Gene targeting in higher plants

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Gene targeting technology (Fig. 1), involving the disruption or substitution of specific alleles, is the most sophisticated tool of reverse genetics. Its use in experimental systems ranging from yeast to mouse allows virtually any cloned gene, even of unknown function, to be specifically mutagenised *in vitro* and reintroduced precisely into its own chromosomal location. Thus, the effects of null, deleterious or even advantageous mutations to that gene can be directly determined. Gene targeting is routinely used in yeast and has been successfully applied to a range of other lower eukaryotes including filamentous fungi, slime mould and trypanosomes. In these organisms the frequency of homologous recombination (HR) is high (over 10%) compared with the chances of random integration by illegitimate recombination (IR). In most eukaryotic cells, however, DNA integration by IR predominates and the frequency of HR is extremely low, making gene targeting infeasible. In a few animal systems, this problem has been overcome by focusing on cell types with the highest HR frequencies and by using powerful negative selection systems. Mouse embryonic stem cells, for example, can now be fairly routinely used to generate targeted gene knock-outs, although the ratio of HR to IR in these cells is still 10 to 1000-fold lower than in yeast. Gene targeting has therefore become an invaluable tool for both animal and microbial geneticists for the elucidation of gene function and for the generation of improved strains by genetic manipulation.

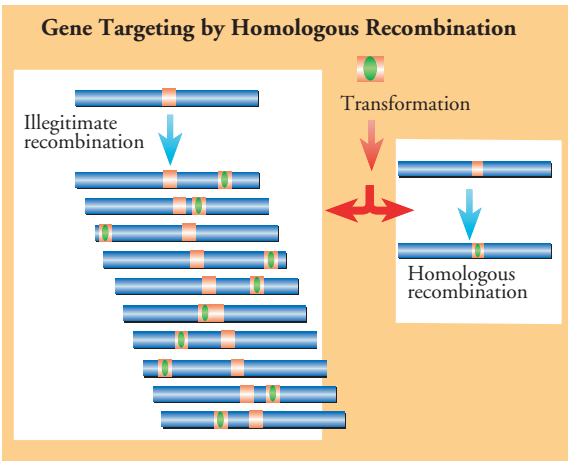

Figure 1 Genetic transformation in higher plants occurs by illegitimate recombination between unlike DNA sequences - in each transformation event the transgene integrates at a different site in the genome. This can result in unwanted effects such as disruption of endogenous gene function or inappropriate transgene expression. Several hundred transgenic lines may have to be generated before a suitable candidate line with the desired phenotype is identified.
In contrast, targeted recombination at a specific site mediated by homologous DNA sequences has no collateral effects and expression level is similar to that of the endogenous gene. Knock-outs, knock-ins and allelic replacements become possible.

By comparison, it has so far proved impossible to establish a feasible gene targeting system for higher plants. This is despite the fact that gene targeting is a key technology both for functional genomics and for the rational, accurate and safe exploitation of plants through genetic manipulation. Current plant genetic manipulation strategies rely on random integration of foreign DNA into the plant genome by illegitimate recombination. Neither the exact site of transgene integration nor the number and organisation of DNA molecules inserted can be experimentally controlled. These factors influence the level, pattern and stability of transgene expression and the likelihood of the transgene eventually becoming silenced. The unpredictability of these processes is one of the major concerns underlying opposition to the use of genetic manipulation for crop improvement. Gene targeting could reduce this concern by offering a more precise, predictable and cleaner method for genetic manipulation of plants. Transgene integration could be directed to particular locations in the genome where unstable expression or other 'position effects' would be minimised. The DNA to be integrated could be very exactly delimited at each end, because integration would be strictly homology-dependent. Moreover gene targeting offers the potential to completely knock-out expression of target genes or to make specific changes to gene function, objectives that cannot be achieved by conventional transgenesis. Given this importance it is clear that commercial biotechnology is active in this area. It is unlikely however that successful gene targeting approaches will be made freely available through this avenue. It is therefore imperative that such research be publicly funded - the importance in the coming years for a clean gene technology

emanating from public laboratories cannot be overestimated.

Development of a gene targeting system for plants is likely to depend on the identification or development of cell types where the ratio of homologous recombination (HR) to illegitimate recombination is maximal. One of the most hopeful discoveries of recent years has been the finding that HR is highly efficient (over 90%) in haploid tissues of the moss *Physcomitrella patens*. This may be because, during the haploid growth phase, random integration of foreign DNA would be immediately mutagenic and is therefore protected against, while constraints on HR to prevent recombination between allelic sequences are unnecessary. If these assumptions are correct, then haploid higher plant tissues might also offer high HR frequencies and thus be the best substrate for gene targeting experiments.

