crawley, 1992).

The programme at the Institute of Plant Science Research, has included a series of field studies to determine the extent of cross pollination from plants

containing marker transgenes to plants of the same crop or related species (Dale *et al*, 1992a; Dale *et al*, 1992b; Scheffler *et al*, 1993). It is important to emphasise, that the genetic isolation of transgenic varieties used in agricultural production will, for the most part, be impractical and unnecessary. The value of data on the frequency of pollination at different distances is for the isolation of new kinds of transgenic plants during their initial assessment and evaluation. It is anticipated that it will be possible, in the majority of cases, to move rapidly to genetic isolation conditions no more stringent than is currently used for non-transgenic varieties.

The part of the PROSAMO research programme described here involves a study of gene dispersal by pollen in oilseed rape.

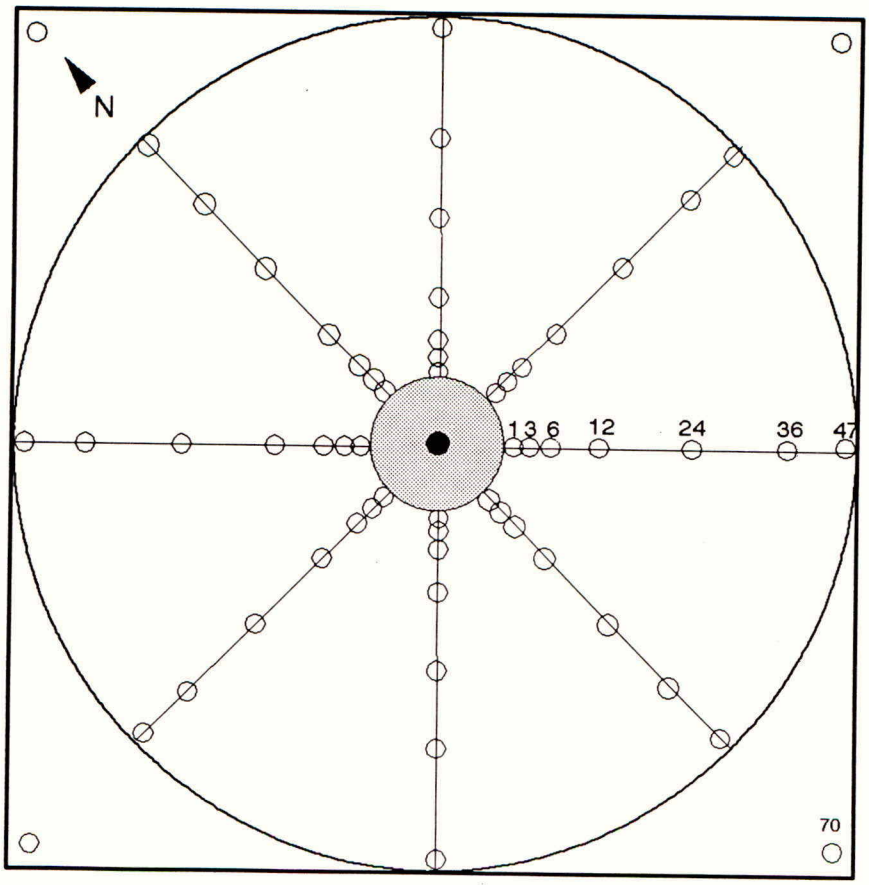
A GENE DISPERSAL EXPERIMENT WITH OILSEED RAPE

Oilseed rape plants of the spring variety Westar contained a gene (*bar*) conferring resistance to the herbicide glufosinate-ammonium (Trade names Basta^R and Challenge^R, Hoechst). Because of the way the seeds had been multiplied, 65% of the seeds carried the herbicide resistance gene (called the transgenic seed stock). A progeny test established that 95% of the resistant plants were homozygous for the resistance transgene. The transgenic seed stock was sown by hand (6 kg ha^{-1}) in a 9m circle at the Institute Farm. In the centre of the transgenic circle was a 1m diameter circle sown by hand with non-transgenic Westar seeds (6 kg ha^{-1}). On the outside of the transgenic circle was a machine-drilled plot of non-transgenic Westar (Figure 1). Six honey bee hives were included in the plot to facilitate pollen transfer. Four hives were placed alongside each of the 4 straight edges of the plot. A further two were placed at a distance of 450m from the plot in an approximately northerly direction (Figure 3 and 4). The proportion of plants flowering in both the transgenic and non-transgenic areas was monitored throughout the flowering season to confirm that there was good flowering synchrony and, therefore, the opportunity for pollination to occur between the transgenic and non-transgenic plants.

All seeds were harvested from the transgenic area and samples of seeds were harvested from the non-transgenic area at distances of 1m, 3m, 6m, 12m, 24m, 36m, 47m and 70m distance from the edge of the transgenic plot. As the probability of finding seeds carrying the herbicide resistance marker gene decreased with distance from the central transgenic plot, the sample size was increased so that approximately 20% of the plants in a 1m strip were harvested at each sampling distance. Sampling was by taking seeds from multiples of 1m areas at each distance. The seed sample at 70m was smaller because it was taken from the corners of the plot. Harvested seeds were screened, both in the glasshouse and in a field experiment, by spraying seedlings with the herbicide when they had 1-4 true leaves. Spraying was carried out twice, with spray applications at an equivalent of 4 l ha^{-1}.

FIGURE 1.

Non-transgenic plants were sown in a 1m circle at the centre of the experimental plot (black). The transgenic seed stock was sown in a 9m circle (grey) and non-transgenic seeds were sown in the remainder of the plot. Seeds were harvested from the non-transgenic plants at distances of 1 to 47m from the edge of the transgenic circle. Seeds were also harvested from the corners of the 105m x 105m plot, at a distance from the transgenic circle of 70m.



Samples of plants surviving spray treatment were moved to a containment glasshouse for seed production. Plants grown from these seeds were screened to confirm that the plants were heterozygous for the herbicide-resistance transgene. Samples of plants were also confirmed to contain the marker transgene by Southern hybridization using the *bar* gene as a probe. Some plants surviving spray treatment under field conditions were found not to contain the transgene. This was believed to result from a high plant density at spraying and the possibility of plant shielding. The growth conditions at spray treatment also influenced the efficiency of herbicide action.

Estimates of pollination frequencies were based on numbers of plants that were confirmed to contain the herbicide-resistance transgene.

FREQUENCY OF CROSS POLLINATION AT DIFFERENT DISTANCES

The frequencies of pollination by the transgenic plants at different sampling distances are given in Table 1. Because the sample sizes from the field screen were much larger than from the glasshouse screen, only data from the field screen are included (except for the central plot). There was good agreement between the data from both glasshouse and field screenings (Figure 2).

The frequency of progeny containing the transgene was estimated from different regions of the plot to determine whether there was evidence of directional effects caused by wind. The wind direction was predominantly towards the east, but there was no evidence that wind direction influenced the direction of pollen travel.

POLLINATING INSECTS

Observations were also made on the relative abundance of honey bees compared with bumble bees. The number of both classes of insects increased as the proportion of oilseed rape plants in flower increased. The number of both insects decreased as flowering was completed, but the number of honey bees followed the flowering pattern more closely than the number of bumble bees (Scheffler *et al,* 1993). To give ample opportunity for cross pollination within the plot, six honey bee hives were included. Approximately 1% of the bees from each hive were marked with different coloured spots on their thorax, so their hive of origin could be identified. Observations of bees were made throughout the season and the location of spotted bees was recorded. It would be necessary to enlarge the sample size to obtain a comprehensive view of honey bee movement in an experiment of this kind. However, observations of bees from the six hives (5 locations; Figure 3), indicate that bees from hives on the immediate edge of the plot moved freely over the whole hectare area. Conversely, bees from hives 450 m away, worked the edge of the plot nearest their hive and hence hive distance influenced their pattern of foraging (Figure 4).

TABLE 1.

The frequencies of plants pollinated by the transgenic oilseed rape plants at distances from 1 to 70m.

Distance from the transgenic oilseed rape plot	Estimated number of plants sprayed (x 1000)	Estimated frequency of seeds containing the transgene(%)
Centre	7	4.8[1]
1	>12	1.6
3	>29	0.4
6	>29	0.11
12	>110	0.016
24	>117	0.0041
36	>179	0.0011
47	>295	0.00034
70	>12[2]	0

[1] The estimated frequency of progeny containing the *bar* gene from the central non-transgenic plot. This figure was obtained from the glasshouse herbicide screen only. The other data were obtained from the field herbicide resistance screen.

[2] The sample size was smaller at 70m because seeds were taken from the extreme corners of the plot.

RECENT EXPERIMENTS

The nature of pollen movement will be influenced by the experimental design. In order to obtain data that will be useful for the evaluation of novel transgenic plants in research and plant breeding programmes we are currently testing gene flow between plants in 20m x 20m plot sizes. A plot containing plants with a marker transgene was positioned in the centre of the Institute Farm, and non-transgenic plots were planted at 200m and 400m distances. Seeds from the non-transgenic plot were allowed to shed directly on the same plot for herbicide screening. Following irrigation the seedlings germinated *in situ* and could be screened with the herbicide. This has enabled us to screen many millions of progeny, and the data are currently being analyzed.

FIGURE 2.

Estimated number of plants containing the *bar* gene, per one million progeny, at distances from 1-47m from the edge of the plot containing transgenic plants. Data are presented for both the glasshouse (dashed line) and the field screening (solid line) for herbicide resistance.

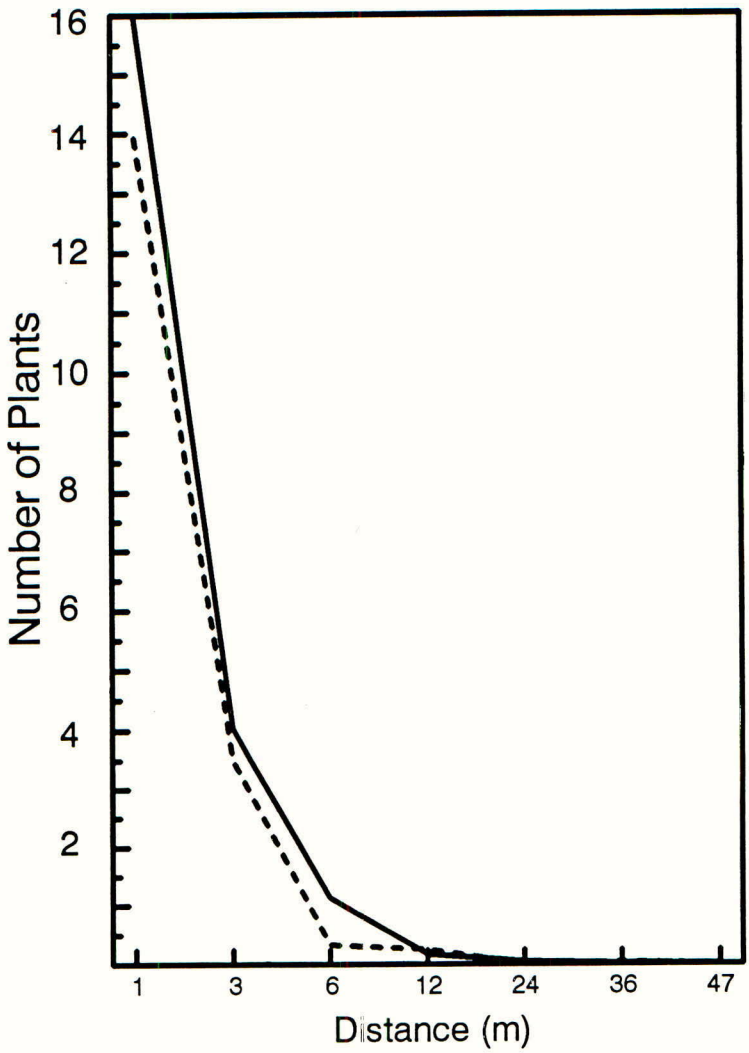


FIGURE 3.

Location of bee sightings made at various times throughout the flowering season. Hives were positioned at the four edges of the plot, as indicated. Approximately 1% of the honey bees from each hive were marked with different coloured spots (pink, blue, yellow and green) on their thorax.

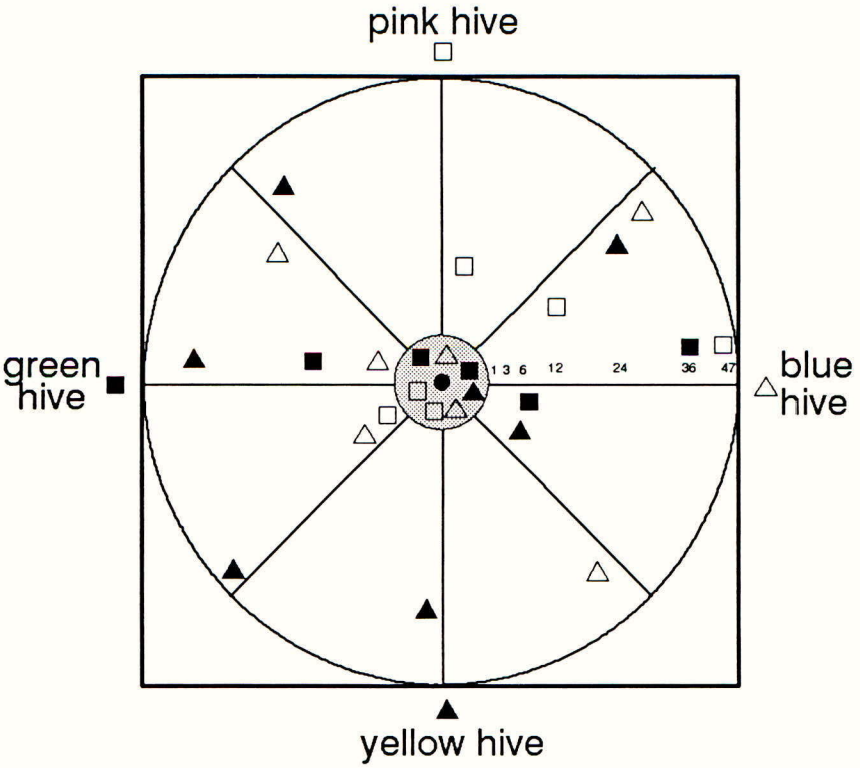
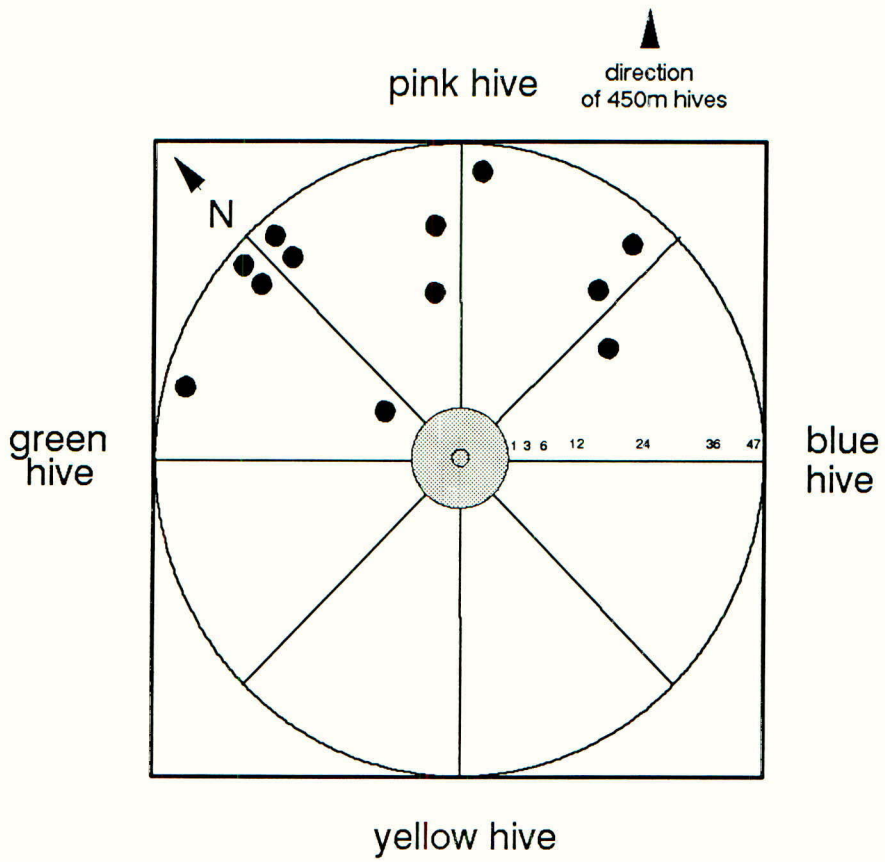


FIGURE 4.

Location of the sightings of bees (black circles) from two hives placed 450m from the plot in an approximately northerly direction.



