PREFACE

At the time this volume goes to press the British Government is reported to be considering a three year moratorium on the commercial production of transgenic crops in the UK. Such considerations have the support of several ecological and conservation organisations but are opposed by the agrochemical companies that have made a huge investment in the development of the technology. The British public has many concerns over the industrialisation of food production which result from recent food safety scares including BSE; they remain sceptical of the benefits of the new technologies in plant breeding. In this background, the research scientist uses molecular biology and transgenic technologies as powerful tools for understanding plant defences to pest and diseases attack and host parasite/pathogen interactions that may lead to transgenic plants with new forms of resistance or to the identification of novel bioactive compounds. Understandably, those involved in agricultural research and development believe these approaches to be an important component in how farming will continue to feed an ever increasing population without agriculture spreading to marginal lands causing further loss of natural habitat and biodiversity.

The speakers in this Symposium have been selected for their research and development experience or their knowledge of risk assessment for regulatory purposes and of the public perception of these risks. This volume briefly reviews the progress made in research and the commercialisation of transgenic crops and the concerns these developments have raised. The papers aim to inform the reader of the issues involved and the potential of the technologies in food production. Some current concerns, such as the use of antibiotic markers, will undoubtedly soon be resolved by technological advances. Others, such as the impact on biodiversity in farmland will take longer and require that the crops are grown on an appropriate scale. In 1998, about 4m ha of crops expressing a toxin from the bacterium *Bacillus thuringiensis* were grown in the US. This led to significant reductions in the application of pesticides to control the main lepidopteran and coleopteran pests but it remains to be seen whether other insects on the crops not controlled by the toxin increase to pest status. Effects of transgenic plants expressing insecticide resistance genes on non-target organisms such as ladybirds and lacewings have been reported in experimental conditions. However, there is a need to assess whether these natural enemies were affected indirectly by poor quality pest hosts on the transgenic plants or directly by the expressed genes and the impact of the observed effects on population dynamics. In either case the effects were small compared to the impact of most pesticides on natural enemy populations. Current experience has already demonstrated that the expression of transgenes and the control achieved are affected by environmental conditions, the physiological age of the crop and pest and disease pressure. As with pesticides, the use of transgenic crops expressing disease and pest resistance genes will need careful integration with other control strategies to ensure that their potential is realised and sustained.

If public pressures prevent the use of transgenic crops in Europe, farmers in the region will be at a competitive disadvantage compared to those elsewhere in the world where these crops are already widely grown. Even if the technologies can be transferred to the resource-poor farmer, governments in less developed countries, where significant increases in agricultural production are much needed, will find it difficult to accept technologies considered inappropriate in Europe. As a result, scientists will need to identify other approaches to pest, disease and weed control. Continued advances in existing technologies and in their application will result in significant improvements in pest and disease control but these do not have the potential of increasing the average yields of crops that transgenic technologies appear to offer. It is therefore, essential that scientists in industry and independent organisations continue to develop the dialogue with those expressing concerns and expand research on risk analysis and the effects on non-target organisms to address current public worries.