Previous work in tobacco (*Nicotiana tabacum* L.) indicated that microspores at late uninucleate/early binucleate stages can be isolated from flower buds and *in vitro* culture methods optimised for their maturation to fully functional viable pollen which, after application to the stigma of emasculated plants *in situ*, led to the generation of large numbers of seed. We have established efficient protocols for the biolistic introduction of a construct containing a reporter gene and selectable marker into these microspores and hence, after *in vitro* maturation and *in situ* fertilisation, for the generation of transgenic plants. Stable transformants of low copy number were generated by this procedure. The efficiency of transformation achieved allowed the production of large numbers of transgenic plants without selection, dispensing with the requirement for a selectable marker in plant transgenesis (Table 1).

DNA delivery method	Target microspores	Maturation protocol	Transformation efficiency
biolistics	late uninucleate/ early binucleate	to pollen used for fertilisation	20%
biolistics	early/mid binucleate	via embryogenesis to regenerate plants	21%

Table 1 Transformation of the haploid male microspore.

Microspores at various stages of development were further isolated from flower buds of tobacco (*Nicotiana tabacum* L.) and *in vitro* culture methods optimised for their switch to an embryogenic developmental pathway, resulting in the high-throughput production of haploid and double haploid plantlets. Biolistic transformation as above was applied to these microspores and, after *in vitro* embryogenesis, transgenic plants were regenerated. Again, stable transformants of low copy number were generated by this procedure. The efficiency of transformation achieved also allowed the production of homozygous transgenic plants without selection (Table 1).

Having developed highly efficient transformation systems for haploid cells, we next developed a selection system which would allow the detection of, potentially rare, homologous recombination events. In reciprocal experiments (Fig. 2), two related gene constructs were prepared as targets. In the first, three stop codons were introduced at the start of the *aphIV* gene, encoding hygromycin resistance, rendering the mutated gene non-functional. In the second, a second non-functional *aphIV* gene was prepared by the introduction of three stop codons near the middle of

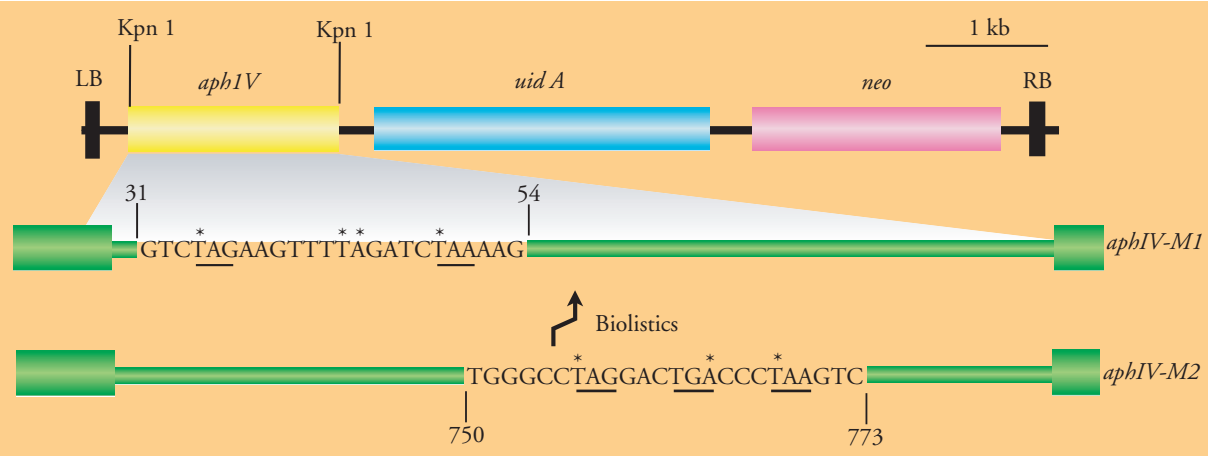

Figure 2 Microspores from transgenic lines carrying either of two mutated hygromycin-resistance genes were bombarded with the reciprocal mutant construct, matured to pollen *in vitro* and used to fertilise emasculated recipient plants. Resulting seeds were germinated and hyromycin-resistant seedlings selected.

the gene. Two sets of tobacco lines were generated (by *Agrobacterium*-mediated transformation) carrying either of these constructs and microspores harvested from these lines. Microspores carrying one mutant gene were then bombarded with the second, and matured to pollen that was subsequently used to fertilise emasculated wild-type tobacco plants. Seed from these fertilisations were germinated in the presence of hygromycin to detect the presence of a rescued hygromycin resistance gene. Initial results from these experiments indicate a ratio of homologous to illegitimate recombination in haploid cells that is two orders of magnitude greater than that observed in diploid tissues. Current experiments are determining whether such a frequency can be achieved using an endogenous gene as a target, to rule out the possibility that the high HR frequency observed resulted from the use of a transgene as a target.

If endogenous genes can be targeted at the frequency observed for transgenes, then gene knock-outs, knock-ins and allele replacements will be possible, using simple PCR-based screening for allelic constitution. No selectable marker is required. The approach is fast and requires no *in vitro* regeneration of plant tissues hence minimising somaclonal variation. It should be applicable to other species (e.g. cereals) that have developed microspore maturation protocols and will be invaluable for functional genomic studies, as well as more precise genetic engineering (with fewer collateral effects) for germplasm enhancement.