CONCLUSIONS

To be able to evaluate novel transgenic plants under standard field conditions, it is important to estimate the degree of genetic isolation that can be obtained. This is necessary information for the Regulatory Authorities, and valuable information for maintaining an acceptable level of genetic purity in research and breeding lines. Pollination frequency falls sharply over a distance of 12m and was negligible at 47m. Evidence from insect observations indicated that, in this experiment, the honey bee was the principal pollinating insect, and the patterns of bee behaviour (and therefore pollen) can be influenced by the position of the hives. The presence of a buffer strip of non-transgenic oilseed rape, surrounding the plot of transgenic plants, may reduce the probability of transgenic pollen being taken by bees from distant hives.

ACKNOWLEDGEMENTS

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RELEASING TRANSGENIC PLANTS

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ABSTRACT

To date, trials of transgenic plants have taken place at several hundred sites throughout the world. At least 1400 of these are in the US, and although the majority have been commercial trials, almost twenty non-commercial instructions have been involved. In contrast, very few UK academics have conducted such tests; this is probably a consequence of the different regulatory attitudes in the two countries.

All the results so far obtained suggest that transgenic material is substantially equivalent to non-transgenic material in terms of its agronomic performance and that there are no specific or peculiar problems associated with its testing. The increasing experience gained from such trials suggests that they will rapidly be adopted as an inherent part of plant breeding.

INTRODUCTION

It has been estimated that the available agricultural land in the world could support a population of 49.8 billion people, some few times the predicted world population in the year 2100. However, these calculations take no account of several constraints on production such as the reduction of soil fertility due to increased cultivation. In addition, it is not certain that large irrigation schemes, seen as some as a simple means to increased production, will be considered acceptable.

There is therefore a gradual increase in interest, both for political and economic reasons, in lower input systems for agricultural production. With the cost of developing and marketing a new active ingredient for agricultural chemical products now approaching £100m, improving the quality of crops through the application of gene transfer techniques to conventional plant breeding, is attracting increased attention.

In the last decade since such techniques were developed, the introduction of genes into plants has been accomplished by a variety of methods, transgenic plants are now relatively commonplace, and their utility has been widely discussed (Dunwell & Paul, 1990).

MOTIVATION FOR FIELD RELEASES

Initially, and inevitably, there was attraction in the mere novelty of transgenic plants and their performance in the field, but as the years have passed and the number of trials reaches the hundreds around the world (425 permits for 1400 sites in the US alone) (TABLE 1), this novelty value has declined, to be replaced by more careful considerations of commercial or

academic value. To some extent, familiarity has generated boredom, as the more exaggerated predictions of "novel life forms" have failed to materialise. Such boredom, of course, does not imply complacency but rather it reflects the reassurance born of experience.

TABLE 1. Crop species for which USDA Experimental Use Permits have been granted.

Crop	No. permits	Crop	No. permits
maize	73	rice	less than five each
tomato	72	sunflower	
potato	58	walnut	
soyabean	58	apple	
cotton	39	cucumber	
tobacco	38	poplar	
melon	17	chrysanthemum	
squash	14	papaya	
rapeseed	12	petunia	
lucerne	11	plum	
TOTAL			425

From the commercial perspective (TABLE 2), field trials are seen as part of the developmental pipeline of products generated in the laboratory but destined for the breeding programmes of the future and thence to the consumer, either directly to the farmer or indirectly to the customer further along the agricultural produce chain.

TABLE 2. Companies which have been granted USDA Experimental Use Permits.

Agracetus	Calgene	Du Pont	Northrup King
Agrigenetics	Campbell	Frito-Lay	Peto Seeds
Agripro	Canners Seed	Harris Moran	Pioneer
Agritope	Cargill	Heinz	Rogers NK
American Cyanamid	Ciba-Geigy	Hoechst-Roussel	Rohm and Haas
Amoco	Crop Genetics	Holdens	Upjohn
App. Starch Tech.	De Kalb	ICI/ZENECA	Sandoz
Biosource Genetics	DNAP	Intermountain Canola	Stine Seeds
Biotechnica	Dow	Monsanto	

Turning to the academic area, there has been, at least in the UK, very limited experience in the use of transgenic material in field trials. Whether this regrettable lack of interest is due to the perceived difficulty (and associated expense) of regulatory applications, is not known. With the notable exception of the large scale ecological study (PROSAMO) coordinated from Imperial College (Crawley *et al.*, 1993), only at the University of Reading, and in a more limited way at Exeter, have academic researchers taken advantage of the benefits provided by the use of

this type of material. Some details of these trials, conducted for biometrical and pollination studies respectively, will be given below.

In the USA, many more academics have conducted trials (TABLE 3), on a wide range of crops, and it is to be hoped that this level of interest, no doubt aided by a positive regulatory attitude (witness the free telephone hotline to obtain advice, and the no-cost simplified procedures available) will spread elsewhere. A report of a recent meeting to discuss some of these issues has been published by Ball (1993).

TABLE 3. Universities/Institutes which have granted USDA Experimental Release Permits.

Auburn	Kentucky	North Carolina State
California/Davis	Louisiana State	Pennsylvania
Cornell	Michigan State	Purdue
Florida	Montana State	Washington State
Hawaii	New York State Exp. St.	Wisconsin
Idaho	Nobel Foundation	Wisconsin/Madison
Iowa State		

INTRINSIC PROBLEMS

These are few in number since field trials of transgenic crops are - or at least should be so considered - no different from those of non-modified crops; in every significant regard, except in the modified character, they are identical, particularly if the introduced trait has been backcrossed into the 'control' line.

In summary, there are no inherent characteristics of transgenics that make their trialling problematic.

EXTRINSIC PROBLEMS

These problems are those imposed on the researcher because of the nature of the transgenic material with which he or she works, and can be summarised as cost. This year has seen the formal adoption, in this country, of the EC directives on deliberate release EC90/220, and with it the charges imposed on the applicant. In the days of government deficit the desire for such income generation is perhaps understandable but it does nothing to encourage the development and extension of trials in this country, rather than in locations where burdens are being removed, not being imposed. For the academic, of course, there is no chance to move location and in all likelihood research budgets will have to include the significant cost of application fees. The research will suffer accordingly.

AGRONOMIC PERFORMANCE OF TRANSGENIC CROPS

To date there is little published information on the results from breeding-scale field trials, since for obvious reasons much of this information is of commercial significance. However, the results from a number of smaller scale studies have been published, though they are of limited value in the applied context. For example, Jefferson (1990) reported preliminary results from expression of the glucuronidase (GUS) gene (a marker detected by histochemical or fluorometric methods) in various transgenic potato clones. These results quite clearly showed the complexities of genotype x environment x time interactions which were well known to plant breeders but which still surprised molecular biologists! In a more recent potato trial, Kuipers *et al.* (1992) reported data from a number of clones transformed with granule-bound starch synthase antisense constructs. These authors also found levels of enzyme actively which varied between clones. As with these results from vegetatively propagated material, there are a limited number of published studies from seed derived material. Possibly the first to describe morphological traits was that on various lines of tobacco transformed with *Agrobacterium rhizogenes* constructs (Mariotti *et al.*, 1989), although there were others in which transgenic plants have been evaluated for expression of herbicide resistance (DeGreef *et al.*, 1989; McHughen *et al.*, 1990), virus resistance (Nelson *et al.*, 1988; Kaniewska *et al.*, 1990; Slightom *et al.*, 1990) or insect resistance (Koziel *et al.* 1993) in field tests. One of the most detailed agronomic studies was that on flax by McHughen and Rowland (1991) who compared five transgenic sulphonylurea resistant lines with four standard commercial cultivars and 24 other breeding lines in a multiple location trial. They concluded that the transgenic lines did not appear to be affected by T-DNA for any quality or agronomic parameter tested, including yield.

More recently, Arnoldo *et al.* (1992) reported on the field performance of eleven oil seed rape (canola) lines transformed with a neomycin phosphotransferase (*npt*) gene which provides resistance to kanamycin. The agronomic characters assessed, which included maturity, yield, and oil and protein content, were found not to differ between control and transgenic material.

In a related, though more specific genetic study, Caligari *et al.* (1993) examined a number of agronomic characters in various lines and hybrids of transgenic tobacco carrying *npt* and GUS (see above) marker genes. Although the lines showed significant differences as estimated by these morphological characters, it could not be concluded that these were due to the presence of the introduced genes.

The difficulty of extrapolating from small numbers of samples has been shown recently in a report on transgenic rice (Schuh *et al.*, 1993). These authors examined the progeny of three transgenic plants, one protoplast-derived plant and 25 non-transformed seed-derived plants; and observed that the transgenic lines were generally smaller, took longer to flower and had reduced fertility. These findings have limited practical value, since in a realistic breeding programme, larger number of transgenic lines would be generated and if unwanted (? somaclonal) variation were observed, material would be backcrossed to remove these deleterious side effects.

EXPRESSION OF TRANSGENES IN THE FIELD

Most gene cassettes introduced into plants contain at least two elements, first a suitable selectable marker allowing identification of the transgenic cell(s) *in vitro*, and secondly a gene of agronomic interest. In a few instances, namely herbicide resistance, a single gene may fulfil both functions, but this is exceptional. Therefore, in most instances, the level of expression of the non selected gene is of absolute importance in terms of commercial development. There is, however, no simple means of predicting the level of expression of such a gene. It is known, for example, on studies of GUS constructs that plants may be produced which show a thousand fold difference in expression levels. In addition, the inheritance of such expression over generations is also not always predictable. In one detailed study (Caligari *et al.*, 1993) on tobacco, it was shown that in a line expressing GUS at high level the introduced gene appeared semidominant with the homozygote showing twice the expression of the hemizygote, while in a low-expressing line the gene appeared dominant, with the homozygote and hemizygote showing the same level (TABLE 4).

TABLE 4. GUS expression (nmoles MU/mg protein/h) in leaves of various tobacco lines and hybrids grown in the field at Reading University.

Male parent	Female parent			
	Xanthi control	Xanthi GUS	Samsun Control	Samsun GUS
Xanthi control	0	250	0	1336
Xanthi GUS	391	305	553	1890
Samsun control	0	284	0	1398
Samsun GUS	1284	1815	1450	2650

This phenomenon has been explored further at the molecular level by Hobbs *et al.* (1993) who showed, also in tobacco, that the high-expressing types had a single copy of T-DNA at a single locus while the low-expressing types tended to have inverted repeats at a locus. For a successful breeding programme it will be necessary to identify, at an early stage, those lines giving the required expression level and showing predictable inheritance. Where the crop is a hybrid one, it will also be necessary to determine whether the gene need be introduced into one or into both parents.

ECOLOGICAL ASPECTS OF TRANSGENIC CROPS

This topic, which is considered elsewhere in this volume, will not be dealt with in detail here. However, there are a number of recent reports which are worthy of note. First, the issue of invasiveness of transgenic material has been investigated in a most extensive study by Crawley *et al.* (1993) and discussed by Kareiva (1993). No evidence has been found which would suggest that the transgenic oil seed rape lines examined had any

greater competitive or invasive tendencies than conventional cultivars, when grown in a range of ecosystems.

The issue of cross pollination, for which transgenic material has been used in academic studies of reproductive biology (Paul *et al.* 1991; Cresswell, pers. comm.), has also been investigated in a more applied context (Raybould & Gray, 1993). These latter authors, in a detailed desk study, considered the relatedness of crops and their wild relatives to be the issue of greatest significance when considering the release of transgenic crops (Wrubel *et al.*, 1992).

CONCLUSION

Field trials are an inevitable and an indispensible part of the plant breeding process, whether or not the material being tested is transgenic. Efficient breeding programmes are those that identify rapidly the advantageous combination of characters required of any new variety and the need for a considerable range of material to be tested applies equally well to transgenic lines. At present, the exact level of expression of an introduced gene cannot be predicted prior to testing and therefore the breeder has to select, as with any other character, the line which best suits his or her specification. This testing, or trialling, process is no more difficult with transgenic than with non-transgenic material, as the breeder is well able to eliminate those off-types or lines which do not maintain the desired standards of performance from generation to generation. In all the trials conducted to date there is no evidence to suggest that the transgenic material should not be considered as substantially equivalent to the non-transgenic control.

As the utilisation of transgenic plants extends from tomato (Schuch, 1993) and maize (Wilson *et al.*, 1993) to a broader range of crops (see TABLE 1) then many of the considerations discussed above will cease to attract attention. It will be at that time that the integration of transgenic techniques into plant breeding can be considered complete.

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MANAGEMENT OF RESISTANCE TO TRANSGENIC PLANTS

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No paper submitted

4.
Progress in Major Crops

Chairman: L.G. COPPING

NEW OPPORTUNITIES IN THE BREEDING OF WHEAT AND BARLEY THROUGH MOLECULAR BIOLOGY- The implications of the Primeval Gramineae Genome concept.

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ABSTRACT

Recent developments in cereal genome analysis including the generation of RFLP maps (for wheat, rye, barley, oats, rice, maize, sorghum and millet) and availability of information on the distribution of genes in the large cereal genomes; provides us with a clearer insight into cereal chromosome structure. A composite map of the ancestral grass genome can now be produced. Comparative genome mapping is likely to have a major impact on plant genetics and hence cereal breeding. These developments will be reviewed.

INTRODUCTION

Wheat is one of the world's most important crops and therefore the small grain cereal crop sector is potentially very open to agricultural biotechnology-derived inventions. Genome analyses have an important role in this research field. They provide molecular markers for use in breeding, particularly in marker-aided selection. They aid in the characterisation of genes controlling traits, indicate where these genes lie on chromosomes and how strategies can be devised for isolating them. However, cereals such as wheat have very large genomes, those of barley (Hordeum vulgare) and the 3 genomes of hexaploid wheat (Triticum aestivum) are $5x10^9$ bp and $1.7x10^{10}$ bp (in total per 1C nucleus) in size respectively (Bennett and Smith, 1991). Extensive renaturation studies demonstrate that the vast majority of sequences in these genomes are reiterated (Smith and Flavell, 1975; Flavell et al., 1977; Rimpau et al., 1978). Their large size imposes a substantial constraint for the application of molecular biology techniques, as does the fact that there are few scientists involved in their analysis compared to the numbers and resources committed to the analysis of the human genome. However there are some cereal genomes which are relatively small, for example rice (Oryza sativa, $4x10^8$bp). Clearly there has been a massive expansion in the size of some grass genomes since their speciation from a common ancestor.

GENETIC MAPS AND CHROMOSOME STRUCTURE

The construction of genetic maps of Triticeae species and of rice has progessed rapidly (Saito et al., 1991; Gale et al., 1990; Chao et al.,1989; Heun et al., 1991; Devos et al., 1992 & 1993a). The current RFLP map of rice constructed by NIAR Rice Genome Program contains more than 500 cDNA markers. Comparative genetic maps have demonstrated that homoeologous single copy sequences/ genes are co-linear on the RFLP maps of wheat, barley and rye except where translocations have occurred (for example, Devos et al., 1993b). Studies on the distribution of recombination along a cereal chromosome using physical markers all show that recombination is predominantly confined to the distal regions (Lawrence and Appels, 1986; Linde-Laursen, 1982; Dvorak and Chen K-C, 1984; Snape et al., 1985; Wang et al., 1991). The distribution of landmarks for genes in the wheat and barley genomes, indicates that these regions also contain a higher concentration of coding sequences (low/single copy) (Moore et

al., 1993a). The analysis also indicates conversely that a significant proportion of the repetitive sequences have arisen by amplification of sequences in the proximal regions of the chromosomes of the large genome cereals (Moore et al., 1991& 1993a). Although rice chromosomes also have these blocks of repetitive sequences, clearly they have not been subject to the same extensive amplification as observed in the large cereal genomes (Moore et al., 1993a). These observations are consistent with the reconstructions of meiotic nuclei from hybrids of two rye grass species whose chromosomes differ in length. Meiotic pairing between homoeologous chromosomes of differing length is achieved in these hybrids by the generation of hairpin loops in the proximal regions of the longer bivalents (Jenkins et al., 1985). This suggests that the difference in length may be accounted for by an expansion of the proximal (but not distal) regions.

IMPLICATIONS OF CHROMOSOME STRUCTURE

During the speciation of rice and wheat, what was the effect of the amplification process on single copy sequences/genes? More than 70 rice RFLP markers (random single copy sequences) have been cross-hybridised to wheat and barley DNA. 60% of these markers cross-hybridise as single copy sequences in the wheat genome and 80% of the wheat homoeologous probes on the wheat RFLP map also cross-hybridise to single copy rice sequences. Therefore, although there was substantial amplification of repetitive sequences in the wheat genome but not in the genome of rice, there has been little amplification of single copy sequences in both genomes. This enables the wheat and rice RFLP maps to be compared (Moore et al., 1993b). In fact the genetic and physical maps of sorghum, rye, barley, wheat, maize and rice can all be compared (Whitkus et al., 1992; Devos et al., 1993b; Moore et al., 1993b). On current evidence, it is likely that portions of these maps are co-linear. This will enable the reconstruction of an ancestral map of the primeval grass from which all these species evolved some 60 million years ago (Wolfe et al., 1989; Martin et al., 1989). The conservation of the co-linearity of genes within the chromosomal segments during speciation is depicted in Figure 1. Although the gene order is maintained within chromosomal segments, these segments themselves will have been subject to translocation, expansion, deletion and inversion, thereby creating the variation in chromosome structure seen in today's grass species. This predicts that all or most genes will be present in the each of the grass species, in related positions on homoeologous chromosome segments.

