GENETIC ENGINEERING OF PLANTS

J. A. Thomson
Department of Microbiology, University of Cape Town, South Africa

Keywords: *Agrobacterium tumefaciens*, biolistics, transgenic plants, transformation, gene silencing

Contents

Summary

Genetic engineering can be used to introduce specific traits into plants. It will not replace conventional breeding but can add to the efficiency of crop improvement. It is possible due to the fact that plants are totipotent, enabling regeneration of a new plant from an isolated cell. Transformation of dicots is usually carried out using the bacterium, *Agrobacterium tumefaciens.* Genes are cloned into plant expression vectors that carry the right and left border sequences. They are introduced into plants with the aid of a disarmed Ti plasmid whose virulence gene products allow the genes to be transferred to the plant nucleus where they are integrated into the genome. Monocots are usually transformed by a biolistic process, using a "gene gun". In both cases callus tissue is regenerated on media containing an antibiotic or herbicide to select for transformants. The exception is transformation of the common wall cress, *Arabidopsis thaliana*, where tissue culture is not required. Gene silencing, whereby plants shut off the expression of multiple copies of a gene, can be a problem when attempting to introduce a new trait into a plant. Although the process is not fully understood it can, at the level of transcription, be due to methylation or ectopic DNA pairing. At the post-transcriptional level, the transgene RNA could be specifically degraded if tagged by a small complementary RNA molecule. It is often advantageous for plants to express the introduced transgenes in specific tissues or under specific conditions. As a result many

genes are cloned downstream of tissue-specific or inducible promoters. High expression of a transgene may be required under certain circumstances. The 35S promoter of the cauliflower mosaic virus is commonly used in dicots while the maize ubiquitin promoter is the monocot promoter of choice. Targetting genes to organelles such as chloroplasts can also enhance expression. Little is understood of the way in which genes are integrated into the plant chromosomes. In many cases multiple inserts occur at one locus. The field of plant genetic engineering is a fascinating one and will continue to grow in efficiency and sophistication in the years to come.

