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Mini-review

Gene replacement

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Introduction

Gene replacement (also called gene targeting) refers to a technique whereby an endogenous gene is modified at its original locus in the genome. The technique is based on recombination between the target locus and a cloned homologous DNA fragment containing the desired modification. This cloned DNA fragment is known as the targeting vector and is introduced into the target cell by transformation techniques. Gene replacement technology was first demonstrated in yeast [see 5 for review]. The technology has now found use in mouse embryonic stem cells [6, 7 for review], where it is possible to introduce various modifications including complete knockout of gene function [e.g. 1, 8, 42, 71], deletions of defined regions [44] and the introduction of designed point mutations into the target locus [e.g. 3, 19, 76, 79]. The development of this technology for use in plant systems is still in its infancy. Experiments show that the homologous recombination events required for gene replacement do occur in plant cells but that the frequency of these events are currently too low to allow practical application of gene replacement. If this problem can be overcome, the potential applications of the technique are great and extend to both basic plant research and crop improvement. Applications in basic research will involve use of the technique to perform reverse genetic analysis of the function of newly identified genes. Crop improvement applications will include the inactivation of genes responsible for undesirable traits, the modification of existing genes and the introduction of new genes into defined locations in the genome.

Here we summarize the gene replacement technique as it applies to animal cells, discuss the progress towards applying gene replacement to plant cells and outline the possible approaches to make gene replacement a technique generally applicable in plant biology. (For another recent review of this subject see [51].) Although gene replacement in plant plastids is becoming a practical reality [see 37, 38 for reviews], in this review we wil only discuss replacement of nuclear genes.

DNA introduced into animal and plant cells integrates randomly into the genome by illegitimate recombination. The frequency of this illegitimate recombination is usually much higher than the frequency of homologous recombination. Developing gene replacement technology therefore not only means maximizing the frequency of homologous recombination, but also improving selection for the rare homologous integration event. The general methods used to approach these problems will be discussed in the following sections.

Maximizing the frequency of homologous recombination

Amount of homology between targeting vector and target

A positive relationship between length of homology and targeting frequency has been found in

mammalian cells by many workers [20, 61, 70]. Deng and Capecchi [14] found an exponential relationship between targeting frequency and the length of homology. At a homology of 2 kb the absolute targeting frequency (ATF = number of recombinants/number of transformed cells) was 10^{-6}. This value is similar to the ATF obtained in plant gene replacement experiments so far (Table 1). Note from this table that, so far, the maximum amount of homology that has been used in these plant experiments is 3.6 kb. Larger amounts of homology are routinely used to obtain targeted replacements in mammalian cells.

Quality of homology

It has been observed in mammalian systems that the frequency of gene targeting is highest if the homologous segment in the targeting vector is isolated from the same cell line as is to be used as the recipient in the targeting experiment [14, 69, 77]. Thus, it appears that the small sequence differences between DNAs from different cell lines lead to decreased frequencies of gene targeting. Most plant gene targeting experiments so far have used artificial target loci, thus guaranteeing perfect homology between target locus and targeting vector. However, to maximize targeting frequency of endogenous plant genes it will be important to use preferably isogenic DNA.

Vector design

In general, two different types of targeting vector are used in gene replacement experiments: replacement vectors and insertion vectors. These vectors are shown in Fig. 1a/1b as used for the introduction of a mutation into the genome. Both vectors need the integration of a positive selection marker. This may be removed in a later step using advanced strategies such as those called 'hit and run' (Fig. 1c) and double replacement (Fig. 1d).