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ABBREVIATIONS

Where abbreviations are necessary the following are permitted without definition

acceptable daily intake	ADI
acid equivalent	a.e.
active ingredient	a.i.
approximately	c.
body weight	b.w.
boiling point	b.p.
British Standards Institution	BSI
by the author last mentioned	*idem.*
centimetre(s)	cm
Chemical Abstracts Services Registry Number	CAS RN
compare	cf.
concentration x time product	ct
concentration required to kill 50% of test organisms	LC_{50}
correlation coefficient	*r*
cultivar	cv.
cultivars	cvs.
day(s)	d
days after treatment	DAT
degrees Celsius (centigrade)	°C
dose required to kill 50% of test organisums	LD_{50}
dry matter	d.m.
Edition	Edn
editor	ed.
editors	eds
emulsifiable concentrate	EC
freezing point	f.p.
for example	e.g.
gas chromatography-mass spectrometry	gc-ms
gas-liquid chromatography	glc
gram(s)	g
growth stage	GS
hectare(s)	ha
high performance (or pressure) liquid chromatography	hplc
hour	h
infrared	i.r.
integrated crop management	ICM
integrated pest management	IPM
International Standardisation Organisation	ISO
in the journal last mentioned	*ibid.*
Joules	J
Kelvin	K
kilogram(s)	kg
least significant difference	LSD
litre(s)	litre(s)
litres per hectare	litres/ha
mass	*m*
mass per mass	*m/m*
mass per volume	*m/V*
mass spectrometry	ms
maximum	max.
melting point	m.p.
metre(s)	m
milligram(s)	mg
milligrams per litre	mg/litre
milligrams per kg	mg/kg
millilitre(s)	ml
millimetre(s)	mm
minimum	min.
Ministry of Agriculture Fisheries and Food (England & Wales)	MAFF
minute (time unit)	min
molar concentration	M
no observed adverse effect level	NOAEL
no observed effect concentration	NOEC
no observed effect level	NOEL
nuclear magnetic resonance	nmr
number average diameter	n.a.d.
number median diameter	n.m.d.
organic matter	o.m.
page	p.
pages	pp.
parts per million	ppm
pascal	Pa
percentage	%
post-emergence	post-em.
power take off	p.t.o.
pre-emergence	pre-em.
pre-plant incorporated	ppi
probability (statistical)	*P*
relative humidity	r.h.
revolutions per minute	rev/min
second (time unit)	s
standard error	SE
standard error of means	SEM
soluble powder	SP
species (singular)	sp.
species (plural)	spp.
square metre	m^2
subspecies	ssp.
surface mean diameter	s.m.d.
suspension concentrate	SC
systemic acquired resistance	SAR
technical grade	tech.
temperature	temp.
that is	*i.e.*
thin-layer chromatography	tlc
time for 50% loss; half life	DT_{50}
tonne(s)	t
ultraviolet	u.v.
United Kingdom	UK
United States Department of Agriculture	USDA
vapour pressure	v.p.
variety (wild plant use)	var.
volume	*V*
weight	*wt*
weight by volume	*wt/v*
(mass by volume is more correct)	*(m/V)*
weight by weight	*wt/wt*
(mass by mass is more correct)	*(m/m)*
wettable powder	WP

less than	<
more than	>
not less than	≮
not more than	≯

Multiplying symbols-		Prefixes
mega	($\times 10^6$)	M
kilo	($\times 10^3$)	k
milli	($\times 10^{-3}$)	m
micro	($\times 10^{-6}$)	μ
nano	($\times 10^{-9}$)	n
pico	($\times 10^{-12}$)	p

TECHNIQUES FOR THE DEVELOPMENT OF TRANSGENIC CROPS IN CROP PROTECTION

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Techniques for the development of transgenic crops in crop protection

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ABSTRACT

Plant transformation techniques have developed rapidly in the last ten years and most of the major crops are now amenable to genetic modification. However, there are still several areas of crop transformation technology which require further development to produce efficient and reliable methods which can be used across a range of crop species. Improved selectable marker genes with neutral environmental impact and techniques for the removal of markers from genetically modified (GM) crops are under development. More sophisticated control of transgene expression, both in terms of spatial and temporal regulation is desired. There is still limited understanding of longer-term stability of introduced traits in most transgenic crops and methods for stabilising transgene expression are being sought. Plant breeding will be increasingly influenced by genetic modification technology and new approaches for the introgression of GM lines into breeding programmes must be developed.

INTRODUCTION

Genetic modification technologies offer important new approaches in the field of crop protection, by their application in the breeding of new varieties with improved pest and disease control and by the use of transgenic plants and pathogens in basic research aimed at understanding the biology of crop - pathogen / pest interactions. For the potential of these new approaches to be fully exploited, efficient crop transformation technology is required. This must be broadly applicable to current germplasm which yields transgenic plants of high agronomic quality and with stable, predictable and heritable expression of the introduced traits. In addition, the novel GM crops and the technology used in their production must be accepted as safe for human and livestock use / consumption and as not having negative environmental impact.