However an important question then arises, will the genes controlling morphological adaptive or disease resistance traits exhibit the same strict co-linearity between the homoeologous chromosome segments as the RFLP markers do? In the Triticeae, there are many cases where such traits are determined by single genes, such as isoenzymes, storage proteins and genes with basic developmental roles. It is likely in these cases that function and co-linearity will be conserved. However, other more complex characters are likely to be the cumulative result of one or more biochemical pathways, each containing several steps controlled by independent genes. Anyone of the gene products in these pathways could be the limiting factor in different varieties or different species. Similar phenotypes can be produced by independent single genes within the genome (for example, gibberellic acid-sensitive dwarfing genes in maize (O'Brien, 1993). Genes with similar effects on height and gibberellin response have been identified in wheat, barley and rye, but all at (apparently) non-homoeologous loci (Hoppe et al., 1982; Plaschke et al., 1993). The situation is even less clear when the genes which control performance are considered. The genes on barley chromosomes 1H and 4H and wheat homoeologous groups 5 and 7 controlling spring/winter habit probably are not homologous, although they have a similar phenotypic effect (Gallagher et al., 1991; Law et al., 1993). Wheat geneticists have identified a major gene controlling photoperiod-insensitivity on

homoeologous group 2, while barley geneticists also recognise loci of major effect on chromosomes 1H, 3H, 4H and 6H (Takahashi and Yasuda, 1971). During the speciation and selection of these grasses, different alleles of non-homoeologous genes in various biochemical pathways may have emerged to produce similar phenotypes. The collation of the map positions of all the loci controlling common phenotypic effects is now urgently required for a range of related grass species to evaluate the extent of co-linearity and the genetic origins of phenotypic variation which ocurred during the speciation of these grasses. This requires extensive genetic analyses. However, it is particularly important as the allelic forms could be substantially different in terms of their contribution to the adapted phenotype in any of the grasses. Alleles determining a phenotypic variant in one species may not, therefore, necessarily be homologous to the alleles responsible for the major control of similar variants in another. In fact some alleles may only be recognisable in specific species or only in certain genotypes within a species.

ANCESTRAL MAP

Combining the genetic maps of all the grass species into a single map will reveal considerable information on the genetic control of grass phenotypes. It will be a very powerful tool for fundamental plant science, bringing together under one system plant geneticists worldwide who currently work on a very large number grass species. Comparative genome analysis will provide for breeding purposes, an indication of the sources of potential traits for manipulation and DNA markers which will aid their selection. It will be possible to trace the origins of allelic variation responsible for agronomic traits selected when grasses are grown under different environmental conditions.

GENE ISOLATION

If as indicated above, the chromosomal location of a gene controlling an agronomic trait can be identified, then it is possible to consider isolating (cloning) the gene. Once isolated, the gene and its regulatory sequences can be studied, modified and then retransformed directly into the variety of choice. In cases where such traits are controlled by single genes and where co-linearity and function is maintained, model systems such as rice with small genomes can be used to clone the genes. The homoeologous genes isolated from these simpler genomes then can be used to study their counterparts in other grass species. But, however useful certain model species such as rice are for identifying and isolating specific genes (alleles), it will also be necessary to be able to isolate genes from large genome cereal species themselves to obtain specific desirable alleles for application in these and related crops. If inverse genetical strategies are to be applied to large cereal genomes, a number of technical advances are required to make such strategies cost-effective. The generation of very high density genetic maps is extremely important for such strategies. They provide markers flanking genes of interest in wheat. Physical mapping can then to be undertaken of a particular chromosome region containing the gene. Genomic libraries can be created in yeast artificial chromosomes (YACs) containing large fragments of plant DNA (several hundred Kb). Markers flanking the gene can be used to identify YACs containing the corresponding sequence to the marker. By selecting sequences at or near the ends of each YAC, it is possible to identify further YACs containing the adjacent plant DNA (termed chromosome walking). In this manner it is possible to build up a physical map of the region containing the gene of interest as a series of overlapping YACs. A very high density map can reduce or avoid altogether any extensive chromosome walking within these regions, by simply providing probes with which to identify the yeast artificial chromosomes (YACs) carrying the genes of interest cloned within them. The development of a number of techniques has facilitated the production of large numbers of RFLP markers for

specific cereal chromosomes, including the flow sorting of hexaploid wheat chromosomes and the generation of flow-sorted chromosome libraries (Wang et al.,1992), and the generation of libraries from micro-dissected individual chromosomes (Houben et al., 1991). The ability to produce and identify RFLP markers from specific chromosome regions has also recently been achieved. Viable deletion mutants involving particular chromosome regions are relatively easily generated in hexaploid wheat. A protocol has recently been described to enhance the cloning of the single copy sequences deleted in these genetic stocks (Clarke et al., 1992). Therefore, it is now possible to produce and identify rapidly RFLP markers derived from specific regions of wheat chromosomes as well as from particular chromosomes.

As described above, YACs containing large DNA inserts can be used for chromosome walking if genes are relatively closely spaced in the genome because it is then probable that the ends of the YACs are single copy sequences. However, if genes are widely spaced amongst blocks of repetitive sequences, then it is probable that the ends of each YAC are repetitive sequences and that each YAC will contain few genes or single copy sequences. In genomes which possess a substantial amount of repetitive sequences between their genic regions (such as those of the Triticeae) it is necessary to devise a walking strategy which avoids the repetitive regions. YACs containing segments of the rice genome may help. Although their genomes are markedly different in size, if rice and wheat genomes still have conserved linkage groups and have maintained gene order within them, single copy end-probes to rice YACs could be used to jump or walk between homologous single copy sequences in these wheat linkage groups, thereby avoiding the intervening repetitive DNA. Homologous sequences in the wheat genome to the ends of a rice YAC are likely to be spaced substantially further apart than the corresponding sequences in the rice YAC. If wheat markers known to flank a gene of interest reside in the same segment of rice DNA cloned in a YAC, this YAC could be used to generate a physical map of the homoeologous region in the wheat genome. Clusters of unmethylated recognition sites for restriction enzymes on the wheat physical map provide an indication of the locations of genes (Moore et al., 1993a) within the stretch of DNA cloned in the rice YAC. This will direct the choice of which DNA segment to sequence in order to identify the open-reading frames that define active genes.

In summary, in the short term, molecular biological studies will generate the cereal ancestral map which will provide breeders with an indication of potential sources of traits for manipulation as well flanking markers to aid in their selection. In the long term, prospects are greatly enhanced for being able to recognise and isolate genes in a cost-effective manner, even from some of the largest plant genomes by the use of the small rice genome.

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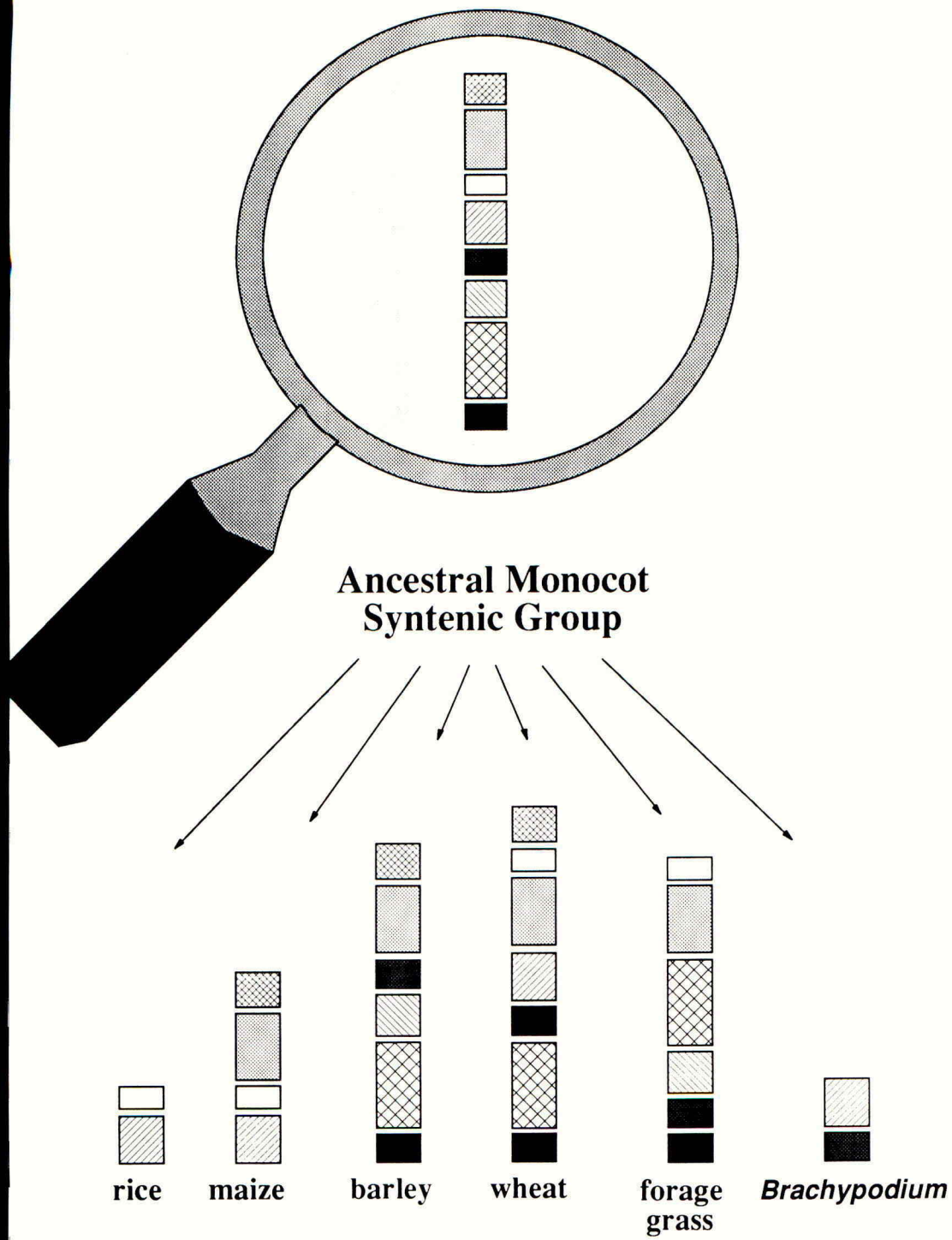
LEGENDS

Figure 1. The concept of the ancestral map.
A map of the primeval monocotyledonous genome can be reconstructed from the segments of linkage groups identified in the genomes of the cereals and grasses. These segments are indicated by the different colours. The amount of DNA in each of the segments varies from species to species, although colinearity of gene order is maintained within the homoeologous chromosome segments.


Ancestral Monocot
Syntenic Group
rice
maize
barley
wheat
forage
grass
Brachypodium

PROGRESS IN THE BREEDING OF CORN

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INTRODUCTION

Modern hybrid corn (*Zea mays* L.) is the product of thousands of years of breeding. This breeding process began with selection by Native Americans and was continued by European Colonists and early American settlers. Farmers and seedsmen in the 19th century developed, through crossing and selection, the strains such as Reid Yellow Dent, Krug, and Lancaster Sure Crop which served as the foundation of modern U.S. corn belt germplasm (Goodman and Brown, 1988). These farmer-developed varieties serve as the genetic foundation for most of the hybrid seed planted for the multi-billion dollar U.S. corn crop (Darrah and Zuber, 1986).

Selections of corn from the Americas were taken to other tropical, sub-tropical, and temperate areas of the earth where they were adapted for regional growing conditions by local farmers, seedsmen, and scientists. This worldwide reservoir of germplasm now serves as the raw material to which modern corn breeding practices are applied for the purpose of developing hybrid corn.

Scientific pioneers such as Charles Darwin and William Beal experimented with the phenomenon of hybrid vigor. However, it was the careful work of East (1908) and Shull (1909) that gave rise to the principles of heterosis and inbreeding depression. Jones (1918) suggested a process for making hybrid corn a practical commercial invention and the era of modern corn breeding was born.

The first decades of corn breeding brought forth the basic principles of ear-to-row selection and early testing that form the basis of what is known as pedigree breeding (Sprague and Eberhart, 1977). Developments in the statistical analysis of field plot data by Fisher and others led to great progress in plant breeding. In the 1940s and 1950s quantitative genetic theory was applied to corn breeding which gave rise to alternate methods of corn improvement called recurrent

selection or population improvement (Hallauer *et al.*, 1988). A combination of pedigree breeding and recurrent selection has been used to develop superior corn hybrids for seven decades. Duvick (1992) has shown that the genetic improvement in corn grown in the U.S. cornbelt has been steady and striking.

STATE OF THE ART

Modern corn breeding is the result of applying the basic principles of genetics to cross breeding and inbreeding systems of mating and the development of field plot and analysis of variance techniques which make possible the accurate evaluation of large numbers of new strains derived from these mating systems.

The goal of modern commercial corn breeding, for developed agriculture, is to provide hybrids which maximize the farmers' return on investment. The hybrids which best meet these goals are most sought after and thus serve as a successful products for the seed industry. Presently, corn hybrids are grown mostly to supply the commodity grain market and grain from different hybrids is not differentiated after harvest. There are some markets for identity preserved corn and these will be addressed later.

Modern corn breeding is thus focused on breeding for hybrids with maximum yield of grain; hybrids that resist lodging; and hybrids that mature during the growing season of the targeted market area. Yield is likely governed by an unknown number of genes interacting in complex ways and whose effect on the phenotype is environment dependent. Specific manipulation of individual genes has not been important in corn breeding. Instead, breeders rely on programs where very large numbers of strains are evaluated across a large number of representative locations over several years. The hybrids which are most suitable for production over a large geographic area are those that will yield the highest over this multi-environment testing. These hybrids are assumed to have the best genotype for the intended market even though little or nothing is known about individual genes.

The genetic basis for the corn breeders success in raising yields is not well understood at the biochemical or molecular level but the process of screening and identifying the best hybrids has been honed to a fine edge. Breeders cannot control the processes of random assortment of chromosomes, crossing

over, and recombination. However, they can cross strains with desired characteristics which increases the probability of identifying a desired offspring. Thus, more emphasis has been placed on the process of evaluation of strains. The technologies that have made modern corn breeding more effective are information management and process automation. A contemporary corn breeder will make about 200 new breeding crosses annually from which new strains will be selected. From these 200 breeding families about 2000 new strains will be tested as experimental hybrids at multiple locations. At each stage of testing a severe culling level is employed so that seven to ten years after this process begins an inbred line derived from this process may become a parent of a commercial hybrid.

Speed is of the essence in breeding and winter nurseries are routinely used so that two generations per year can be achieved. Planting and harvesting of research plots are done with modified versions of commercial farm equipment which allow two to five skilled technicians to plant or harvest several thousand plots per day. Data are recorded in computer databases that allow breeders to conclude harvest at night and have a statistical analysis of the results the next morning. Breeding strains can thus be selected on the basis of analyzed data and sent to a winter nursery for advancement.

NEW TECHNOLOGIES APPLIED TO BREEDING

The discoveries of DNA being the material of genes, the structure of the double helix, and the cloning of DNA have had little or no impact on modern corn breeding. However, this appears to be about to change as successes are occurring in the field of biotechnology.

Biotechnology is being applied to corn improvement through two ways. One is the insertion of genes of agronomic value from plant and non-plant species. The other is the use of DNA markers such as RFLPs to track genome segments for the purposes of germplasm or gene identification.

Resistance to specific herbicides is being introduced into otherwise susceptible corn through transformation or *in vitro* selection (Schulz *et al.*, 1990). Resistance to imidazolinones, glyphosate, sulfonylureas, sethoxydim, and others are being developed or deployed.

Disease resistance is an obvious target for biotechnological manipulation. Virus resistance has been obtained by inserting viral coat protein genes (Abel, *et al.*, 1986). Resistance to ear molds and the development of associated toxins is a target for genetic engineering. There is valid concern that since the biotechnological approach is limited to single genes that the classic problems of pathogen races overcoming resistance genes will be a chronic problem.

Insect resistance may be improved by inserting insecticidal proteins such as lectins (Chrispeels and Raikhel, 1991). Also, genes from non-plant species such as insecticidal toxin genes from *Bacillus thuringiensis* have already been inserted into crops (Gasser and Fraley, 1989). The concern of new biotypes developing exists in this area as well (Tabashnik *et al.*, 1990).