In replacement-type vectors the DNA is linearized outside the region of homology before introduction into the cell. Homologous recombination (double cross-over or gene conversion) causes the sequence at the target locus to be replaced by the sequence in the targeting vector (Fig. 1a). In insertion type vectors the DNA is linearized within the region of homology. Homologous recombination (single cross-over) at this double-stranded break (DSB) causes the entire targeting vector to be inserted at the target locus and results in a duplication of the target region (Fig. 1b). Intra-chromosomal recombination (ICR) between the duplicated segments can lead to a resolution of this structure, leaving behind a target locus with the desired mutation (Fig. 1c). The combination of insertion and subsequent selection of an ICR-mediated deletion of one copy of the target segment using a negative selectable marker (see below) forms the basis of the so called 'hit and run' or 'in-out' gene replacement strategy [19, 60, 76]. Using this strategy it is possible to introduce even subtle base pair mutations in the genome. A second way to introduce subtle mutations in the genome is by the 'double replacement' strategy shown in Fig. 1d [3, 79]. Here in the first step the gene of interest is 'tagged' with a replacement-type vector. This event is selected for with a positive selective marker. In the second step a second replacement-type vector (containing the subtle mutation) is introduced. Selection against cells expressing the negative selectable marker (see below) results in enrichment for cells in which the subtle mutation has been introduced by a second gene replacement event.

Theoretically, insertion-type vectors should be more efficient for targeting than replacement-type vectors since the former requires only one, and the latter two cross-over events to achieve gene targeting. However, it was found that the efficiency of these vectors differs for certain loci only [14, 21]. It was proposed that this may be indicative of a different DNA sequence or chromatin structure preference for the different targeting pathways [21].

Published plant gene targeting experiments (Table 1) have exclusively used replacement-type vectors. However, we are currently assessing a protocol based on targeting at an endogenous *Arabidopsis* locus using 18 kb of homology with

Table 1. Lengths of homologous overlaps and absolute targeting frequencies (ATF) observed in plant cells.

First author (reference)	Homology (kb)	Cells surviving	Targeted recombinants	ATF
Paszkowski [52]	0.4	7.7×10^{6} [a]	8	1.0×10^{-6}
Lee [35]	1.9	1.1×10^{6}	3	2.8×10^{-6}
Offringa [47–49] Risseeuw (pers. comm.)	3.6	1.1×10^{7}	9[c]	0.8×10^{-6}
Halfter [17, 18]	1.1	3.5×10^{5} [b]	2	5.7×10^{-6}

[a,b] Obtained by multiplying the number of treated cells with a plating efficiency of (a) 10% [46] and (b) 0.5% [11].
[c] A summary of four different experiments.

an insertion-type vector and screening by polymerase chain reaction (PCR).

Detecting the rare homologous recombination event

Screening for the recombination event

Two screening methods have been employed in mammalian cells. PCR has been used to detect the junction fragment produced by homologous recombination [26, 28, 65, 81]. One PCR primer is specific for a unique vector sequence (the positive selectable marker for example) and the other primer specific for a site in the chromosomal locus not contained in the targeting vector. A prerequisite for this type of screen is that the selectable marker be placed near one end of the targeting vector since PCR amplification is not efficient for long PCR products. This type of screen has the advantage that it is sensitive, can be performed on rather crude extracts and pooling of clones allows screening of large numbers. On the negative side this method may be unreliable with significant false-negatives or false-positives [6]. False-positives may be explained by recombination occurring between homologous DNA sequences during the PCR reaction itself [43]. Southern analysis can also be used to screen for unique junction fragments produced by gene targeting. The screen is reliable and does not restrict the positioning of the selectable marker within the targeting vector. Although it is a labour-intensive method [63], with the use of genomic DNA micro-extraction procedures [33, 58], it has been successfully utilized to detect targeted recombinants in mammalian cell lines [79].

Direct selection for the targeting event

Initial gene replacement experiments with mammalian cells involved the use of defective antibiotic resistance transgenes which were then repaired by targeting constructs and the resulting recombinant cells selected on antibiotics [64, 72]. Experiments then moved on to endogenous genes in which a gene targeting event could be selected for. For example, it was possible to disrupt the hypoxanthine phosphoribosyl transferase (HPRT) gene in mice by selecting for this event on 6-thioguanine [70].