While transformation technology is essentially the tool kit for the production of GM crops with enhanced disease and pest resistance, the specific technology used, such as the transgene delivery system, the selectable marker gene and components of the transformation vector, may affect the quality of the transgenic lines produced, via factors such as the stability of the introduced trait, the environmental acceptability of a new variety and also the economics of production of the GM line.

In this paper, the technologies used in the production of GM crops are reviewed with consideration of the current status of differing methodologies and their application in the major crop species. Technological constraints which call for further developmental research are discussed, as well as aspects of transgene expression and stability, and GM crop breeding.

GENE TRANSFER METHODS

A technique for the introduction of a new gene into the genome of a recipient cell (transformation) is the primary requirement for the production of GM crops. Two basic methods for plant transformation are currently in use; *Agrobacterium*-mediated gene transfer and direct gene transfer (DGT) techniques (Gheysen *et al.*, 1998, Barcelo & Lazzeri, 1998). *Agrobacterium* transformation is based on the ability of the bacterium to transfer a segment of an extra-chromosmal plasmid (the Ti plasmid) called the T-DNA into a host plant cell where it integrates into the recipient genome (Gheysen *et al.*, 1998). In nature, *Agrobacterium* delivers the T-DNA genes which cause host tissue to proliferate to form a tumour and to synthesise novel metabolites which are used by the pathogen as carbon and nitrogen sources. For use as a vector for plant genetic modification, the T-DNA genes causing tumour formation are removed and replaced with genes allowing the selection of transgenic plants (selectable and scorable markers) and genes coding for traits of interest. As a plant pathogen, *Agrobacterium* has evolved to enter plants at wound sites on intact plants, but for genetic modification work the bacteria are "co-cultivated" with plant tissue explants under sterile conditions. The explants are then cultured *in vitro* for the regeneration of new transgenic plants. The natural host range of *Agrobacterium* includes a range of plant species, although its interaction tends to be most efficient with broad-leaved herbaceous species (Gheysen *et al.*, 1998). However, the great interest in applying genetic modification techniques to a very wide range of plant species, many of them not natural hosts to the bacterium, has led to the development of sophisticated methodology for increasing the efficiency of the bacterial x plant cell interaction and thus transformation frequency. Recently, attention has focused on modification of the virulence (*vir*) genes of *Agrobacterium*, which influence host-range and it has been possible to extend the range of plants which can be transformed to include a number of species previously considered highly recalcitrant, foremost among these being the major cereal crops (e.g. rice, Hiei *et al.*, 1994.).

Direct gene transfer (DGT) methods for plant transformation involve the use of a range of physical, chemical or electrical stimuli to introduce DNA into recipient cells. The idea of transforming plants with "naked" DNA has received attention from the 1960s, and early attempts focused on the transformation of intact plants, either by mixing DNA with pollen and pollinating with the mixture or by injecting DNA into developing floral structures. While the concept of such relatively "low-tech" *in vivo* transformation approaches is attractive, there is still no unequivocal evidence that they actually function, despite having been repeatedly revisited with improving levels of technological sophistication over some thirty-five years. However, because *Agrobacterium* transformation technology was until recently confined to the natural hosts of the pathogen, efforts were made to develop alternative DGT techniques. This resulted in the establishment of a number of methods for the transformation of plant cells *in vitro*. These include microinjection of DNA into cells, electroporation of cells or protoplasts (cells with their walls removed), chemically-induced DNA uptake into protoplasts, puncturing

cells with microscopic silicon carbide fibres and particle bombardment (Barcelo & Lazzeri, 1998). Each of these techniques has been used in crop species, but most require sophisticated cell culture and regeneration procedures to function efficiently and this has limited broad application. The exception is the technique of particle bombardment, in which gene transfer is effected by precipitating DNA onto microscopic gold particles which are then accelerated into target tissues. The DNA is then released from the particle and, if in the vicinity of the cell nucleus, may be integrated into the host cell genome giving a transgenic cell, from which a plant may subsequently be regenerated. The significance of particle bombardment technology is that it allows DNA delivery to tissue cultures amenable to regeneration *in vitro* and its advent permitted the transformation of important crop species recalcitrant to *Agrobacterium* methods.