Grain yield is a poorly understood trait and will not likely be directly enhanced by single gene transformation. DNA markers may be useful in selecting for multiple chromosome segments that are correlated with yield (Stuber *et al.*, 1987). Of course, improving disease or insect resistance can have a large indirect effect on yield.

Successful application of transformation as a crop improvement tool requires that it be fast and applicable to elite germplasm. Modern corn breeding programs are delivering new hybrids annually and the lifetime of a hybrid may be only five to seven years. Progress in transformation of elite corn germplasm has been reported recently (Koziel *et al.*, 1993) and is expected to continue.

THE FUTURE

As mentioned earlier, most corn grown now enters the commodity market and grain from different hybrids is not differentiated after harvest. Some special markets have existed for several years. These include waxy endosperm types, high amylose types, white endosperm, and hard textured kernel types used for certain foods (Glover and Mentz, 1987). However, there is expectation that corn grain can be modified further to meet special needs for protein, oil, or starch and that end-users will demand these specialized varieties. This will require an identity preservation system.

Breeding of speciality corns might be accomplished through selection of variants endemic to corn or by genetic

engineering. In either case these breeding programs will require support from analytical chemists and biochemists whose skills have not traditionally been widely used in corn improvement.

Modern corn hybrids provide farmers with a crop that will yield much more than the crops of years past. Also, modern corn hybrids tolerate environmental stresses such as drought much better. The finished corn hybrid is a result of quality germplasm as a starting material; well planned and executed breeding programs; multi-disciplinary support teams of agronomists, chemists, entomologists, pathologists, physiologists and biometricians; and computer supported data management.

Hybrids are on the market now with biotechnologically selected herbicide resistance. During this decade we will likely see hybrids with biotechnologically developed resistance to maize dwarf mosaic virus, maize chlorotic mottle virus, maize chlorotic dwarf virus, European corn borer, corn rootworm, and other pest resistances. Also, we will see corn hybrids with modified protein, oil, or starch content for special industrial or feeding markets. These special characteristics must be built upon the foundation of a high yielding, lodging resistant hybrid that is adapted to the growing season of the targeted market. This foundation will continue to come from the whole plant breeding process. Whole plant breeding may also be used to develop some of the aforementioned traits but these traits will often come from biotechnology. This will allow the resources for whole plant breeding to be focused on the complex, multigenic traits such as yield while biotechnology resources are focused on traits that can be affected by single genes.

It is exciting and encouraging to see the accumulation of knowledge of molecular biology being applied to modern corn breeding. The result will be a greater choice of hybrids for special markets or environments.

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RICE BIOTECHNOLOGY: PROGRESS AND PROSPECTS

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ABSTRACT

Considerable progress has been made in the development of molecular biology techniques that can be applied to the genetic improvement of rice. Molecular genetic maps and markers of the rice genome and for the genomes of major rice pests and pathogens are available. They are providing the basis for more efficient and effective selection and deployment of resistance genes, and should make significant contributions toward breeding for durable resistance. Genetic engineering of rice is now a reality with transgenic plants containing useful alien genes at the stage of greenhouse and field testing. This paper reviews progress to date and describes several promising research projects.

INTRODUCTION

Rice is the most important food crop in the world serving as the major staple for about three billion people. Most rice is used in the country where it is produced with over half consumed by the people who grow it. To meet projected demand, rice production will need to more than double by the Year 2030, and most of the increase must come from productivity improvements since there is limited opportunity for increasing the area sown.

Genetic improvements have played a major role in increasing rice output over the past three decades. High-yielding varieties have spread to over 50% of the world's rice area and provide additional output sufficient to feed more than 600 million people with an estimated economic value of $3.5 billion/yr. (Lampe, 1993). However, the rate of growth in rice yields in the highest-yielding areas of Asia has slowed significantly, suggesting that a yield plateau has been reached. The major scientific challenges facing rice researchers are to raise the maximum yield potential, close the yield gaps between potential yields and those achieved in farming practice, and sustain current high yields.

Biotechnology holds much promise as a new set of tools which can enable scientists to successfully meet these challenges, particularly by strengthening rice breeding. Rice

breeding involves two phases: the evolutionary phase where variable populations are produced; and the evaluation phase where desirable genotypes are selected.

Variability has been created traditionally by hybridization and to a lesser extent by mutations. Wide hybridization through embryo rescue or somatic hybridization, somaclonal variation, and genetic engineering are biotechnology tools which can significantly expand the range of variability available to breeders. Genetic engineering especially should bring a much greater degree of predictability to the process of generating desirable variability and help in attaining goals not feasible using conventional techniques. Biotechnology tools which can improve the evaluation phase are: anther culture to produce double haploids and eliminate dominance variance; molecular maps and markers of the rice genome to tag and follow the inheritance of genes for important traits, particularly quantitative traits and those that are difficult to score; and molecular genetic maps and markers of pests and pathogens that can be used to characterize and monitor their population structure and dynamics thereby allowing for more effective selection and deployment of resistant plants. This paper will review progress and prospects for utilizing molecular genetic maps and markers of both rice and rice pests and pathogens, and for rice genetic engineering.

RICE GENOME MAPS and MARKERS

Genome research in general and rice genome research in particular have advanced rapidly over the last five years. New techniques for detecting DNA polymorphisms, such as the polymerase chain reaction (PCR), combined with restriction fragment length polymorphisms (RFLPs) have made it practical to develop and utilize molecular genetic linkage maps of important crop plants. For use in breeding these DNA-based maps have inherent advantages over classical maps. The markers are discrete, co-dominant, non-deleterious characters that are unaffected by the environment and free of epistatic interactions. There is essentially an unlimited number of such markers allowing saturated coverage of the genome.

Among crop plants, rice (*Oryza sativa* L.) has attributes which make it especially amenable to genome research. It has a DNA content smaller than any other grain species (C = .45 pg of DNA) and among the smallest of any crop plant (Arvmanagathan and Earl, 1991). Rice is a true diploid (2n = 24) with a relatively high percentage (ca.75%) of single copy DNA (Deshpande and Ranjekar, 1980; McCouch, et al., 1988). Still there are sufficient repeat sequences to select species-specific repeat sequence probes (Zhao, et al. 1989; Cordesse, et al., 1992) and microsatellite markers (Zhao and Kochert, 1993;Wu and Tanksley, 1993b). A vast reservoir of germplasm (more than 200,000 accessions) exist worldwide for genetic and breeding research.

Status of Maps - There are two major independently developed molecular genetic linkage maps of rice. The first, generated in Steve Tanksley's laboratory at Cornell University, now has over 600 markers (McCouch, et al., 1993). This level of saturation represents an average of one marker approximately every 2 cM and in physical terms translates to one marker approximately every one million base pairs. It is sufficient for detecting linkage to genes governing both monogenic and polygenic characters.

The Cornell map has been correlated with the classical and isozyme maps of rice. Three rice telomers have been genetically and physically located on the map at the distal ends of RFLP linkage groups (Wu and Tanksley, 1993a). They were shown to be genetically and physically linked to the terminal RFLP markers.

Close to a hundred cloned rice genes and cDNAs of known genes have been placed on the map (Gary Kochert, pers. comm.). Selected markers which give good polymorphisms across breeding lines are being converted into sequence-tagged sites at the International Rice Research Institute (IRRI) to facilitate dissemination and use in breeding (John Bennett, pers. comm.). In addition, since the degree of polymorphisms with RFLP markers is often low within a subspecies of rice, microsatellite markers are being developed and mapped as alternatives. Rice contains an abundance of both dinucleotide (e.g. $(GA)_n$ and $(GT)_n$) and trinucleotide (e.g. $(GGC)_n$) repeats (Wu and Tanksley 1993b; Zhao and Kochert, 1993). The degree of polymorphisms for rice microsatellite markers is significantly higher than that of RFLP markers and genetic mapping indicates random genomic distribution. Thus, the probability of finding a polymorphic microsatellite marker in a given region is much higher than that of finding a RFLP marker in a cross involving parents within a subspecies (Wu and Tanksley, 1993b). Moreover, analysis of microsatellite polymorphisms is based on the PCR reaction which requires only tens of nanograms of DNA or even crude DNA extracts, and involves only amplification and electrophoresis without radioactive labelling.

The Cornell rice map and markers have been widely distributed throughout the world as part of a kit which contains the most recent version of the map and protocols for using the markers. These materials are available free of charge to scientists who request them. Repositories of mapped clones are maintained at Cornell (c/o Steven Tanksley), the University of Georgia (c/o Gary Kochert), IRRI (c/o Ning Huang), CIAT in Cali, Colombia (c/o Joe Tohme) and The Institute of Genetics in Beijing (c/o Zhu Lihuang).

The second independently developed rice RFLP map is a product of the Rice Genome Research Program (RGP) at the Japanese National Institute of Agrobiological Resources in Tsukuba, (Saito, et al., 1991). As of October 1992 this map had 418 markers, three-quarters of which are sequence-tagged DNA clones (Antonio, et al., 1992). By reciprocal clone exchange,

the Tsukuba and Cornell maps have been correlated with each other and show colinearity for almost all markers (Xiao, et al., 1992). Together they provide over 1,000 mapped markers. IRRI has received a full set of clones from both groups for use in its research programs.

The Rice Genome Research Program at Tsukuba is a major scientific undertaking involving over fifty full-time scientists and excellent facilities with the goal of determining the organization and genetic machinery of the complete rice genome (NIAR, 1992). A physical map based on YAC, cosmid and other large DNA clones is being developed and will be used for map-based cloning of important genes. Numerous cDNA libraries from different organs, tissues and stages of development are also used to identify and characterize hundreds of rice genes. Cloned genes are sequenced and analyzed. The Japanese scientists intend to share the results and most of the probes and clones with others, and welcome visiting scientists from foreign countries to work with them. The RGP should become an important contributor to and resource base for further advances in rice biotechnology.

Applications - Molecular markers are now routinely used to study rice genetic diversity, classification, and phylogeny for purposes of germplasm management (Wang et al., 1992). Scientists at IRRI use mapped probes to survey the molecular diversity of rice and identify genetic trends that occurred during domestication and modern breeding (Lampe, 1993). This has validated the hypothesis that cultivated rice has a hybrid origin with two distinct wild ancestors which were domesticated independently. These findings are important for gene tagging strategies. From a single cross many gene differences can be detected with additional allelic diversity then sought in the wild species. In today's cultivated rice, the indica/japonica subspecies and their recombination appear to be the main structure of genetic diversity. Within subspecies greater genetic differentiation has occurred in indicas than in japonicas (Ishii, et al., 1993b).

At a recent rice biotechnology meeting in Thailand scientists from over a dozen countries reported on their progress in finding markers tightly linked to rice genes of agronomic/economic interest and on using the markers to follow inheritance of the genes in breeding programs. Table 1 summarizes these and other reports. At IRRI scientists are now using the markers to pyramid multiple genes for resistance against bacterial blight into elite breeding lines. Those lines containing combinations of resistance genes show greater resistance than lines carrying a single gene (Lampe, 1993). The durability of the resistance is now being tested. Eventually IRRI hopes to provide breeders with a "superdonor" line which will allow multiple genes for resistance to bacterial blight to be selected in a single cross (McCouch, 1992).

Table 1

Rice Genes of Economic Importance that Have Been Tagged with RFLP Markers

Gene Symbol	Character	Donor Variety	Chrom Location	Reference
Xa-1	BB res.	Kogyoku	4	Yoshimura, et al., 1992b
Xa-3	BB res.	Chogoku45	11	Yoshimura, et al., 1992b
Xa-4	BB res.	IR20	11	Yoshimura, et al., 1992b
xa-5	BB res.	IR1545-339	5	McCouch, et al., 1991
Xa-10	BB res.	CA S209	11	McCouch, 1992
Xa-21	BB res.	*O.longistam.*	11	Ronald/Tanksley, 1991
Pi-1(t)	blast res.	LAC23	11	Yu ZY, 1991
Pi-2(t)	blast res.	5173	6	Yu, et al., 1991
Pi-4(t)	blast res.	Tetep	12	Yu, et al., 1991
Pi-5(t)	blast res.	Moroberekan	4	Wang, et al., 1992
Pi-?(t)	blast res.	IRAT13	4	Tohme, et al., 1991
Pi-zh	blast res.	Zhaiyeqing	8	Zhu, et al., 1993
BR14	blast res.	Apura	12	Yu ZY, 1991
BR26	blast res.	Apura	12	Yu ZY, 1991
Hbv	hoja bl.res.	Fanny	12	Tohme, et al., 1991
Wph-1	WBPH res.	N-22	7	McCouch/Tanksley, 1991
Wph-2	WBPH res.	ARC10239	4	Chung, et al., 1993
Bph-?	BPH res.	*O. austral.*	12	Ishii, et al., 1993a
Gm-2	gallmidge res.	Phalguna	4	Mohan, et al., 1993
sd-1	semidwarf	TN-1	1	Yu ZY, 1991
sd-g	semidwarf	Guiyangai	5	Zhu, et al., 1993
sd-?	semidwarf	Ar-10	4	Zhuang, et al., 1993
Se-1	photoperiod	Puang Rai2	6	MacKill, et al., 1993
fgr.	fragrance	Basmati 370	8	Ahn, et al., 1992
cooked-kernel elongation		Basmati 370	8	Ahn, et al., 1993

Disease and insect resistance portion from McCouch, 1992

Both qualitative race-specific and quantitative race non-specific genes for blast resistance have been tagged (Yu, et al., 1991; Tohme, et al., 1991; Zhu, et al., 1993). Using these markers, a traditional rice upland variety having durable resistance to blast was shown to have genes for complete (qualitative) and partial (quantitative) blast resistance (Wang, et al., 1993). Three of the markers associated with quantitative effects in this variety had previously been shown to be linked to qualitative blast resistance genes in other cultivars. These results suggest that genes controlling quantitative resistance may be identical to or allelic with those conferring qualitative resistance in other genetic backgrounds and/or in response to different isolates of the pathogen (Wang, et al., 1993).

For a few quantitative traits of rice that are relatively easy to score, such as cooked-grain elongation, markers which detect QTLs have been identified (Ahn, et al., 1993). For more complex traits, like drought tolerance, crosses which give significant genetic variation for component traits (e.g., osmotic adjustment, epidermal conductance, root/shoot ratio, root length, root thickness, root penetration) are being used for QTL analysis (Lilley and Ludlow, 1993; Yu, et al., 1993, Champoux, et al., 1992).

Two large research projects are under way in China using markers to better understand the genetic basis of heterosis in rice and to more effectively exploit it in hybrid rice production (Gao, et al., 1993; McCouch, 1992). By using markers to select parental lines which give excellent hybrid performance and genetic engineering to introduce male sterility/restorer gene systems into these lines, hybrid rice has the potential to provide for significant increases in maximum yield potential across most rice producing regions.

Continuing collaborative research at Cornell and IRRI is aimed at developing rice molecular genetic mapping technology into a practical breeding tool. As an example, a Thai graduate student at Cornell has developed a simple and rapid PCR-based method for determining the genotype of seeds before germination and without DNA isolation (Chunwongse, et al., 1993). Single half-seeds of rice are preincubated, without grinding, in an aqueous extraction buffer. When the supernatants are used for PCR reactions with primers for rice single copy sequences, the products are of identical size to the PCR products produced from DNA isolated from leaf tissue. Since there is F_2 segregation, the amplified DNA must be from endosperm and not maternal tissues. Seedlings derived from the remnant half-seeds grow vigorously and survive transfer to soil. Japanese scientists have also recently reported a rapid and simple method for PCR-based marker analysis using crude extracts from rice leaves (Yoshimura, et al., 1992a).

The linkage maps of *O. sativa* may also have applications beyond rice. Comparative mapping of cDNA clones with maize and rice, and wheat and rice demonstrate conservation of linkage relationships across substantial regions of chromosomes in these distantly related monocot genera (Ahn and Tanksley 1993; McCouch, et al., 1993). In some instances entire chromosomes or chromosome arms are nearly identical with respect to gene order and gene content. Interestingly, greater than 72% of the single copy loci in rice are duplicated in maize supporting the theory of ancestral polyploidization in maize. This work suggests that it may be possible to develop a common reservoir of markers and linkage information for several of the important Gramineae crop species and to gain new insights through comparison.

DNA ANALYSIS OF PESTS AND PATHOGENS

Molecular genetic maps and markers can also be used to analyze and monitor genetic diversity and variability in populations of insect pests and pathogens of rice, thereby facilitating more effective use and deployment of resistance genes. Repetitive DNA elements are especially well suited for generating informative markers because they allow analysis of a large portion of the genome with one probe (Hamer, et al.,1989)

The Asian rice gallmidge, *Orseolia oryzae*, is a major pest of rice. In India there are at least four biotypes of gall midge which differ in host range depending upon the resistance genes present in the rice cultivar. Ehteshan et al. (1993) selected a DNA clone from a genomic library of gall midge which generated DNA fingerprints that were biotype-specific. The DNA extracted from a single insect was sufficient for making biotype determinations. This combined with rice RFLP probes tagged to genes for resistance to specific biotypes should allow breeders to use resistance genes when and where they will be most effective.