Early experiments in plant cells have also involved repair of defective antibiotic resistance genes. These experiments have shown that gene targeting events can occur in both tobacco [48, 49, 52] and *Arabidopsis* [18] cells. Targeting vectors delivered by *Agrobacterium* or using polyethylene glycol (PEG) are capable of causing gene replacement. Homologous recombination in plants is high-fidelity with a very low frequency of mutations introduced during the process [24, 50]. It has also been possible to induce a selectable modification in an endogenous tobacco gene using an *Agrobacterium*-delivered targeting vector [35]. The targeting frequencies obtained in these experiments have been low (Table 1), discouraging attempts at targeting non-selectable genes. The main reason for this may have been the use of homologous segments of suboptimal length.

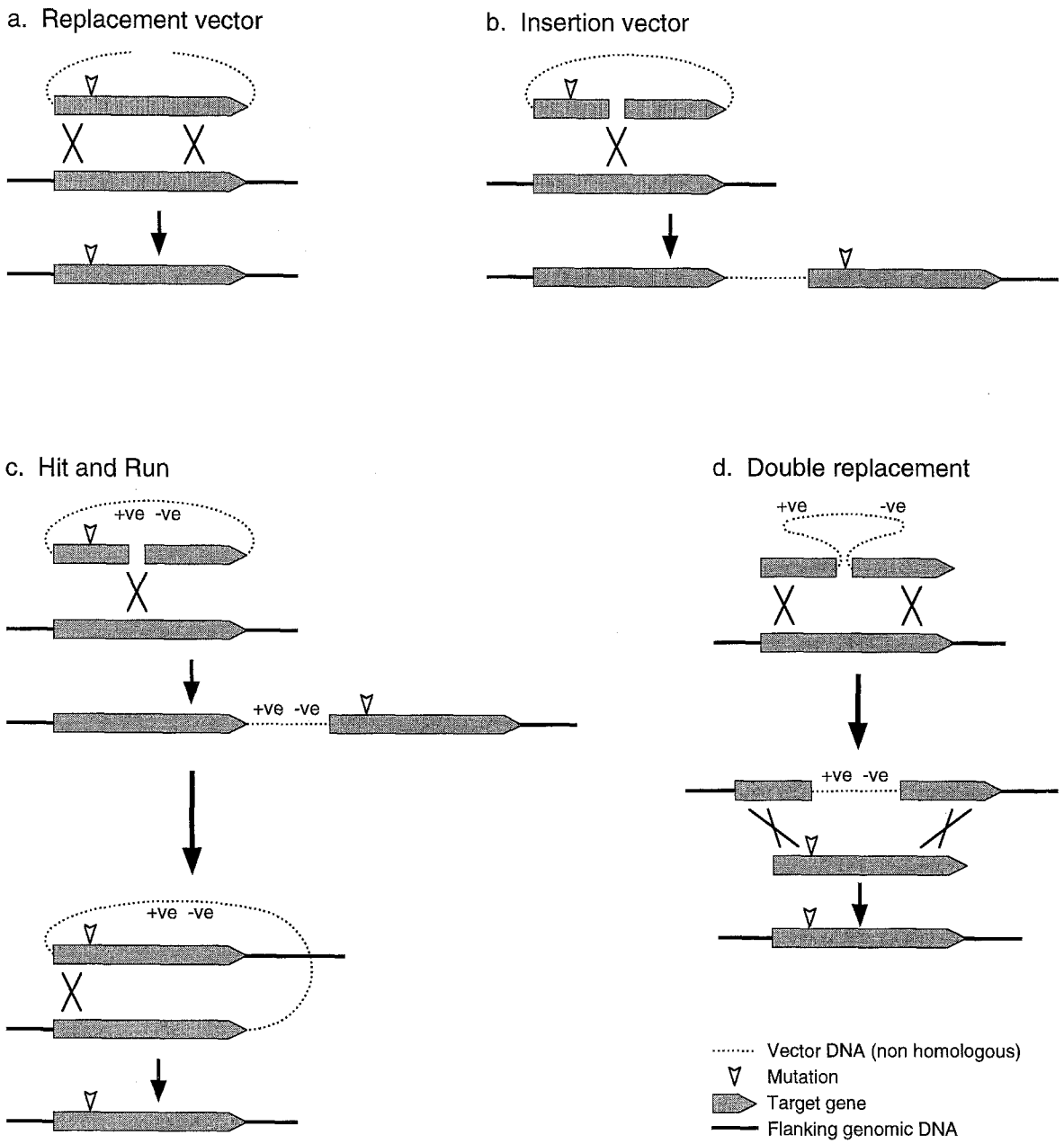

Fig. 1. Different types of vector systems used for gene targeting. + ve = positive selective marker; – ve = negative selective marker.

Marker fusion

Positive selectable markers missing either a promoter or a terminator sequence but flanked by sequences homologous to the target gene have been used for targeting in mammalian systems. Integration by homologous recombination at the target locus provides the missing promoter or terminator to the selectable marker making it functional (Fig. 2a) while disrupting the function of the target gene. Using this approach, up to a 100-fold enrichment for gene targeting has been obtained [1, 29, 39, 40, 42, 71]. The use of this strategy is limited by the requirement that the