Comparing *Agrobacterium* and particle bombardment methodology for crop genetic modification, the general preference is for the former because of its relative technical simplicity, the potential for very high efficiency from optimised procedures and the fact that very similar techniques are employed for a range of crop species. *Agrobacterium* transformation is also perceived as a more "directed" gene transfer method as the T-DNA is delivered to, and integrated into the host genome by a highly evolved mechanism which appears to target active regions, increasing the chance of subsequent transgene expression. Further, control processes appear to operate to limit the number of T-DNA molecules integrated per host genome generally to less than four, with one or two being typical (Gheysen *et al.*, 1998). In contrast, integration of DGT transformation vectors is thought of as essentially a random process which may result in the integration of large numbers of transgene copies and also the integration of partial and rearranged inserts. For incorporation into breeding programmes, lines containing single, intact transgene insertions are preferable as their genetics are less complicated. There is good evidence that the stability and heritability of transgene expression is influenced by the physical integration pattern and complicated patterns of integration are suggested to be inherently less stable. However, there are few studies in which populations of plants produced by DGT or *Agrobacterium* have been compared directly to determine the influence of transformation method on stability (see section on Stability and Heritability below).

At present, the cereals are the major crop group in which particle bombardment is the primary transformation method, but within this group *Agrobacterium* transformation is now efficient in rice (Hiei *et al.*, 1994), is applicable in a range of maize germplasm (Ishida *et al.*, 1996) and functions in limited barley and wheat germplasm (Tingay *et al.*, 1997, Cheng *et al.*, 1997). There is at present intensive effort world-wide to improve *Agrobacterium* technology in the latter three cereals, because of the perceived advantages of the system. Against this background, however, there are a number of crops for which DGT methods are more productive. An important example is sugar beet, in which *Agrobacterium*-mediated transformation is highly labour-intensive and for which an efficient protoplast-based procedure has been developed (Hall *et al.*, 1996). The question in this crop, however, is whether the typically greater transgene copy numbers associated with the method will prove problematic.

SELECTION SYSTEMS

Even in the highly efficient transformation systems the frequency of transformed cells among the treated population is very small, so that some mechanism is needed to identify or select the desired transgenic individuals. This is achieved by the use of "marker" genes which are

inserted along with the genes conferring the desired new trait. Markers may either be selectable, typically conferring resistance to a toxic selection agent, or scorable, giving a visible phenotype to transformed cells. During developmental research it is common to use both selectable and scorable markers, but in crop genetic modification work it is normal for only a selectable marker to be used. The two types of selectable marker commonly in use today are antibiotic resistance genes (e.g. *neo* and *aph*, conferring resistance to kanamycin, / G418 and hygromycin, respectively) and herbicide resistance genes (e.g. *bar* / PAT conferring resistance to Basta and CP4 / GOX conferring resistance to glyphosate).

The current antibiotic and herbicide resistance genes may be considered the first and second generations of selectable markers and there is currently considerable activity in developing alternative selectable markers. This is driven in some cases by intellectual property considerations causing companies to seek their own marker genes which give them freedom to operate, but also by public and governmental concern about potential environmental impact of existing markers. Such concern has stimulated research to develop "benign" markers. Some benign markers already exist, such as the xylose isomerase positive selection system which allows transgenic tissues to use xylose as a carbon source (Haldrup *et al.*, 1998).

The situation regarding the desirability of selectable marker genes remaining present in a GM plant line depends on the strategy for exploitation / commercialisation of the crop. In some cases, herbicide resistance may be the primary trait desired or may be part of a package of traits (e.g. in combination with a pathogen resistance or quality trait), while in others it may be preferable if the GM crop does not contain a selection marker. The latter situation can be envisaged where a GM crop has close weed relatives with which it readily out-crosses, such as the case of cultivated and wild oats.