For the rice blast fungus, *Magnaporthe grisea*, RFLPs generated by DNA probes for dispersed repeated sequences have provided a new fingerprinting mechanism for understanding population dynamics of the fungus and variation in virulence (Hamer, 1991). Based on such DNA fingerprinting, blast pathogen populations can be organized into a discrete number of families with similar genetic backgrounds, referred to as lineages. In the USA the rice blast population was shown to be composed of eight well differentiated genetic lineages with defined pathotype associations (Levy, et al., 1991). Isolates of the same pathotype, collected from different areas at different times over a 30-year period had diagnostically similar DNA fingerprints. Thus, the major U.S. pathotypes were composed of specific clonal lineages identifiable by consensus DNA fingerprints. The probe was not only suitable for identifying pathotypes and distinguishing various isolates, but could also

be used to assess the phylogenetic relationships within and between various pathotype groups.

Using the same probe, Levy, et al. (1993) conducted fingerprinting analysis for nearly 1,000 field isolates of the blast fungus collected from over a dozen countries throughout the world. They concluded the following: 1) the blast pathogen populations in each country are clonal and composed of a small number (6-18) of lineages; 2) where pathogenicity testing was available, each lineage was associated with one to several closely related pathotypes, or had a limited cultivar range, and these relationships are generalized over several years; 3) rice pathogens and grass pathogens are generally host-limited and easily distinguished by DNA fingerprints; and 4) globally, the majority of lineages appear to be indigenous, but there are at least four lineages that are widespread in the Americas and at least two other lineages are present both in Asia and the Americas, suggesting human-assisted migration. These findings have led to the strengthening of hygienic treatments and protocols used to assure that rice seed stocks received from other countries are free of pathogens.

The relationships between lineage, pathotype, and cultivar are now being examined in greater detail at several locations with the aim of developing specific schemes for durable resistance breeding which emphasize lineage-exclusion strategies. Lineage analysis is already being used for evaluating the diversity of pathogen populations in blast screening nurseries. In the Philippines, for example, only five lineages were found at the IRRI blast nursery, while twelve lineages were detected at an upland site elsewhere in the country (Leung, et al., 1993).

Similar research on the use of DNA probes to analyze population genetic diversity and variability is being conducted on the brown planthopper, and the pathogens of bacterial blight, bacterial leaf streak, tungro, and other rice diseases.

RICE GENETIC ENGINEERING

Transgenic cereal plants were first obtained in rice using protoplast-based transformation systems (Toriyama, et al., 1988; Zhang and Wu, 1988; Yang, et al., 1988; Peng, et al., 1992). In these systems uptake of DNA by protoplasts is usually mediated by treatments that increase the permeability of the cell membrane and regeneration is dependent upon delicate manipulations of both protoplasts and embryogenic cell suspension cultures. While efficiencies remain low and many of the resulting transgenic plants are pollen sterile, these techniques have provided the basis for the first field tests of transgenic cereals and for production of numerous transgenic rice plants containing agronomically useful alien genes (Datta,

et al., 1992,; Hayakawa, et al., 1992; Rathore, et al., 1993). Protoplast uptake and regeneration continues to be the most widely employed and one of the most reliable techniques for rice transformation.

Biolistic techniques are now also used for rice transformation with promising results, particularly since they appear to be genotype independent. Christou, et al. (1991) subjected 12 - 15-day old rice immature embryos to electric discharge, particle-mediated transformation with a selectable marker gene. When placed on selective media, the embryos produced chimeric embryogenic callus having both transformed and non-transformed sectors. From this callus, fully transformed plants, transformed tissues (e.g.,roots and shoots), and non-transformed plants were obtained. Germ-line transformation frequency was 1 - 2 % with 45% of the transgenic plants containing a single copy of the marker gene which segregates in a Mendelian fashion (Christou, 1992).

Cao, et al. (1992) used a helium gas particle gun to transform cells in an embryogenic suspension culture with a selectable marker gene. Transformed calli could be selected and regenerated into plants.

More recently, Li, et al., 1993, used a homemade particle gun similar to the one described by Klein, et al., 1987, to bombard rice immature embryos and pieces of calli. On selection medium, the explants initially turn brown. However, within two weeks white cell clusters formed on the surface of the bombarded tissue. These cell clusters were transferred to selection medium and then regeneration medium. Approximately 30% could be regenerated into plants which were shown to be transgenic by Southern blot hybridization reactions. There was Mendelian inheritance of the marker gene. This protocol has since been used to produce transgenic rice plants containing various gene constructs for resistance to rice tungro virus which are being evaluated at IRRI (Fauquet and Beachy, 1993). The details of this transformation protocol have been published (Li, et al., 1993) and it has been successfully transferred to other laboratories (Li Lingcai, pers. comm.)

Other techniques for the production of transgenic rice have been reported. Dekeyser, et al., (1990) developed a procedure to electroporate DNA into intact and organized rice tissues and reported transient gene expression. This technique has since been refined to give stable transformation of rice and other crops (Marc Van Montagu, pers. comm.).

Agrobacterium is able to transfer its T-DNA into rice as measured by Agroinfection (Nester, et al., 1993). Moreover, high level transient expression of marker genes in rice has been reported using *Agrobacterium* vectors that do not express in bacteria (Li, et al., 1992). Other reports of *Agrobacterium*-mediated transformation of rice calli have appeared (Raineri, et

al., 1990; Chan, et al., 1992), but convincing data of stably transformed rice plants via *Agrobacterium* has yet to be presented. Like the earlier reports of success using pollen tube methods (Luo and Wu, 1988), *Agrobacterium*-based methods appear to be plagued by false positives. Data demonstrating incorporation of vector DNA into high molecular weight rice DNA as well as data demonstrating stability and Mendelian segregation of the alien genes is needed before any of these techniques can be considered reliable. To date, such data has been published only for protoplast-based and biolistic procedures.

Using these two reliable techniques, chimeric gene constructs can now be integrated into the nuclear DNA of rice plants and passed on to subsequent generations as part of the rice genome. The added gene may encode a new protein or it may alter the level or location of expression, or both, of existing proteins. The site of integration appear to be random. Techniques for targeting genes to a particular site on the rice genome, or for replacing an existing gene with an engineered alternative are being developed, but are not yet available.

The coding sequence of these chimeric genes can come from any source - rice, wild relatives of rice, other plants, microbes, animals, or chemical synthesis. In the case of nonplant coding sequences, it may enhance and stabilize expression of the foreign gene if codons that do not occur often in plants are substituted with an equivalent codon commonly used by monocot plants during translation of mRNA to protein (Huang, et al., 1990). The regulatory sequences will need to function in rice and often will come from rice. Many of the more sophisticated and powerful uses of genetic engineering will involve highly regulated genes that are expressed at desired levels in particular cells, tissues, or organs at particular stages of development, or in response to particular environmental stimuli. To be useful, these added genes must instill new traits in elite rice lines that further contribute to one or more of the following broad breeding objectives: enhanced yield potential, pest resistance, abiotic stress tolerance, improved product quality, and lowering of production costs. Table 2 lists examples of the types of genes currently being investigated and constructed for transfer to rice. Transgenic plants have been produced with several (e.g., viral coat protein genes, herbicide resistance genes, *B.t.* endotoxin genes, male sterility genes and modified storage protein genes) and are now being evaluated. Within a few years, transgenic rice plants with useful alien genes should provide rice breeders with a new and valuable source of genetic variability.

Table 2

Target Traits/Genes for Genetic Engineering of Rice

Enhanced yield potential
- Male sterility/restorer gene systems
- Enhanced starch biosynthesis
- Dwarfism
- Early maturity
- Apomixis

Insect resistance
- Modified *Bacillus thuringiensis* endotoxin genes
- Inhibitors of insect digestive enzymes
- Lectin genes
- Chitinase genes

Disease resistance
- Coat protein and other viral genes
- Chitinase genes
- Glucanase genes
- Lectin genes
- Antifungal/antibacterial protein genes
- Enhanced biosynthesis of phytoalexins
- Enhanced lignin biosynthesis

Abiotic stress tolerance
- Proline biosynthetic genes
- Betaine biosynthetic genes
- Osmotin genes
- Enhanced root vigor
- Submergence tolerance/anaerobic energy production

Improved product quality
- Modified starch biosynthesis
- More nutritious/digestible storage proteins
- Carotenoid biosynthesis in endosperm

Lower production costs
- Herbicide resistance
- Improved nitrogen use efficiency

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ADVANCES IN OILSEED RAPE USING BIOTECHNOLOGY

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ABSTRACT

Oilseed rape breeding has been subject over recent years to considerable outside influences. These include such diverse factors as demands for improved product quality such as low glucosinolate rapeseed meal and the availibility of radically different breeding technologies such as genetic transformation. Many new products derived from oilseed rape can now be postulated partly in consequence of the growing knowledge of the genetic control of key biosynthetic pathways but largely because DNA engineering and genetic transformation now offer a practical way forward. Genetic modification of oilseed rape is particularly attractive for developing novel products and improving the quality of existing ones. It may also play a role in the development of varieties with improved agronomic performance where low input cropping systems may be encouraged by the introduction of new pest and disease resistances. Enthusiasm for developments in this field of breeding must, however, be tempered by the realities of incorporation into a full breeding programme. The necessary regulations which govern testing of this material not only add costs to the development of varieties but also may delay the selection and breeding process. In addition it is likely that the release of genetically modified varieties of oilseed rape will be further delayed by the need to breed any new trait into an acceptable background.

RFLP and related DNA marker technology represents the other major new development in breeding. Application to oilseed rape means that for the first time in the crop's history linkage maps of the genome are now being produced. Ultimately molecular markers offer opportunities for the routine early screening of segregating material reducing the need for expensive and lengthy field testing. The extent to which markers will be used in the future will depend upon the reliability of the correlations between marker genotype and trait phenotype and the unit costs of the DNA assessments. The role of the new molecular technologies in oilseed rape may become substantial but the techniques should be kept in context and viewed as new tools for the breeder to use alongside traditional methods.

INTRODUCTION

In contrast to most arable crops, oilseed rape (*Brassica napus* L.) has a relatively short history of widespread intensive cultivation. In many ways this crop has proved itself to be one of the most malleable of species and is an ideal crop to benefit from the advances in molecular genetics. With EC support the use of oilseed rape expanded greatly during the 1970s and with the introduction of low erucic acid varieties around 1976 this expansion

continued well into the 1980s. The introduction of varieties with reduced glucosinolate quantities (to below 25μ g/ml), allowed more meal to be used in animal feed compounds and hence increased the total crop value. Current world production is in excess of 24 M tonnes, most of which is crushed for use in blended vegetable oil or hydrogenated for margarine manufacture. In recent years there has been an increasing industrial demand for rapeseed oil (principally for high erucic acid types), where it is used as a rubber additive and for high temperature lubricants.

The development of varieties to date has almost entirely exploited existing variation within the *B. napus* species, using this to improve the crop by conventional crossing and selection techniques. Advances in chemical analysis (for fatty acid composition and glucosinolate content) allowed breeders to identify and exploit low erucic and (later) low glucosinolate lines. More recently desirable traits have been identified and introgressed into *B. napus* from *B. oleracea* L. or *B. rapa* L. by making synthetic *B. napus* (= *B. oleracea* x *B. rapa*, followed by chromosome doubling). In common with many other crops mutagenesis has been used as a tool to create novel variation with fatty acid composition as a popular target. The desired change has often been achieved but, unfortunately, the resulting associated mutational damage is generally quite considerable and requires several breeding cycles to remove.

In its broad sense biotechnology encompasses developments such as improved analytical instrumentation and genetic modification by mutagenesis. Tissue culture is a further example in oilseed rape of a new technology which has become established as part of most breeding programmes. Developments in molecular technologies such as genetic transformation and genetic marker techniques are relatively new and in this paper I shall attempt to outline how these may contribute to the oilseed rape crop.

Variety development is influenced by breeding objectives (product development and agronomic performance) and by the techniques available to the breeder to achieve these (Fig 1). Conventional breeding is central to this structure since the breeding programme will be based upon the essential breeding ingredients of crossing and selection.

BREEDING OBJECTIVES

Agronomy objectives

Traditionally the most important of the breeding objectives is the agronomic performance and foremost of the agronomic traits is seed yield. This is as true as ever with the qualification that reduced subsidies (particularly in the EC) has moved the demand for high yielding varieties *per se* to a demand for high yielding varieties using lower inputs. The balance is shifting particularly in favour of varieties with improved pest and disease resistance, a trend which is likely to continue into the future. The identification and isolation of several candidate pest resistant genes (e.g. trypsin inhibitors and chitinases), together with the ability to introduce these into oilseed rape by genetic transformation, makes this one of the most exciting new developments in the breeding of this crop. Reliability of a variety under a wide range of environmental conditions is an important consideration, therefore, good standing ability is a vital characteristic of a sound variety. Herbicide tolerance of oilseed rape varieties

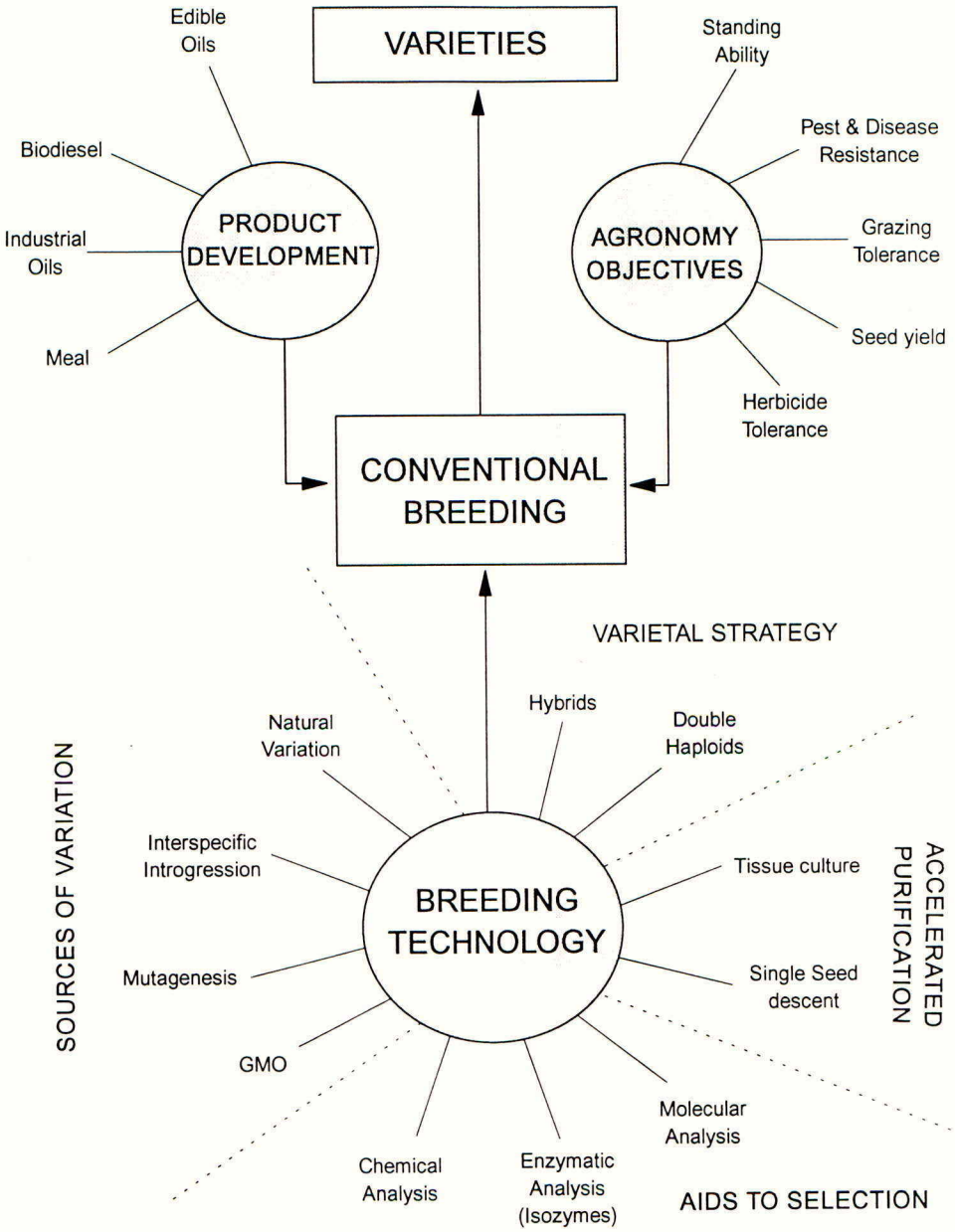

Figure 1.
Diagramatic representation of the major factors influencing variety development in oilseed rape.

may prove to present unacceptable environmental problems but the successful introduction of this characteristic could expand the agricultural range of oilseed rape perhaps enabling rape to be used in rotations where it is currently inappropriate. More interesting, if more speculative, is the prospect of producing varieties of rape distasteful to grazing pests, particularly pigeons (Magrath *et al* , 1993).