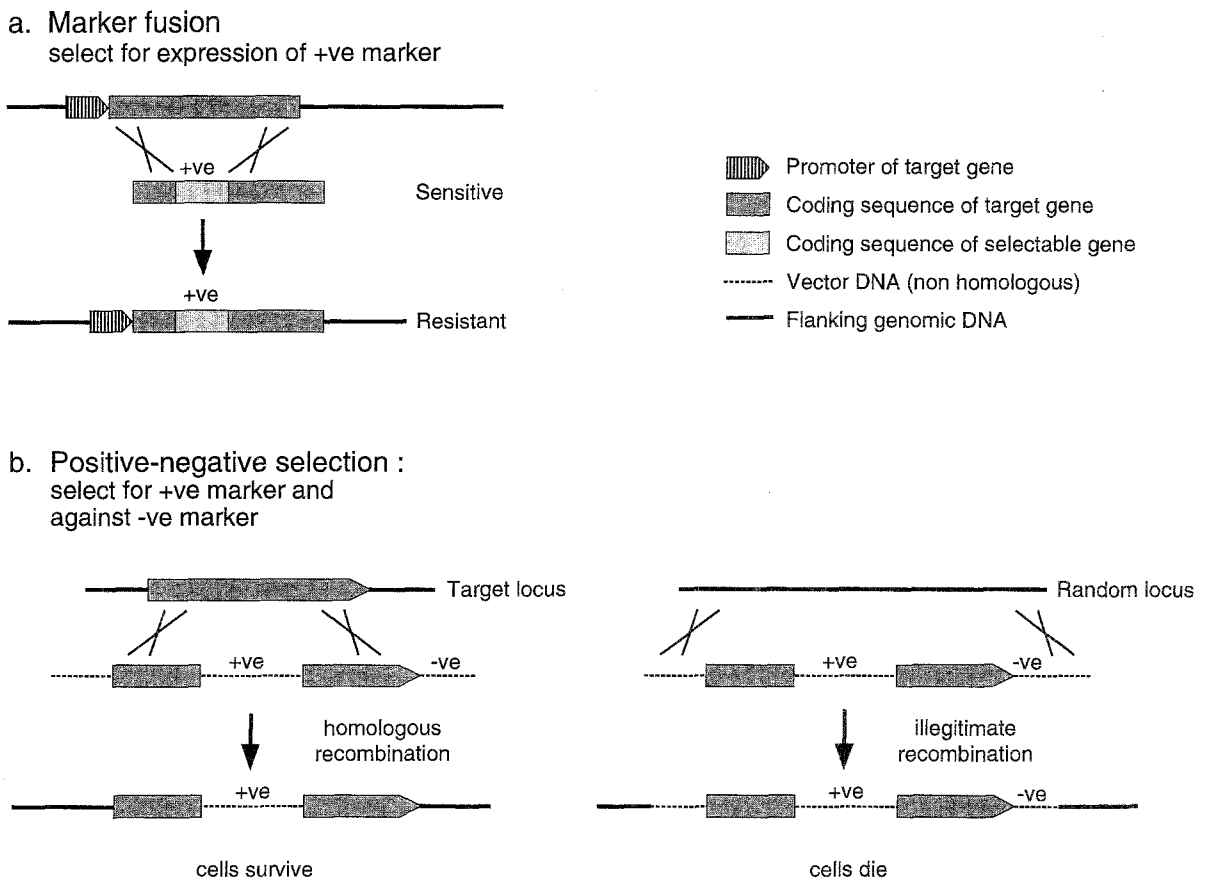

Fig. 2. Principles of (a) marker fusion, (b) positive-negative selection. See text for further details.

target gene be expressed in the tissue type used for the targeting experiment.

There are no published reports of successful use of this method for achieving gene targeting in plant cells. This method was evaluated in our laboratory with an experiment designed to target the *rbcS* (ribulose-1,5-biphosphate carboxylase small subunit) gene of tobacco. However targeting was not achieved, possibly because expression from the *rbcS* promoter in callus tissue was too low to allow selection [12, 47]. These experiments also confirmed that promoterless transgenes fuse with endogenous promoters at remarkably high frequency [22, 27, 32]. Another problem relevant to this system is the observation that defective genes in the targeting vector can fuse to other promoters also contained in the vector and cause a high background of antibiotic-resistant plants [13].

Positive-negative selection

In this system a positive selectable marker with its own promoter is included within the homology to the target. This provides selection for integration of the vector. A negative selectable marker (a gene which will kill the cell if expressed) is included in the targeting vector flanking the homology region. Cells in which random integration of the entire targeting vector has occurred can then be selected against using the negative selectable marker. Homologous recombination, however, results in integration of the positive selectable marker into the target locus but not of the negative marker. Cells in which this has occurred survive the negative selection (Fig. 2b). This strategy typically achieves enrichments from 10-fold to 10000-fold [1, 29, 39, 40, 42, 71]. Our group investigated the use of the *aux-2* gene as a negative