The use of *Agrobacterium*-mediated transformation offers a relatively simple mechanism for the removal of marker genes *via* the delivery of the gene-of-interest and the marker on separate T-DNA molecules. This leads to their integration at different genetic loci in around a quarter of lines recovered, allowing their separation by segregation in progeny (Komari *et al.*, 1996). A second approach for the removal of marker genes, applicable also in DGT-produced transformants is the use of molecular excision systems such as cre / lox technology by which marker genes may be removed from the crop plant genome when they are no longer needed (Stuurman *et al.*, 1996). A third methodology for protection against the risk of undesired gene dispersal from GM crops is chloroplast transformation (Svab & Maliga, 1993). With this strategy, the selectable marker gene would be integrated and expressed from the chloroplast genome, so that in most crop plants, which show maternal plastid inheritance, dispersal of the marker gene *via* pollen would not occur. The extent to which the removal or fixation of marker genes will be part of future GM crop breeding is at present not clear and will depend in part on public perception and government reaction to perceived risks to consumers and the environment from GM crops and in part on the data amassed from experimental studies on GM releases and monitoring of the first commercial GM crops.

CONTROL OF TRANSGENE EXPRESSION

Beyond the integration of a transgene into the genome of a target crop plant, the next priority is its pattern of expression. The primary level for the regulation of expression of a transgene is exerted by the use of a promoter sequence cloned into the transformation vector upstream of the structural gene. The promoter controls the level of transcription of the gene, but its activity may in turn be influenced by further regulatory molecules termed transcription factors (Verrijzer & Tjian, 1996).

In general, the level of expression of a transgene is likely to be of priority, but in addition spatial distribution of expression (at organ, tissue and possibly cell levels), temporal patterns of expression, and regulation in response to external stimuli and by internal signals may also be of importance. The pattern of expression desired for a particular transgene will depend on the gene's function. Where, for example, the protein or starch content or composition of a crop is to be modified, high levels of expression should be targeted to an appropriate storage organ (e.g. seed, taproot or tuber). Alternatively, if the aim is protection of a crop against a phytophagous insect then expression should be targeted to the aerial parts of the plant where insect attack occurs. In the first GM crop lines produced, transgenes were generally expressed under the control of active constitutive promoters (most commonly the Cauliflower Mosaic Virus 35S promoter) which leads to high levels of the gene product to be produced in most tissues. In later work, emphasis switched to the use of organ or tissue-specific promoters such as those active in photosynthetic tissues (e.g. Kyozuka et al., 1993) or seeds (e.g. Barro *et al.*, 1997). Currently there is movement towards the use of regulated and inducible promoters whose activity may either be switched on by the addition of exogenous compounds such as herbicide safeners or alcohol (Gatz & Lenk, 1998), or are activated by pest or pathogen attack on the crop plant. There are very clear advantages to the temporal and spatial regulation of transgene expression; the metabolic cost of expressing a gene product unnecessarily is avoided, as is the possibility of the transgene product interfering with metabolism in non-target cells. In the case of protectant molecules, which may be toxins directed at pests or pathogens, these may be excluded from plant parts subsequently used for animal or human consumption. However, while our ability to control transgene expression has become more sophisticated, we are still some way from the sensitivity of control which will ultimately be required. For example, there may be opportunities to control crop plant nutrient use efficiency by manipulating uptake, transport and partitioning (Clarkson & Hawkesford, 1993). For such applications in a field situation, promoters / regulatory elements which respond to nutrient status will be required. Classes of genes whose expression is regulated by nutrient status are known, but so far there is little experience in the use of their promoters in engineering nutrient-responsive systems in transgenic plants.

STABILITY AND HERITABILITY OF GM TRAITS

The stability of expression of a GM trait under differing environmental conditions and over different seasons, and the reliable transmission of the trait are clearly of central importance to the success of the GM crop line. This is equally true of traits introduced by conventional plant breeding, but we have many years of experience with the latter technology and while reasons for instability are not always understood, there are established assessment procedures to ensure

that new varieties released behave predictably. To date there is far less experience of GM crops, and we are still at the stage of learning about longer-term stability of introduced traits.