1. Introduction

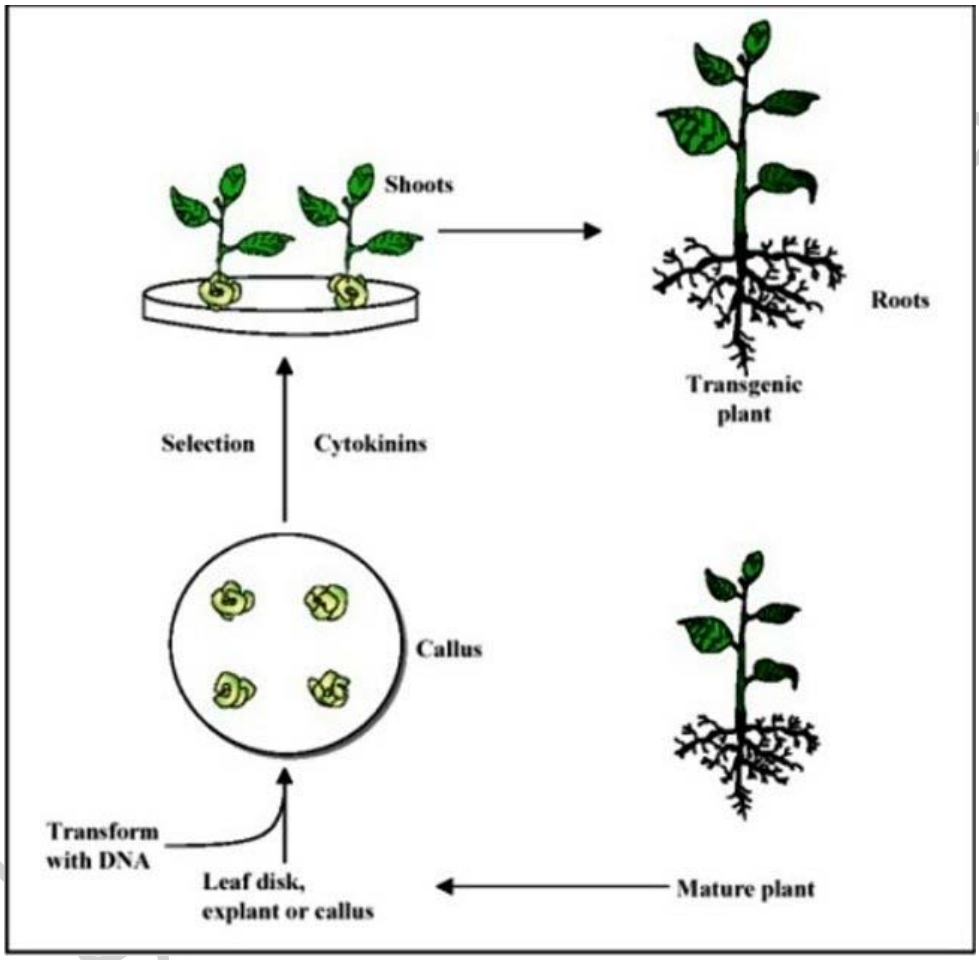

Figure 1: Plant transformation. Plant material is transformed with DNA carrying a selectable marker and the gene of interest. Callus tissue develops on selective media. Shoots develop on the addition of cytokinins. Withdrawal of cytokinins promotes root development.

Conventional plant breeding has succeeded in producing a wide variety of commercial plants and crops with a range of important agronomic traits. It has succeeded in converting a Mexican grass into maize and Middle East grass into wheat. However, it is to a large extent a hit-or-miss process, combining large parts of parental genomes in a rather uncontrolled fashion, although this is currently being improved due to the modern

technique of marker assisted breeding. Genetic engineering, on the other hand, allows scientists to transfer very specific genes into plants, resulting in the introduction of one or more defined traits into a particular genetic background. This process is called transformation and the genes involved are expressed to form a protein responsible for the particular trait. The traits involved include herbicide and drought tolerance, and resistance to viral, bacterial and fungal pathogens as well as to herbivorous insects. The added advantage is that the transferred gene(s), or transgene(s), can come from any organism as long as its expression is compatible with its new host.

Plant transformation is possible due to the fact that plants are totipotent, enabling regeneration of a new plant from an isolated cell. Thus if a gene is transferred to a plant genome in a cell the regenerated plant will contain that gene in every cell. In practice the gene(s) of interest are introduced together with a selectable marker such as resistance to a herbicide or antibiotic to which the plant is sensitive. Cells and regenerating plants are grown in gel-like media containing that herbicide or antibiotic and only plants expressing the genes for resistance will grow. The hormone auxin is used to initiate and maintain callus. Once cells have been transformed, cytokinin hormones are incorporated into the medium to allow shoot development. Withdrawal of cytokinin promotes root growth. Once plants are fully developed they are taken out of the media and planted in soil for "hardening off". The process is shown diagrammatically in Figure 1.

2. Transformation of dicotyledonous plants

Dicotyledonous plants are those which develop from two cotyledons in the seed. They can be recognized by the branching veins in their leaves. Dicots of commercial value include many horticultural plants such as petunias, and crops such as tobacco, tomatoes, cotton, soybean and potatoes. Petunias have been engineered to produce a range of attractive flower colors and patterns. Tobacco, due to its ease of transformation, initially became the workhorse of plant genetic engineering, but more recently the common wall or thale cress, *Arabidopsis thaliana*, has become very popular. It has the advantage of not requiring tissue culture during its transformation. Tomatoes have been transformed to delay their ripening, cotton to insect resistance and herbicide tolerance, soybeans to improved oil quality and herbicide tolerance, and potatoes to resist viruses.

2.1 Transformation using *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is a plant pathogenic soil bacterium. It makes (tumefacient = swollen: Latin = tumefacere) a tumor on plants it infects and as these are often on the crown region where the stem meets the roots, the disease is called Crown Gall. Scientists were amazed to discover that the bacteria transfer part of their DNA to the plant nucleus where it becomes integrated into the plant genetic material. The transferred DNA, or T-DNA, is part of a large tumor-inducing (Ti) plasmid. The T-DNA carries an *onc* (oncogenic) region, which, by coding for the production of plant growth hormones, results in the proliferation of plant cells forming a tumor or gall. It also codes for the production of unusual derivatives of arginine, such as nopaline or octopine, which the bacteria can use as growth substances. This bacterial-plant interaction is known as genetic colonization. It was not long after this discovery that

scientists realized that the introduction of a foreign gene into the T-DNA would enable its transfer to the plant cell nucleus. This led to the development of plant transformation using a disarmed, *onc*-, version of the Ti plasmid that could transfer DNA into plants without causing the production of a tumor.