selectable marker in gene replacement experiments. This *Agrobacterium*-derived gene converts an inactive auxin, α-naphthalene acetamide (NAM), to an active form, naphthalene acetic acid (NAA), which is toxic to cells at high concentrations. The use of this negative selectable marker caused a 3- to 4-fold reduction in false-positives in targeting experiments [47]. However, a major drawback of this system was that the *aux-2* gene has non-cell-autonomous effects, because NAA excreted from the dying *aux-2*-positive cells also killed rare *aux-2* minus cells. This is also known as the 'innocent bystander effect'.

The DT-A gene encodes the A-chain of diphtheria toxin which is highly toxic to cells but acts in a cell-autonomous manner. This gene has been successfully used as a negative marker in gene replacement experiments in mammalian cells [41, 80]. The DT-A gene is a non-conditional negative marker for plants as well [9, 31, 74]. Transient expression of the DT-A gene in plant cells causes cell death ([9] and unpublished observations by E. Risseeuw in our laboratory). Such transient expression from a targeting vector would result in cell death before a targeting event has a chance to occur. Therefore, a prerequisite for the use of such a marker is an inducible promoter system. Unpublished observations from our laboratory indicate that expression of the DT-A or 'attenuated' DT-A coding sequence from the tetracycline-inducible 'triple operator' promoter system [78] causes cell killing in tobacco even in the uninduced state. Better results may be obtained by using a tetracyclin-responsive promoter with the same degree of inducibility but with lower absolute expression levels.