Studies in (research) model plant species and with the first GM varieties are yielding information on factors which may lead to instability in transgene expression and to the development of methods for its control. Broadly viewed, transgene expression stability may be influenced by the nature of the transformation construct itself, (e.g. the promoter used to control expression, the degree of similarity of the structural gene with the host genome) by the structure of the transgene insertion (i.e. the number of copies inserted and the extent of transgene rearrangement, truncation, etc.), by the location of the transgene insertion (both at the level of the immediate chromatin domain and of chromosomal location) and probably also by the background variability of the recipient genome. In some of these areas, there are now opportunities to improve transgene expression stability, such as the choice of the most suitable promoters and the construction of transformation vectors incorporating specific DNA sequences (matrix attachment regions - MARS) which have activity in reducing variation in expression due to insertion site (Holmes-Davis & Comai, 1998). In others areas, such as directing the site and structure of transgene insertions, we do not yet have effective techniques for plants.

The accumulated knowledge on the inheritance of transgenes is that, in principle, they follow standard Mendelian rules of inheritance, but that lines showing deviations from normal inheritance patterns are frequently recovered and in some systems may be in the majority. In most situations, it does not appear that the insertion event itself is unstable but that transmission via the gametes does not follow the expected patterns. The situation is to some extent confused by the fact that, in many studies, segregation ratios among progeny are assessed on the basis of expression of the trait which does not distinguish poor transmission of a gene from silencing of its expression. As a general rule, plants generated via *Agrobacterium*-mediated transformation which tend to have simple, low copy number transgene insertions show more normal inheritance patterns than plants produced by DGT methods which tend to have more complicated patterns of integration. However, both methods can produce lines which show normal, stable inheritance patterns and which can be manipulated in breeding programmes.

GENETIC MODIFICATION TECHNOLOGY AND BREEDING

GM crops are increasingly influencing the practice of plant breeding, in that new methodologies are being developed for the introgression of transgenic lines into breeding procedures and that a target of genetic modification technology in several crops is the development of novel engineered male sterility systems (e.g. De Block *et al.*, 1997) to improve the efficiency of hybrid systems or to make what are currently inbred crops into hybrid crops. Further, continued increases in the efficiency of plant transformation have opened the way for the use of transformation-based breeding tools. Examples are the creation of mutated populations where the mutated genes are tagged to facilitate their isolation. This may be achieved using *Agrobacterium* transformation to produce T-DNA insertions which disrupt genes or the by introduction of transposable elements via transformation and the use of these elements to create tagged mutations (Choe & Feldmann, 1998; Pereira, 1998). These approaches have hitherto been applied in model plants or selected crops amenable to

transformation, but continued improvements in transformation technology make their application in mainstream crops or closely-related species increasingly feasible. Lastly, the present explosion in activity in the area of plant genomics will greatly increase the pool of genes available for crop improvement as well as generating a great volume of gene sequences whose exact function is unknown (Lee, 1998). The development of automated transformation technology, coupled with automated phenotype screens (for example, assays for changes in a range of metabolites) would allow the identification of novel genes associated with traits of interest for breeding.

CROP GENETIC MODIFICATION TECHNOLOGY - STATUS AND PROSPECTS

The last decade has seen enormous progress in the development of enabling technology for GM crop production. Today most of the world's major crop species are reproducibly transformable, with a few notable exceptions such as the large-seed legumes *Phaseolus vulgaris* and *Vicia faba*. While transformation efficiency or broad genotype applicability still pose limitations in crops such as sugar beet or pea; technologies are developing rapidly and we can expect continued improvements.

The areas of regulation of transgene expression and the stability of introduced traits are now of high priority for research effort. We still have limited information on the performance of GM crops of most species in the field and as the area and range of GM crops grown increases it is inevitable that we will need to modify approaches and practices to achieve optimum performance and that technology will need to evolve as genetic modification approaches become more sophisticated.

REFERENCES

Barcelo P; Lazzeri P A (1998). Direct Gene Transfer: Chemical, Electrical and Physical Methods. In: *Transgenic plant research*, ed K Lindsey, pp 35-56. Harwood Academic: Amsterdam.