The Ti plasmid is very large, in the order of 200 kb, and therefore unwieldy to work with *in vitro*. It was soon discovered that all that is required for a gene to be introduced into a plant are the 25-bp repeat sequences at the borders of the *onc* region, known as the left and right borders (LB and RB), and the virulence genes (*vir*) of the Ti plasmid. It was possible, therefore, to separate these in a system of binary vectors. Genetic manipulation is done in *Escherichia coli* on a small plasmid carrying a multiple cloning site (MCS) downstream of a plant promoter, and a gene coding for resistance to a herbicide or antibiotic that is toxic to the plant of interest, situated between the LB and RB. This plasmid is then transformed into a strain of *A. tumefaciens* carrying a disarmed Ti plasmid, which essentially consists only of the *vir* region and an origin of replication (Figure 2).

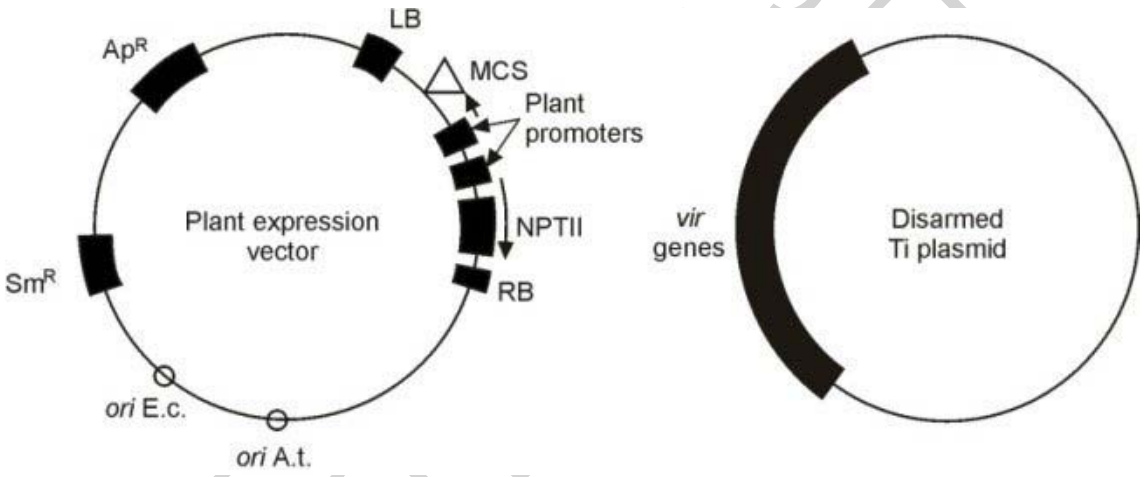

Figure 2: Binary vectors. Ap^R, ampicillin resistance for selection in *E. coli*; Sm^R, streptomycin resistance for selection in *A. tumefaciens*; *ori* E.c, origin of replication for *E. coli*; *ori* A.t, origin of replication for *A. tumefaciens*; RB, right border; NPTII, kanamycin resistance for selection in plant cells; MCS, multiple cloning site; LB, left border

This strain of *A. tumefaciens* is then used to transform plants. The earliest species to be transformed was tobacco, *Nicotiana tabacum*, which rapidly became the model dicot plant. However, more recently the workhorse has changed to *Arabidopsis thaliana* which has a very small genome of 120 Megabases and is easier to transform. In order to transform tobacco, and most other dicots, leaf disks are cut and placed in a Petri dish containing a liquid medium. The *A. tumefaciens* strain is placed on the surface of the disks and co-cultivation carried out for 2-3 days. The cutting of the leaf disks results in the plant producing wound-response compounds, such as acetosyringone, which induces the virulence genes. The leaf disks are then transferred to selection media containing the herbicide or antibiotic of choice. This is often kanamycin as many binary vectors carry the neomycin phosphotransferase gene (NPTII) which codes for kanamycin resistance. Transformation occurs along the cut edges of the disks, resulting in the formation of

callus tissue which carries the DNA between the LB and RB integrated at random into the plant genome. The callus tissue is then transferred to regeneration medium also containing kanamycin, which only allows transgenic plants, expressing kanamycin resistance, to develop. The whole process takes about three to four months.

During the regeneration process care must be taken to inhibit the growth of *Agrobacterium* as false positive results could be due to the expression of the T-DNA-carrying genes in the bacteria rather than in the plant. This is often found despite the fact that the genes are expressed from eukaryotic promoters. Antibiotics such as carbenicillin or cefotaxime can be used to eliminate the bacteria but they are not always sufficient. Another strategy is to introduce into the T-DNA a GUS gene, coding for ß-glucuronidase, which carries a plant intron. The enzyme is very easy to detect histochemically and fluorometrically and will only be correctly spliced if it is expressed within the plant and not in *A. tumefaciens*.