Product objectives

Yield, pest tolerance and disease resistance have always been top of breeders' priorities but increasingly in oilseed rape there is a need to be aware of potential product developments. The main market is likely to always be for edible oils but the development of varieties will be directed more specifically at the two main uses, namely, frying and margarines. Frying oils ideally require a long shelf life and good stability at high temperatures, both these properties would be improved by a reduction of the proportion of linolenic acid in the oil from the current level of 8-11% shown by most varieties. Spring oilseed rape varieties have been introduced which have levels of linolenic acid down to 2% (Eskin, 1988) but these varieties have had limited success owing to their poor agronomic performance. The margarine industry may also be happy to see a lower proportion of linolenic acids in rape oil but would also like to work with an oil that is higher in saturated fatty acids, preferably palmitic acid. This would enable a greater proportion of rapeseed oil to be used in blended margarines. From a public health point of view this could be highly desirable since rapeseed oil is extremely high in monounsaturated fatty acid (rapeseed oil contains approximately 60% oleic acid). Looking further into the future research may show that the health issues depend upon specific triglyceride structures and not simply on total fatty acid compositions. However, any developments in breeding specifically for 'healthy eating' will need to be driven by a detailed nutritional understanding of the role of oils in the onset of conditions such as heart disease.

Industrial use of rapeseed oil concentrates on the utilisation of high erucic acid varieties. Current varieties deliver about 45% of the total oil as erucic acid but many industrial uses would benefit from an oil as close to pure erucic acid as possible. Unfortunately the construction of the triglyceride molecule in oilseed rape currently limits the long chain ($C_{22:1}$) to positions 1 and 3 with the shorter C_{18} fatty acids occupying position 2. This leads to a theoretical upper limit of 67% for the proportion of erucic acid. Other species such as *Limnanthes alba* Benth. are not subject to this limitation and may provide an enzyme system that could be used to drive erucic acid levels beyond the current limit (Hills & Murphy, 1991). Targets for basic research include the production of lines able to produce usable amounts of specific fatty acids such as racinoleic acid, petroselenic acid or lauric acid. These could supply a wide range of industrial uses as lubricants, plasticisers, ingredients in cosmetics and pharmaceuticals and as precursors for plastics and detergents.

Rapeseed oil can be methylated to produce rape methyl esters (RME) popularly known as biodiesel. One of the disadvantages that has been identified is that the freezing point of this biodiesel is a little higher than for conventional diesel which means cold starts in a hard frost can be a problem. Breeders may be able to solve the problem by producing lines with a modified fatty acid composition where the main concern is the physical property of freezing point/viscosity.

Rapeseed meal (the cake left after crushing and oil extraction) is a high protein feed currently used in cattle, pig and broiler poultry compounds. There are limits to the proportion

that can be incorporated owing to poor palatability to ruminants (caused by glucosinolates) and to 'fishy' flavoured eggs when it is fed to laying hens. Glucosinolates are defensive compounds produced by the plant to provide protection against grazing pests and also to some diseases. This leads to a conflict for the breeder. The current trend towards varieties with progressively lower glucosinolates is certainly desirable for the feed industry but may be leaving crops more susceptible to grazing and diseases. Ideally varieties should possess leaf glucosinolates capable of conferring useful protection against grazing pests and zero (or very low) levels of glucosinolates in the seed. Recent research has suggested that only some of the glucosinolates are a problem in the meal and a serious objective for breeders is to attempt to manipulate the composition and not simply the total level of glucosinolates (Magrath *et al*, 1993a). Further improvements to rapeseed meal could be gained if the fibre content and concentration of tannins could be reduced. This has led to efforts to introduce the yellow seed trait from related Brassica species.

BREEDING TECHNOLOGY

That we can now consider achieving these objectives is itself a testimony to the strides made in technological developments. There are four major areas in which improved technology can assist the breeder:

1. Expanding the source of genetic variation.
2. Improvements in analytical tools to aid selection.
3. Development of tissue culture to accelerate breeding programmes.
4. Development of alternative seed production strategies for varieties.

The source of genetic variation

'Traditional' sources of variation, including interspecific introgression and mutagenesis, can now be greatly increased by the ability to transform oilseed rape with alien genes from virtually any source. The only constraints are the technical limits to the ability to identify and isolate genes of known function and, since (in the UK) such products fall within the scope of the Genetically Modified Organisms (GMO) Act 1992, the requirement to produce a modification which will be environmentally acceptable and safe to use. In practice most examples of transgenic oilseed rape currently in the public domain have involved modifications aimed at improving agronomic performance such as the introduction of insect, disease or herbicide resistance. These developments are generally aimed at producing varieties which will be better able to give a reliable performance in the absence of costly (both in monetry and environmental terms) chemical treatments and sprays. The development of male sterility systems (aimed at hybrid production) by genetic modification is a further compelling reason to take this technology seriously. Genetic transformation could prove of particular value, however, in the area of product development. Manipulation of the biochemical pathways involved in fatty acid synthesis is one obvious target which is currently being investigated by several groups worldwide. In general the power of this technology is that, in principle, a modification need only be made once since conventional breeding can subsequently be used to transfer the new trait throughout the gene pool of the crop. The

payback period is, therefore, very long which hopefully will lead to the successful fruition of this young technology which necessarily involves very high development costs.

Analytical aids

Selection is the essence of plant breeding and when breeding for quality traits, chemical analysis has been part and parcel of the breeders tool kit. Modern instrumentation has almost eliminated wet chemical analysis from the laboratory and has enabled the screening of thousands of samples each year. Many of these tests, such as X-ray Fluoresence (XRF), measure an indicator character (in the case of oilseed rape usually sulphur content) which is highly correlated to the trait required for selection (glucosinolate content). Selection of lines based on the indicator character leads in turn to the breeding of varieties with improved genotypes. DNA markers are now developed to the stage where we can begin to test alternative selection methods which reduce the need to measure phenotypes directly. The principle is to replace a phenotypic measure (such as glucosinolate content) with an assessment of marker genotypes known to be linked to the gene(s) responsible for the selection trait. RFLP (Restriction Fragment Length Polymorphism) maps are now being produced for oilseed rape (Parkin *et al*, 1993) and the genes responsible for various agronomic and quality traits are beginning to be mapped. The advantage of the technique is that mapped traits can be screened and selected, using the molecular markers and a few grammes of tissue from each plant (or line), eliminating the need for expensive field measurements of phenotype. In practice such marker assisted selection would be best carried out in the early generations whilst retaining conventional trialling and phenotype assessment in the more advanced breeding material.

The supply of markers is rapidly increasing with the development of RFLPs, RAPDs (Random Amplified Polymorphic DNA) and more recently AFLPs (Amplified Fragment Length Polymorphisms). Two important aspects need to be overcome to permit marker techniques to be used on a large scale in breeding programmes. Firstly, reliable correlations between marker genotypes and the desired phenotype need to be established. This will only be achieved as a result of large scale trials. Secondly, faster, cheaper DNA techniques need to be developed to permit large numbers of individuals to be screened; RFLP conversion to a PCR (polymerase chain reaction) primer is one of the options available.

Tissue culture

Tissue culture has been available to oilseed rape breeders for 10-15 years and is now well established particularly in the routine production of haploid plants by pollen microspore culture (Chuong & Beversdorf, 1985). Nevertheless, improvements remain to be made since even now many varieties prove resistant to culture techniques and are, therefore, excluded from programmes involving tissue culture. Recent work with genetic transformation of oilseed rape has encountered the same problem at the tissue culture phase with some genotypes proving resistant to the best efforts to induce regeneration of transformed tissue.

Varietal strategy

All the above technological advances in oilseed rape have been applied, so far, to the production of pure line varieties (either self pollinating pedigree lines at F_7 onwards or doubled haploid lines) or synthetics. The immediate future of oilseed rape is, however, likely

to be dominated by hybrid varieties. Various systems exist and may be used commercially. These have been developed by several different technologies, (Table 1). Regardless of the system which will eventually prove to be best commercially the problem of finding the optimum parental combinations is common to all hybrid production. As with other hybrid crops, such as maize, predictive methods which can be used to reduce the number of parental combinations to a manageable level will be extremely valuable. Molecular markers may prove cost effective in this role, initially to estimate genetic distances, (and hence, by inference, heterosis) and later, as mapping data improves, attempts may be made to match parents for complementary gene action in regions of known importance for the trait(s) being considered.

TABLE 1. Summary of methods used to develop the major hybrid systems available in oilseed rape.

Hybrid System	Genetic Source	Techniques Used
Cytoplasmic male sterility		
- Polima	*B. napus*	Conventional only
- Ogura	*Raphanus sativus* Vilm.	Protoplast fusion
	B. napus	+ Interspecific introgression
Nuclear male sterility		
- PGS	Engineered gene	Transformation
Self incompatibility	*B. oleracea*	Interspecific introgression
	B. rapa	

The Integration of Molecular Technology into Oilseed Rape Breeding

At Cambridge Plant Breeders our view of oilseed rape breeding retains conventional techniques (crossing, selfing and visual/randomised trial selection) as the central element for any full programme. Fig 1, summarises the more important elements that the present day breeder must consider. The advent of oilseed rape transformation has enabled breeders to broaden their horizons and see practical solutions to the development of new products and improved crop protection. It is important, however, to put this advance into perspective. At best a new trait may be introduced into *B.napus* which by further conventional breeding will lead to a commercial variety but, in all probability, this will be no sooner than nine years after the original transformation. Early release (after 4-5 years) of the original transformant line may be possible for a novel product, but otherwise the recipient variety will probably be superceded, on agronomic grounds, before the GMO version could reach the market place. Furthermore, this technology is subject to environmental scrutiny and safety checking

governed by EC regulations. Whilst not stopping work in this area these factors increase the early costs in an already expensive field. Clearly in such a climate breeding targets need to be well focused and, therefore, in the foreseeable future GMOs are not likely to comprise more than a small proportion of most breeders' elite material.

Molecular markers on the other hand are here now and, in one form or another, are likely to become a routine screening tool in oilseed rape breeding. In contrast to GMOs, marker techniques will be applied to large numbers and, ultimately, perhaps all lines in a breeding programme will at some stage be screened using molecular markers. The gains per test are likely to be relatively small but immediate. Ideally the technology will be used to impose selection some months earlier than would be possible conventionally. The scale on which this technology will be used will depend on two factors: the cost per marker per individual (still coming down) and the reliability of correlations between marker genotypes and the trait phenotype. In common with GMOs the great attraction is that once the early development work is done the rewards will be extended over a very long period.

If we look forward to the next generation of oilseed rape varieties what might we expect? Hybrid varieties are already available in China and during 1993 are likely to be on sale to growers in Canada. In Europe within the next 2-4 years, we will see competitive hybrid varieties appearing in national trials and it seems likely that by the turn of the century hybrids will dominate the world market in oilseed rape varieties. These varieties will continue to reduce in total glucosinolates (in the main simply in response to conventional selection) and we are likely to see the introduction of varieties with specific glucosinolate compositions providing meal with improved feed quality. Oil quality is likely to improve as the low linolenic acid character is bred into a wider range of varieties. Each of these developments owes its origins to basic research carried out 10-20 years ago, the equivalent of one to two breeding cycles. In this respect the molecular technology offers no panacea and we must expect comparable time periods to bring new products to the market place. The real value of the recent molecular developments is that new opportunities now exist which breeders can add to their repertoire of techniques to achieve new goals.

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PROSPECTS FOR THE MODIFICATION OF METABOLISM.

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ABSTRACT

Modifying metabolism to improve the quality of plant produce is a feasible but not simple goal. In most cases the goal requires an understanding of plant metabolism that is, at present, lacking. However, the modern tools of genetic modification combined with the modern methods of biochemical analysis make it possible to improve crops and further improve our understanding of metabolism. The scientist is now well equipped to continually adapt the crop to the consumer.

BACKGROUND

The substantial advances and refinements in the techniques of molecular biology have allowed the problems of crop productivity and quality to be approached in new ways. One of the factors which limits crop productivity is the efficiency with which the plant stores carbon and nutrients in a form of use to man. Another major influence on crop productivity will be the ability of the plant to withstand damage by pests and pathogens. For example, in the case of the cereal grain or the potato tuber the yield will be affected by the amount of carbon translocated to the storage organ. The proportion of carbon translocated has a finite limit since, ultimately, if too much is translocated, there will be insufficient to develop leaves. In turn this will limit the amount of carbon available for transport. The challenge, therefore, is to define the extent to which the balance can be shifted towards increasing yield without decreasing potential yield. Similarly the amount of carbon in the storage organ available for storage is balanced by the amount needed for growth and maintenance of life. The amount of carbon stored as starch, protein and oils will greatly affect the quality of the harvested crop.

Molecular tools allow all these aspects of crop production to be approached. However, there may be a large difference between the modifications required for yield and quality compared with pest and disease resistance. To modify yield and quality requires an alteration in the balance of metabolism, the proportion of carbon that flows down one pathway compared to another. Improving pest and disease resistance can be achieved by adding something novel to the plant. Therefore, to alter quality, the regulation of biochemical pathways needs to be adjusted, not changed drastically, since too great a change may be as detrimental as no change.

To modify the flux of carbon through a pathway requires one to identify the most important enzyme in the pathway, ie. those enzymes

that are important in limiting the flow of carbon through the pathway. Classically plant biochemists have used three types of information to identify enzymes involved in regulation of a pathway, the properties of the enzyme, the changes in the amount of metabolic intermediates that occur when the environment is altered, and comparative biology. Of these three, the first, the properties of the enzyme has tended to dominate the literature because it is the most tangible. Thus if the activity of the enzyme can be regulated by allosteric effectors or other proteins, the enzyme can catalyse an irreversible reaction and it is at an early step in a pathway biochemists have favoured the step as a point of control. These arguments however confuse the regulation of an enzyme activity with the regulation of the flux through the pathway. The flux across a step in a pathway may be affected by the activity of the enzyme, the availability of substrate for the reaction and the rate at which product is used. Thus, in the extreme, if there is no enzyme or no substrate the step is unlikely to proceed.

While enzymes catalysing irreversible reactions are often favoured as points that regulate flux through a pathway there is no *a priori* reason why the regulation of an enzyme catalysing a reversible reaction could not regulate the flux through a pathway. When the enzyme activity is insufficient to use the substrate at the rate of supply then the enzyme will limit flux. Therefore the particular environmental conditions, the rate of supply and use, could easily alter the influence of any one enzyme on the regulation of the overall pathway. Consequently comparative biology may be used as pointers but should not be used as justification because when the conditions change, the balance changes and, therefore, different steps may assume importance. Kacser (1989) has, since the early 1970s developed a theory for metabolic control which seeks to evaluate the steps in a pathway and rank them in importance in terms of the control of flux through the pathway. For this theory to be applied it is necessary to cause a single change in a step of the pathway and measure the consequences. Genetic modification is a suitable mechanism to do this because it can be precise although usually the changes in enzyme activity that are obtained are larger than ideal. However, because one genetic modification (GM) experiment will provide many progeny with the amounts of enzyme altered to varying extents the GM experiment provides both the means to develop the ranking and the means to test the validity of it over a range of conditions.

STRATEGIES

The synthesis of sucrose and starch and the use of these compounds to provide energy for growth are central to the quality of many crops. We have examined the regulation of these pathways in the potato tuber.

Glycolysis.

Phosphofructokinase (PFK) catalyses the conversion of fructose-6-phosphate (Fru-6-P) to fructose-1,6,-bisphosphate and is generally regarded as the enzyme that regulates the entry of carbon and, therefore, the availability of carbon for respiration. We have introduced and expressed in potato tubers the enzyme of the *E. coli PFKA* gene and altered the enzyme activity upto 20 fold. The genetic modification did not cause any alteration in any of the other enzymes of glycolysis. The effect of the introducted PFK was to cause

a small decrease in the amount of Fru-6-P and other intermediates that are before the enzyme in glycolysis and cause some substantial increases in the amounts of intermediates that occur after PFK in the pathway of glycolysis (Fig. 1). However neither in mature tubers nor in aged discs of tubers has the genetic modification of the amount of PFK caused any change in the rate of respiration. The process of aging discs causes an increase in respiration. Therefore, under two different environmental conditions despite the changes in amount of intermediates we have not detected any change in the flux of carbon through glycolysis.