Barro F; Rooke L; Bekes F; Gras P; Tatham A S; Fido R; Lazzeri P A: Shewry P R; Barcelo P (1997). Transformation of wheat with high molecular weight subunit genes results in improved functional properties. *Nature Biotechnology* **15**, 1295-1299.

Cheng M; Fry J E; Pang S; Zhou H; Hironaka C M; Duncan D R; Connor T W; Wan Y (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiology* **115**, 971-980.

Choe S; Feldmann K A (1998). T-DNA mediated gene tagging. In: *Transgenic plant research*, ed K Lindsey, pp 57-76. Harwood Academic: Amsterdam.

Clarkson D T; Hawkesford M J (1993). Molecular biological approaches to plant nutrition. In: *Plant nutrition - from genetic engineering to field practice*, ed N J Barrow, pp 23-33. Kluwer: Dordrecht.

De Block M; DeBrouwer D; Moens T (1997). The development of a nuclear male sterility system in wheat, expression of the *barnase* gene under the control of tapetum specific promoters. *Theoretical and Applied Genetics* **95**, 125-131.

Gatz C; Lenk I (1998). Promoters that respond to chemical inducers. *Trends in Plant Science* **3**, 352-358.

Gheysen G, Angenon G; Van Montagu M (1998). Agrobacterium-mediated plant transformation: a scientifically intriguing story with significant applications. . In: *Transgenic plant research*, ed K Lindsey, pp 1-34. Harwood Academic: Amsterdam.

Haldrup A; Petersen S G; Okkels F T (1998). The xylose isomerase gene from *Thermoanaerobacterium thermosulforogenes* allows effective selection of transgenic plants using D-xylose as the selective agent. *Plant Molecular Biology* **37**, 287-296.

Hall R D; Riksen-Bruinsma T; Weyens G J; Rosquin I J; Denys PN; Evans I J; Lathouwers J E;

Lefebre M P; Dunwell J M; van Tunen A; Krens F A (1996) A high efficiency technique for the generation of transgenic sugar beets from stomatal guard cells. *Nature Biotechnology* **14**, 1133-1138.

Hiei Y; Ohta S; Komari T; Kumashiro T (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *The Plant Journal* **6**, 271-282.

Holmes-Davis R; Comai L (1998). Nuclear matrix attachment regions and plant gene expression. *Trends in Plant Science* **3**, 91-96.

Ishida Y; Saito H; Ohta S; Hiei Y; Komari T; Kumashiro T (1996). High efficiency transformation of maize Zea *mays* L.) mediated by *Agrobacterium tumefaciens*. *Nature Biotechnology* **14**, 745-750.

Komari T; Hiei Y; Saito Y; Murai N; Kumashiro T (1996). Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. *The Plant Journal* **10**, 165-174.

Kyozuka J; McElroy D; Hayakawa T; Xie Y; Wu R; Shimamoto K (1993). Light-regulated and cell-specific expression of tomato *rbcS-gusA* and rice *rbcS-gusA* fusion genes in transgenic rice. *Plant Physiology* **102**, 991-1000.

Lee M (1998). Genome projects and gene pools: new germplasm for plant breeding? *Proceedings of the National Academy of Science* **95**, 2001-2004.

Pereira A (1998). Heterologous transposon tagging. In: *Transgenic plant research*, ed K Lindsey, pp 91-108. Harwood Academic: Amsterdam.

Tingay S; McElroy D; Kalla R; Fieg S; Wang M; Thornton S; Brettell R (1997). *Agrobacterium tumefaciens*-mediated barley transformation. *The Plant Journal* **11**, 1396-1376.

Stuurman J; De Vroomen M; Nijkamp H J J; Van Haaren M J J (1996). Single-site manipulation of tomato chromosomes *in vitro* and *in vivo* using Cre-lox site-specific recombination. *Plant Molecular Biology* **32**, 901-913.

Svab Z; Maliga P (1993) High frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. *Proceedings of the National Academy of Sciences* **90**, 913-917.

Verrijzer C P; Tijan R (1996) TAFs mediate transcriptional activation and promoter selectivity. *Trends in Biochemical Science* **21**, 338-342.