Arabidopsis transformation is very simple and does not require tissue culture. This is advantageous because during the tissue culture process somatic mutations can occur which may adversely affect the plant of being very simple and not requiring any tissue culture. To transform *Arabidopsis* young flowering plants are inverted into a suspension of *A. tumefaciens* cells under a vacuum. This causes the bacteria to infiltrate into the flowers and transfer the T-DNA into the DNA of the developing seeds, which are collected and germinated on the selected antibiotic. Only transgenic seeds will germinate and although the frequency of transformation is only about 1%, *Arabidopsis* produces such copious amounts of seed that transgenic plants are readily obtained. (Figure 3).

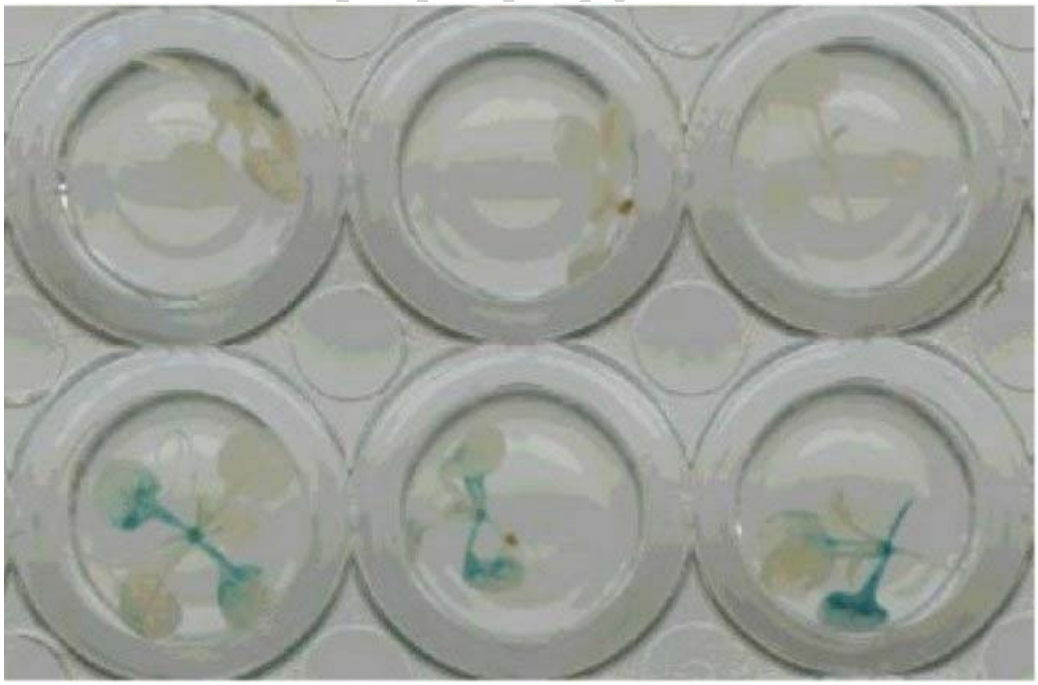

Figure 3: Top row: untransformed *Arabidopsis* plants. Bottom row: transformed *Arabidopsis* plants stained blue due to expression of the β-glucuronidase gene

-
-
-

Bibliography

Burkhardt, P.K., P. Beyer, J. Wünn, A. Klöti, G.A. Armstrong, M. Schledz, J. von Lintig, and I. Potrykus. (1997) Transgenic rice (*Oryza sativa*) endosperm expressing daffodin (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. The Plant Journal 11, 1071-1078. [This paper announces the development of Vitamin A rice which has been widely acclaimed for relieving eye problems in Asia.]

Chen, L., P. Marmey, N.J. Taylor, J-P. Brizard, C. Espinoza, P. D'Cruz, H. Huet, S. Zhang, A. de Kochko, R.N. Beachy, and C.M. Fauquet. (1998) Expression and inheritance of multiple transgenes in rice plants. Nature Biotechnology 16, 1060-1064. [This work outlines ways of introducing a number of transgenes into a plant.]

Christensen, A.H., and P.H. Quail. (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Research 5, 213-218. [This article introduces the ubiquitin promoter, so far the best monocot promoter.]

De Neve, M., S. De Buck, A. Jacobs, M. Van Montagu, and A. Depicker. (1997) T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. The Plant Journal 11,15-29. [This is an important paper dealing with the pattern of insertion of transgenes.]