We have applied metabolic control theory to our results and this indicates that under the conditions of assay at least three other enzymes exert more control over the flux through the pathway than PFK. Although our measurements are of necessity at this stage imperfect, it is clear that the classical view that PFK is of overriding importance in the regulation of glycolysis is no longer tenable for the potato tuber. However out of this experiment we now have the information which allows us to predict which enzymes need to be altered and by how much to alter the flux of carbon through glycolysis.

Starch synthesis.

We have used a similar approach to examine the regulation of starch biosynthesis. It has been argued (Preiss 1988), based on the properties of the enzyme, that Adenosine diphosphoglucose pyrophosphorylase (ADPG PPase) is of overriding importance in regulating the synthesis of starch. However, Neuhaus and Stitt, (1990) studied the regulation of starch synthesis in mutants of *Arabidopsis thaliana*. Under conditions of low and high light intensity only 28 and 65% of the flux control could be attributed to ADPG PPase. We have introduced and expressed in potato tubers the *E. coli GlgC* gene which encodes ADPG PPase and a mutant form of the enzyme $GlgC^{16}$ which is more sensitive to certain allosteric effectors and is therefore likely to be more active per unit of protein *in vivo*. It is clear from our results that under some conditions an increase in enzyme activity is not associated with an increase in amount of starch while under other conditions there is an association. It is likely that ADPG PPase is not the only enzyme to limit starch biosynthesis.

CONCLUSION

It is quite clear that with genetic modification it is possible to alter the balance of metabolism. The power of the technology is that with the correct use of the available biochemical tools metabolism can be finely tuned to the requirements of the market place.

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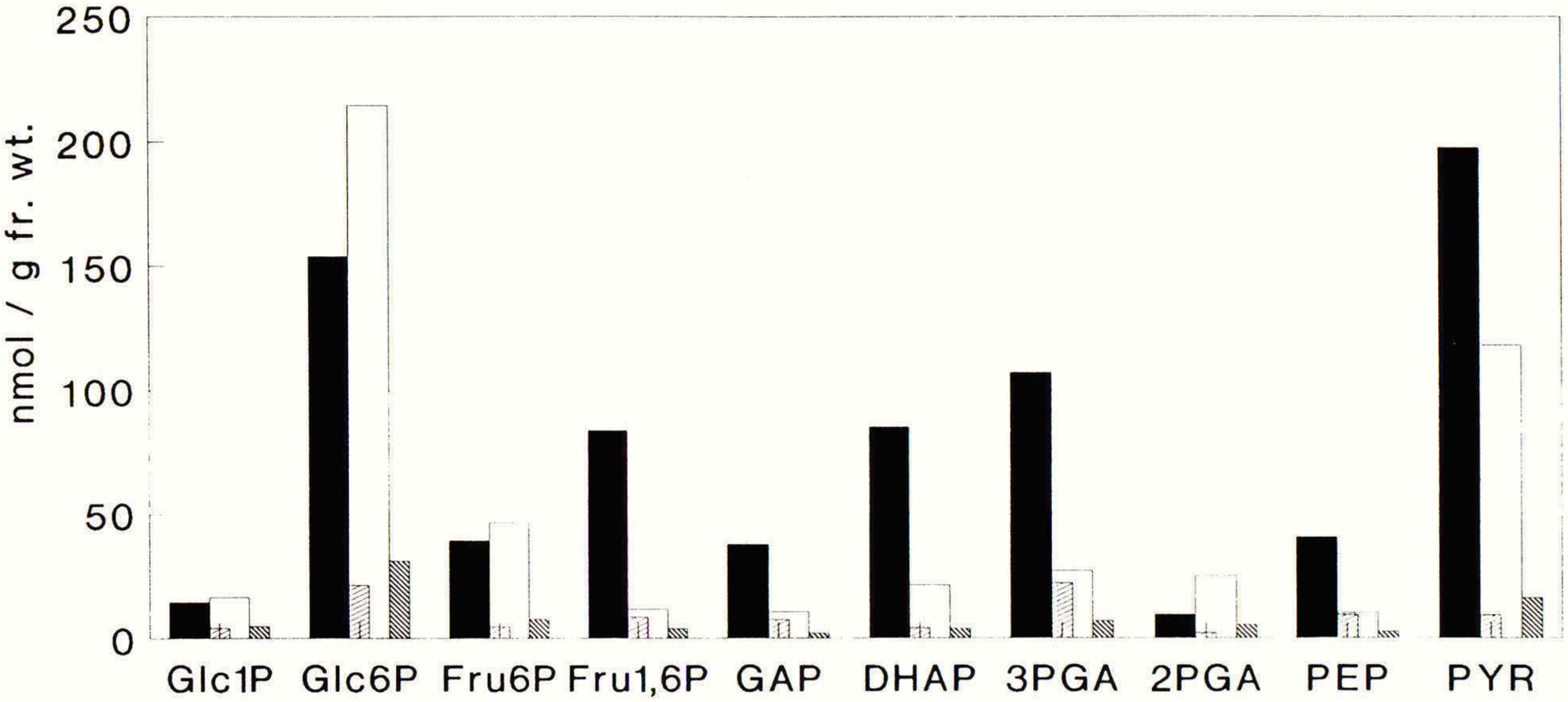
Glycolytic intermediates in aged discs of transgenic potatoes



Intermediates are shown for transgenic plants expressing PFK and controls (GUS). The intermediates are in the order of glycolysis. PFK catalyses the reaction Fru6P to Fru1,6P.

NEW APPROACHES TO SUGAR BEET IMPROVEMENT

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ABSTRACT

Elite sugar beet varieties may yield 83t of storage roots/ha which provide 15t of sucrose. Such varieties routinely accumulate sucrose to fresh weight concentrations of 18% which equate with dry weight concentrations of 75%. These remarkable statistics do not, however, identify the crop as one which is incapable of improvement. The wedge shaped storage roots have their crowns at the soil surface. During mechanical harvesting, large amounts of fertile soil are removed from the field and subsequently water is wasted when the crop is washed and energy is wasted in transporting soil to and from the factory and in sterilising the soil so as to minimise the spread of soil-borne diseases. Gene manipulation to create new elite varieties with globe shaped storage roots which penetrate the soil less deeply will reduce the soil tare and, hence, the environmental impact of sugar beet growth.

INTRODUCTION

Sugar beet (*Beta vulgaris*) is a major temperate zone crop which provides almost 40% of the world's sucrose. In the EC alone some 2.5 million hectares of farmland are dedicated to the sugar beet crop each year and sucrose production in the Community is valued at £6.3 billion ($9.5 billion) annually. The very high yields of storage roots (83t/ha) and sucrose (15t/ha) do not reflect a high thermodynamic efficiency of crop growth. For the sugar beet crop, as for other major crops, high yields per ha have been confused with high "agricultural efficiency". The lack of commitment to efficiency in the real thermodynamic sense means that *inter alia* there is a high environmental impact of crop growth. Reduction of this environmental impact is now an urgent priority.

Elite sugar beet plants have wedge shaped storage roots which have their crowns at the soil surface. When the storage roots are mechanically harvested considerable amounts of soil are removed from the field. Within the EC some 3 million t of soil tare have to be separated from sugar beet at the sugar factories each year. This costs some £35 million ($52 million) per annum. The pure financial impact of this problem is obviously important in a highly competitive world market. The reduction of production costs by minimising soil tare is, therefore, a very important commercial target. This commercial target is paralleled by the need to reduce the environmental impact of culture of the crop. The high soil tare means that fertile soil is lost from the field, water is wasted when the crop is washed and energy is wasted in transporting soil to and from the factories and in sterilising the soil so as to minimise the spread of soil-borne diseases.

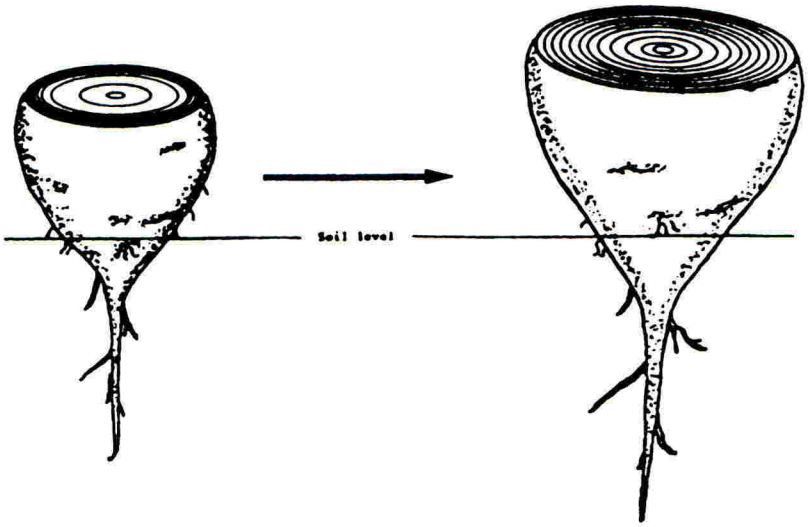
The environmental impact of sugar beet crop growth (and the corresponding production costs) will be greatly reduced by the production of a new generation of sugar beet plants whose storage roots penetrate the soil

less deeply and hence are more easily harvested and suffer a lower soil tare. The new plants will resemble large beetroot (table beet) and will have globe-shaped storage roots without branches or grooves and with a narrower crown. They will benefit the industry (and the environment) by suffering reduced yield losses because the narrow crown will minimise the consequences of over-topping (i.e. reduce top tare) while the modified root shape and absence of grooves and branches will reduce the risk of root breakage and minimise soil adherence to the roots (i.e. soil tare). Traditional plant breeders (e.g. Meskin, 1989) have assaulted these problems but the genetic variation for root shape within the sugar beet gene pool is small. Breeding programmes which sought to combine the desirable characteristics of sugar beet (high sucrose concentration and high juice purity) and table beet (appropriate storage root shape and growth habit) have resulted in some improvement in root shape but the progeny have no commercial value because they have a low sucrose content, poor extractability and inconsistent root shape. For these reasons the gene manipulation approach has been brought to bear upon the problem.

The differences in sucrose content and juice purity between sugar beet (*Beta vulgaris altissima*) and beetroot (*Beta vulgaris conditiva*) reside in anatomical differences between the two types. In each case a transverse section of the storage root reveals a conspicuous ringed structure which is caused by the alternation of vascular and parenchymatous zones. These zones arise from the division, enlargement and differentiation of the derivatives of secondary cambia which are formed very early in development. Each vascular zone contains xylem towards the inside and phloem towards the outside. Sucrose enters the root via the phloem and is transported laterally to the cells of the adjacent parenchymatous zone where it is stored in the cell vacuoles (Elliott and Weston, 1993). The storage root of sugar beet has some 12-15 secondary cambia but only about half of them make significant contributions to the development of the organ. Beetroot appears to have fewer secondary cambia (8-12) but here again only about half of them make significant contributions to the development of the storage organ. Hence the beetroot has fewer relatively broader parenchymatous zones than the sugar beet. The factor which determines sucrose accumulation by the cell is the distance of that cell from its nearest phloem conduit. Hence, sucrose concentration and juice purity will be highest in storage roots with large numbers of vascular zones alternating with similar numbers of relatively narrow parenchymatous zones so that the diffusion paths between the phloem and the storage vacuoles of the parenchymatous cells are kept short (Wyse, 1979; Elliott *et al.*, 1984, 1986; Hosford *et al.*, 1984; Elliott and Weston, 1993).

Essentially the strategy for production of this new generation of sugar beet plants requires the activation of the outer (presently inactive) cambial rings of the beetroot so that all of the secondary cambia contribute to the production of vascular and parenchymatous zones, instead of fewer than half of them, as is presently the case (Figure 1). Such a manoeuvre (Elliott and Weston, 1993) would convert a beetroot (with its small number of very broad vascular and parenchymatous zones) into a high sucrose/high dry matter/low environmental impact sugar beet in which each of the vascular zones supplied sucrose to the vacuoles of a parenchymatous zone made up of small cells close the the relevant phloem sieve tubes which accumulated sucrose more evenly and to higher average levels (Elliott and Weston, 1993). The transformation of table beet plants by chaemeric genes with outer cambial ring specific promoters and appropriate structural genes is at the heart of this strategy.

FIGURE 1. The activation of the outer cambia of the beetroot to produce a low tare sugar beet.



The evidence (Elliott *et al.* 1984; Hosford *et al.*, 1984) that phytohormone profiles determine ontogenetic sequences in *Beta vulgaris* seemed to be in accord with proposals that sugar beet development could be beneficially modified by plant growth regulators (Milford and Lenton, 1978; Elliott *et al.*, 1984, 1986) but the data revealed that the determinative changes in phytohormone levels were so subtle as to render improbable the commercial development of plant growth regulator regimes which could precisely manipulate storage root growth and development (Elliott and Weston, 1993). On the other hand the required subtlety (in terms of level, location and timing of the changes) may be realised by the introduction of *Agrobacterium tumefaciens* T-DNA genes 1, 2 and 4 into the plant genome. T-DNA transcripts 1 (which encodes tryptophan-2-mono-oxygenase) and 2 are involved in IAA biosynthesis in transformed plants while gene 4 of the T-DNA encodes isopentenyl transferase and gives enhanced cytokinin levels in the tranformants. The expression of these T-DNA phytohormone biosynthetic genes with their natural promoters leads to the formation of tumorous growths (Elliott *et al.*, 1988). However, the use of constructions with other (e.g. site-specific) regulatory elements provides the potential to influence storage root growth and development via changes in cell division, expansion or differentiation (Brown *et al.*, 1990; Gartland *et al.*, 1990; Thomas *et al.*, 1993).

The conceptual promise of this approach still exposes several major unsolved problems, not least the need to determine the relationship between the transcriptional activity of the biosynthetic genes and the resulting concentration of active phytohormone. Furthermore, determination of the effects of manipulated phytohormone levels on plant development must still

require a largely empirical approach requiring the examination of a large number of transgenic plants.

To realise the aim of manipulating cell division during sugar beet root development it will, therefore, be necessary to have a much greater understanding of the molecular mechanisms involved in the initiation and regulation of cell division. One approach which has been taken is initially to characterise cell division in the defined and manipulable system which is made available when sugar beet cells are grown in suspension culture. To this end a suspension culture system has been developed in which quiescent cells can be induced to grow and divide.

EXPERIMENTAL

Characterisation of the quiescent state of sugar beet cells in suspension culture

Sugar beet cells derived from leaf tissue have been routinely maintained in suspension culture in MS medium (Murashige and Skoog, 1962) with no added plant growth regulators. The normal batch culture cycle is 17 days from initial subculture until the onset of the stationary phase, at which point cells are normally subcultured into fresh culture medium. The absence of plant growth regulators from the culture medium means that the reinitiation of cell division results solely from the replenishment of depleted nutrients. When cells are maintained in the stationary phase for two days (i.e. a 19 day cycle) they undergo a number of biochemical changes and exhibit many of the characteristics of quiescent cells (Fowler *et al.*, in press). For example, there is a considerable decrease in DNA synthetic activity from 17 to 19 days in culture. The level of protein synthesis is also much reduced in day 19 cells. The proteins synthesised in the stationary phase cells show a much shorter half-life which correlates with an increase in protease activity. Analysis of the total cellular protein composition by SDS.PAGE confirms that many protein bands disappear during the stationary phase. On the other hand, a small number of stationary phase-specific proteins are synthesised in the day 19 cells.

Following subculture of the sugar beet cells into fresh medium there is a lag phase of several days prior to the commencement of cell division. The length of this lag phase is determined by the length of the preceding stationary phase, the longer the cells are maintained in a non-dividing state, the longer it takes for division to recommence. The biochemical and physiological features of these non-dividing sugar beet cells in stationary phase are characteristic of quiescent cells. The sugar beet cell suspensions, therefore, lend themselves to an investigation into the molecular events which occur during the transition from quiescence to cell division. Furthermore, the ability experimentally to manipulate the duration of this transition makes it possible to distinguish those events resulting from the initial subculture from the events related to the onset of cell division.

The transition from quiescence to cell division

Subculture of quiescent cells into fresh culture medium results in a number of rapid biochemical changes. Protein synthesis resumes rapidly (within 6 hours) after subculture of stationary phase (day 17 or day 19) cells and specific changes in the pattern of newly synthesised proteins are

apparent within this period. On the other hand, DNA synthesis does not recommence until 2 days after subculture of day 19 cells, in contrast to the immediate increase after subculture of day 17 cells. It is apparent, therefore, that changes in protein synthesis are an immediate response to subculture, whereas the onset of DNA synthesis is coupled to the onset of cell division, since there is a corresponding delay in both events when cells are held in the stationary phase prior to subculture.

Polyamine biosynthesis and cell division

The changes in DNA and protein metabolism which occur during the transition from quiescence to cell division are, inevitably, related to a large number of other biochemical changes during this period. Since the long term aim of the programme is to reactivate quiescent cells in the outer cambia and regulate the rate of division throughout the development of storage organ, it is necessary to determine which of these molecular events play a causal role in the reinitiation of division. Division of the cells of the hormone-autonomous suspension cultures described above, was not enhanced by adding auxins and cytokinins, presumably because these primary determinants of cell division were already present at optimal levels. The increase in the level of endogenous IAA began before the onset of cell division and reached its maximum level (per cell) slightly after the recommencement of cell division. There are also large increases in the concentration of polyamines, specifically putrescine and spermidine, during the lag phase in these cultures and peak levels are reached before the onset of cell division. Polyamines are considered to be virtually ubiquitous in both prokaryotes and eukaryotes and they are implicated in a wide range of biological processes, including plant cell division (Galston and Sawnhey, 1990). In particular, polyamines are thought to be involved in controlling nucleic acid metabolism and protein synthesis and a number of developmental processes which involve cell division (Heimer *et al.*, 1979, Maki *et al.*, 1991). Putrescine formation, the first step in the synthesis of plant polyamines, can occur by two separate pathways, involving ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) respectively. A key enzyme in the subsequent production of both spermidine and spermine is S-adenosylmethionine decarboxylase (SAMDC). There is a dramatic increase in the level of total cellular putrescine after subculture of quiescent cells towards a peak three days after transfer which precedes the onset of cell division and the peak of DNA synthesis (Figure 2A). The total cellular putrescine is composed of approximately equal amounts of the free and conjugated forms of putrescine. The rise in putrescine is accompanied by a 20 fold increase in ODC activity which occurs within 6 hours of subculture but subsides to the basal level before cell division commences (Figure 3B). In contrast, ADC activity remains lower than that of ODC and variation in ADC activity through the batch culture cycle is not correlated with cell division (Figure 3A). Spermidine also accumulates during the first three days after subculture of quiescent cells, reflecting changes in both free and conjugated forms (Figure 2B). This is accompanied by a rapid 10 fold rise in S-adenosylmethionine decarboxylase activity within 6 hours of subculture, which also declines within two days (Figure 3C). Spermine levels (Figure 2C) do not change in concert with putrescine and spermidine. The inference that both ODC and SAMDC activity are required for cell division is supported by the result of experiments involving specific inhibitors of these enzymes. It is, therefore, of considerable interest to determine whether these changes in ODC and SAMDC are due to changes in gene expression and, therefore, provide an opportunity to modulate polyamine levels by manipulation of ODC and SAMDC gene expression.

FIGURE 2. The cellular concentration of total: A) Putrescine, B) Spermidine, and C) Spermine in sugar beet cells from suspension cultures. Polyamine concentrations were determined in cells before subculture on day 19 of the culture cycle and at various times after subculture. Three replicates were used for each data point. Error bars are ± 1 standard deviation.

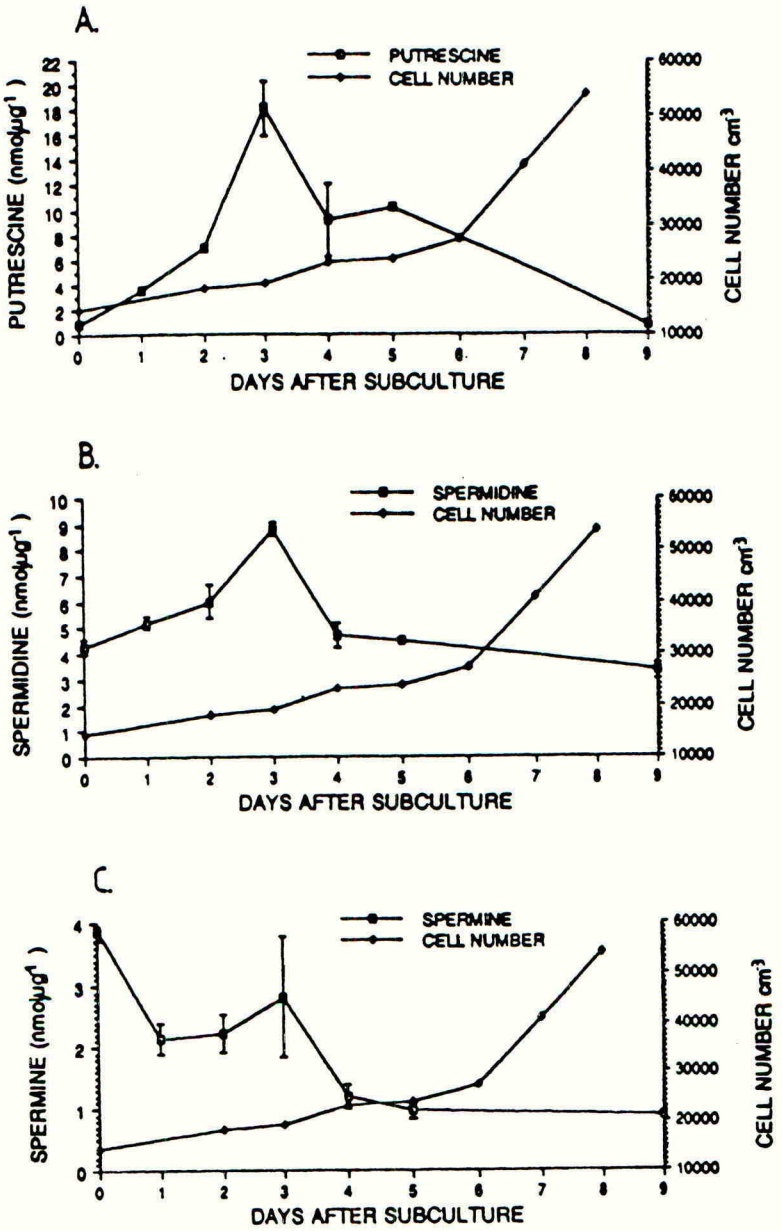
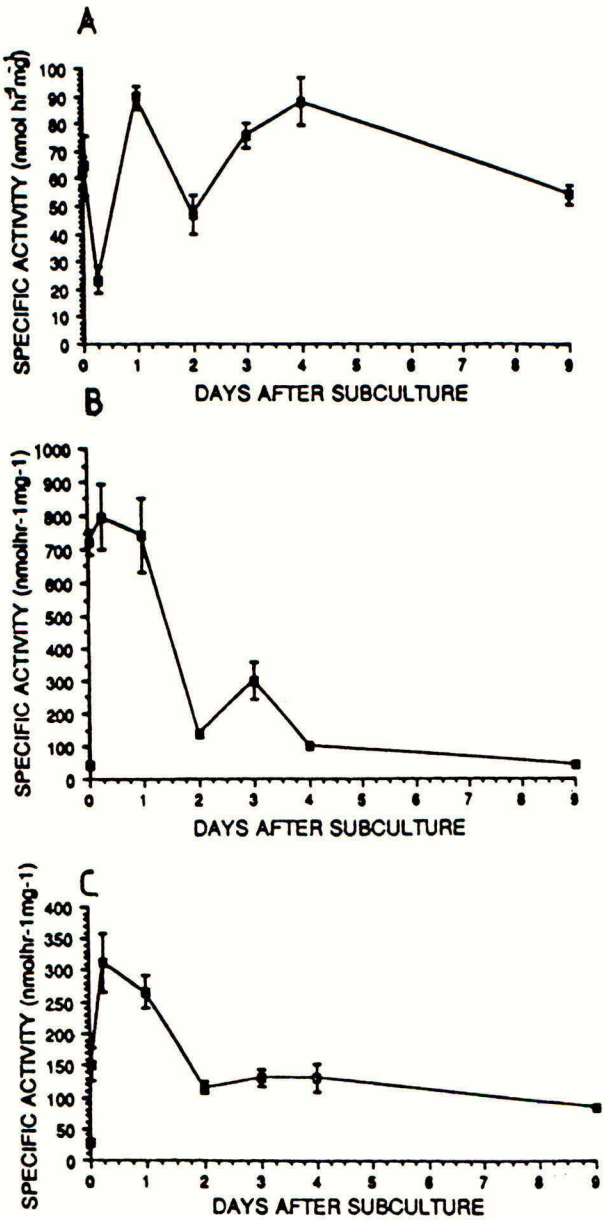


FIGURE 3. Specific activities of: A) Arginine decarboxylase, B) Ornithine decarboxylase, and C) S-adenosyl-methionine decarboxylase. Enzyme activities of extracts from cells before subculture on day 19 of the batch culture cycle and at various times following subculture were assayed *in vitro*. Four replicates were used for each data point. Error bars are ± 1 standard deviation.



Detection of ODC and SAMDC gene sequences in sugar beet

Mammalian ODC cDNA clones were used by Malmberg *et al.* (1985) to detect homologous sequences in tobacco RNA. Since that time there has been no published report of detection of plant ODC gene sequences. In our work mouse ODC cDNA was used to detect homologous sequences in sugar beet genomic DNA by Southern blot analysis. Specific RNA bands also hybridise to mouse ODC sequences on Northern blots. This analysis indicates that levels of the putative ODC sequences are low in the stationary phase and increase within 6 hours of subculture. Using multiple alignment techniques it has been possible to identify conserved regions in four ODC cDNA sequences (human, mouse, rat and *Neurospora crassa*). Degenerate primers complementary to two conserved regions have been used in a PCR to amplify sugar beet cell cDNA sequences. A product of the expected size is generated and hybridises specifically to mammalian ODC cDNA sequences. This putative plant ODC sequence has been cloned and is currently being characterised.

Bovine SAMDC cDNA clones have been used to investigate the presence and expression of homologous sequences in the sugar beet genome. Sequences hybridising to bovine SAMDC can be detected both in genomic DNA and RNA from suspension cultured cells. The pattern of expression of putative SAMDC sequences is markedly different from that of the putative ODC in that the highest levels are detected in the stationary phase.

Sugar beet cdc genes

An alternative approach to the manipulation of cell division during sugar beet storage organ development depends upon the recent dramatic progress towards understanding of the molecular biology of the cell division cycle. Much of the pioneer work was done in the fission yeast, *Schizosaccharomyces pombe* but subsequent advances in the study of the budding yeast and a range of animals from *Drosophila* to humans indicates a highly conserved mechanism. There has been much less progress in the study of the plant cell cycle, but those components which have been studies yield results which appear to confirm the universal nature of the controls. Studies over many years in a range of organisms have indicated that progression through the cell division cycle is regulated at two major control points: one control point in the G_1 phase (called START in yeast) and another at the G_2-M transition (Norbury and Nurse, 1990). The control of transition from the G_2 phase into mitosis has been most extensively characterised (Nurse, 1990). At the core of the control mechanism is a 34kD serine/threonine protein kinase ($p34^{cdc2}$) which is the product of the fission yeast *cdc2* gene. This kinase is activated during the G_2-M transition and is thought to phosphorylate a range of substrates including histone H_1, nuclear lamins and nucleolar proteins leading to the major structural re-organisation which occurs during mitosis, including chromosome condensation and breakdown of the nuclear lamina. In *S. pombe* the active $p34^{cdc2}$ kinase is found in a complex with the *cdc13* gene product which is one of a group of proteins called cyclins. The activity of this complex is regulated by several other gene products. In particular, the $p34^{cdc2}$ kinase is activated by removal of a phosphate group by the *cdc25* gene product. Premature activation is prevented by an inhibitory kinase, the product of the *wee 1* gene and it is this balance of phosphorylation and dephosphorylation which determines the activity of $p34^{cdc2}$. Genes homologous with *cdc2* and cyclins have been detected in a small number of plant species but there are, as yet, few published reports of any of the other regulatory genes involved in this cascade being found in plants.

The accumulated sequence information available for many of these genes and the highly conserved nature of the gene products have made it possible to design primers for PCR amplification of several *cdc* sequences from sugar beet genomic DNA and cDNA. For example, PCR amplification of sugar beet genomic DNA with degenerate *cdc 2* primers results in a small number of products. Southern blot analysis of products using an *Arabidopsis* cdc 2 cDNA probe shows hybridisation to a single band of the expected size. PCR amplification of sugar beet genomic DNA sequences with degenerate primers complementary to conserved regions of *cdc 13* (cyclin B) and *cdc 25* sequences has also been achieved. There are many advantages to amplifying sequences from cDNA rather than from genomic DNA. RNA isolated from dividing sugar beet cells from suspension culture has been used as a template to produce cDNA for specific amplification using paired degenerate primers. Amplification of sequences homologous to *cdc 2, cdc 13, cdc 25* and *wee 1* has been carried out and confirmation of specificity provided by Southern blot analysis. For example, the products of the *cdc 25* amplification reaction hybridise to a fission yeast *cdc 25* cDNA probe. Taken together these results provide evidence that sequences homologous to the main components of the G_2-M regulatory machinery are present in the sugar beet genome and are expressed in dividing cells in suspension cultures. The cloning and characterisation of suitable PCR products is currently underway, as is the isolation of the corresponding full length cDNA clones from a library made from RNA from dividing sugar beet cells using the PCR products as probes. A fuller understanding of the regulation of progression through the sugar beet cell cycle will emerge from this work which also offers opportunities for the precise regulation of cell division by gene manipulation.

FIGURE 4. The amino acid sequence deduced from the structure of the sugar beet RS1 clone aligned with that of the proline-rich tomato protein (TPRP).

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TPRP    1  MHVLIACPY CPYPPSTPKH PKLPPKVKPP STQPPHVKPP
TPRP   40 STPKHPKDPP HVKPPSTPKQ PPYVKPPTTP KHPPHVKPPS
TPRP   80 TPKHPKHPPQ KPCPPPSHHG PKPPIVKPPH VPRPPIVHPP
TPRP  120 PIVSPPSTPK PPKTPPFTPK PPSPIPPIVS PPIVYPPITP
TPRP  160 TPPIVHPPVT PKPPSPTPPI VSPPIVYPPI TPTPPVVSPP
TPRP  200 IIPTPPIVSP PFVPNPPVVI PPPYVPSPPV VTPPIVPTPP

TPRP  240 TPCPPPPPPP AIIPSPPAQP TCPIDALKLG ACVDVLGGLI
 RS1    1 TSPPPPPPVP CPPPSTPVQP TCSIDTLKLN ACVDVLGGLI

TPRP  280 HIGIGGSAKQ TCCPLLGGLV DLDAAICLCT TIRLKLLNIN
 RS1   41 HIGIGSGAKG ACCPILGGLV GLDAAVCLCT TIRAKLLNIN

TPRP  320 IILPIALQVL IDDCGKYPPK DFKCPST
 RS1   81 IILPLALQVL  ADCGKSPPP GFQCPSSY
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Targeting gene expression

Each of the approaches to the manipulation of cell division outlined above (the use of phytohormone biosynthetic genes, polyamine biosynthetic genes or cell division control genes) requires that expression of the potent effector genes must be tightly regulated otherwise the results for the plant will be catastrophic. The strategy for modification of storage organ development requires that gene expression occurs at a specific location within the storage organ (for example, the outer cambial rings) and at a specific stage of development. As a preliminary approach to this problem two storage organ specific/enhanced cDNA clones have been isolated from sugar beet in order to determine their pattern of expression and isolate suitable promoters from the corresponding genomic sequences. These clones have been sequenced and are currently being characterised. One of them, RS1, encodes a protein which shows some similarity (Figure 4) to a proline-rich protein found in tomato fruit (Salts *et al.*, 1992). The pattern of expression of RS1 indicates preferential but not unique expression in the storage organ. The precise location of expression within the storage organ is still unknown. The other cDNA clone, RS2 has also been sequenced, its function is not known but it shows a greater degree of storage organ specificity. The genomic sequences corresponding to both cDNA clones are being defined.

DISCUSSION

Transgenic sugar beet have been produced after *Agrobacterium*-mediated transformation (Elliott *et al.*, 1992). The aminoglycoside transferase IV gene encoding resistance to hygromycin was used to obtain transformed shoots from petiole sections of sugar beet (cv Bella). The binary vector pJIT73 which was used also conveyed glucuronidase (GUS) activity, as demonstrated by a fluorometric assay, to the hygromycin-resistant shoots which were obtained. Refinements of the transformation/selection protocols are being developed and applied to beetroot. In due course these procedures will be used to incorporate gene fusion constructions of site-specific (outer cambial rings of the storage roots) promoters with appropriate structural genes. The production of a low environmental impact sugar beet offers major challenges which have led us to recognise the merit of three related approaches. The data of Elliott *et al.*, 1984 Hosford *et al.*, 1984; Elliott *et al.*, 1986; Elliott *et al.*, 1988 and Brown *et al.*, 1990 suggest that manipulation of phytohormone levels in the outer cambia will produce the desired changes. However, we have been eager to recognise the possibility that more direct approaches involving manipulation of polyamine levels or direct manipulation of cdc genes may realise our objectives at an earlier date.

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