Frame, B.R., H. Shou, R.K. Chikwamba, Z. Zhang, C. Xiang, T.M. Fonger, S.K. Pegg, B. Li, D.S. Nettleton, D. Pei and D. Wang. (2002) *Agrobacterium tumerfaciens*-mediated transformation of maize embryos using a standard binary vector system. Plant Physiology 129,13-22

Hansen, G., and M-D. Chilton. (1996) "Agrolistic" transformation of plant cells: Integration of T-strands generated *in planta*. Proceedings of the National Academy of Science, USA. 93,14978-14983. . [This is an interesting paper which uses aspects of both biolistics and *A. tumefaciens* transformation in a single system.]

Henry, E.C., and R.H. Meints. (1994) Recombinant viruses as transformation vectors of marine macroalgae. Journal of Applied Phycology 6,247-253. . [This paper is one of the few dealing with transformation of algae.]

Hiei, Y., S. Ohta, T. Komari, and T. Kumashiro. (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. . [This is the first paper describing rice transformation using *A. tumefaciens*.]

Kay, R., A. Chan, M. Daly, and J. McPherson. (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236,1299-1302. [This work shows how the 35S promoter can be improved for use in dicots.]

Kohli, A. M. Leech, P. Vain, D.A. Laurie, and P. Christou. (1998) Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. Proceedings of the National Academy of Science, USA 95, 7203-7208. [This paper deals with the mechanisms of DNA integration into plants.]

Köhler, W.R. Zipfel, W.W. Webb, and M.R. Hanson. (1997) The green fluorescent protein as a marker to visualize plant mitochondria *in vivo*. The Plant Journal 11, 613-621. [This important paper introduces the green fluorescent protein, one of the most useful marker genes available.]

Mascarenhas, D., I.J. Mettler, D.A. Pierce, and H.W. Lowe. (1990) Intron-mediated enhancement of

heterologous gene expression in maize. Plant Molecular Biology 15, 913-920. [This paper deals with a method of increasing gene expression in maize.]

Omirulleh, S., M. Ábráham, M. Golovkin, I. Stefanov, M.K. Karabaev, L. Mustárdy, S. Mórocz, and D. Dudits. (1993) Activity of a chimeric promoter with the doubled CaMV 35S enhancer element in protoplast-derived cells and transgenic plants in maize. Plant Molecular Biology 21, 415-428. . [The work in this article discusses the effects of a particular promoter in transgenic cells and plants.]

Ow, D.W., K.V. Wood, M. DeLuca, J.R. De Wet, D.R. Helinski, and S.H. Howell. (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Science 234, 856-859. . [Before the discovery of the green fluorescent protein, luciferase, described in this paper, was a widely used marker.]

Rao, K.V., S.K. Rathore, T.K. Hodges, X. Fu, E. Stoger, D. Sudhakar, S. Williams, P. Christou, M. Bharathi, D.P. Bown, K.S. Powell, J. Spence, A.M.R. Gatehouse, and J.A. Gatehouse. (1998) Expression of snowdrop lectin (GNA) in transgenic rice plants confers resistance to rice broth planthopper. The Plant Journal 15, 469-477. [Lectins can protect plants from insects as described in this article.]

Sandhu, J.S., C.I. Webster, and J.C. Gray. (1998) A/T-rich sequences act as quantitative enhancers of gene expression in transgenic tobacco and potato plants. Plant Molecular Biology 37, 885-896.

[The work in this paper shows how gene expression can be enhanced in transgenic plants.]

Vancanneyt, G., R. Schmidt, A. O'Connor-Sanchez, L. Willmitzer, and M. Rocha-Sosa. (1990) Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Molecular and General Genetics 220, 245-250. [This important paper shows how to distinguish between transgenic cells and contamination by *A. tumefaciens*.]

Vaucheret, H., C. Béclin, T. Elmayan, F. Feuer bach, C. Godon, J-B. Morel, P. Mourrain, J-C. Palauqui, and S. Vernhettes. (1998) Transgene-induced gene silencing in plants. The Plant Journal 16, 651-659. [This is one of the early papers on gene silencing in plants.]

Biographical Sketch

Jennifer Thomson has a BSc in Zoology from the University of Cape Town, an MA in Genetics from Cambridge and a PhD in Microbiology from Rhodes University. She was a post-doctoral fellow at Harvard Medical School. She was a lecturer, senior lecturer and Associate Professor at the University of the Witwatersrand, Director of the CSIR Laboratory for Molecular and Cell Biology before becoming Professor and Head of the Department of Microbiology at the University of Cape Town. She is currently Professor in the Department of Molecular and Cell Biology at UCT. Her main research interest is in the development of maize resistant to viruses and tolerant to drought.