The most commonly used negative selectable marker in gene targeting experiments in mammals has been the herpes simplex virus thymidine kinase gene (HSVtk). The product of this gene catalyses the phosphorylation of ganciclovir (a guanine analogue) converting it to a toxic form that inhibits DNA replication [66]. This thymidine kinase gene was also tested as a negative selectable marker in plants. *Arabidopsis* explants expressing the gene under the control of the 35S promoter showed a 25-fold reduction in shoot formation in the presence of the negative selective agent, ganciclovir [10]. In contrast, others reported that transgenic plants expressing this gene were not reproducibly sensitive to ganciclovir [55].

Another gene which might be used as a negative selectable marker in plant cells is the *Escherichia coli* gene for cytosine deaminase. Cytosine deaminase, which is found in prokaryotes but not in higher eukaryotes, converts the non-toxic 5-fluorocytosine to 5-fluorouracil which is toxic to cells. The *E. coli* gene has been used as a negative marker in mammalian cells [45]. It also works as a negative selectable marker in tobacco [67] and *Arabidopsis* [55] at the seedling level, a situation where cell autonomous action is not important. We are assessing its effectiveness as a negative marker for gene replacement experiments.

It is likely that a negative marker with characteristics useful for gene replacement strategies in plants will be developed eventually. However, it is still an open question as to how useful such a negative marker will be for the direct selection of gene replacement in plants. Negative markers can only enrich for homologous recombination if a majority of recombinant cells do not have additional randomly integrated targeting vectors. Whereas this is the case for the majority of homologous recombinants in animal experiments [15, 25, 62, 73, 81], several of the recombinant plant lines obtained so far had extra random insertions of the targeting vector (unpublished observations in our laboratory by Offringa and Risseeuw). Another problem with negative selection which seems specific for plants, is the occurrence of transgene silencing in plants [23]. Nevertheless, negative selectable markers will probably become an important tool in gene targeting in plants as well as essential components of strategies such as 'hit and run' and 'double replacement'.

Future prospects

Recombination rates may be enhanced in plants and thus targeting frequencies improved. Treat-

ments that induce damage to DNA have been shown to increase rates of intrachromosomal recombination in plant cells [34, 53, 75]. Such treatments may also increase targeting frequencies but they will have an unwanted side-effect of introducing other mutations into the plant material. Such a problem could be dealt with since the targeted mutation should segregate with the selectable marker enabling other induced mutations to be crossed out.

Another possibility for increasing recombination rates is to over-express proteins associated with recombination in plant cells. For example, the well characterized *E. coli* RecA protein (which is involved in homology search and strand exchange) has been expressed in tobacco plants and targeted to the nucleus. Such plants show elevated extra-chromosomal recombination frequencies and are more resistant to compounds that induce double-stranded breaks in DNA (B. Reiss, M. Klemm, H. Kosak, and J. Schell, personal communication). Experiments are in progress to examine if enhanced gene targeting frequencies are obtained with such transgenic plant lines.

Gene targeting is thought to occur mainly according to the double-stranded break repair model [54, 60, 76] proposed by Szostak *et al.* [68]. If this is true then it might be that the induction of double-stranded breaks in the chromosome enhances recombination frequencies. In an elegant experiment, Puchta *et al.* [57] introduced *in vivo* double-stranded breaks into an extrachromosomal recombination substrate at an 18 bp I-*Sce* I site by co-expressing the I-*Sce* I restriction enzyme in cells. *In vivo* induction of this double-stranded break enhanced recombination by 10-fold. Results of recent experiments using the same system but assaying gene targeting frequencies at a chromosomally located recombination substrate containing the I-*Sce* I site indicate that *in vivo* induction of double-stranded breaks in the genome also produces a strong enhancement in recombination (H. Puchta and B. Hohn, personal communication). The next question is then how double-stranded breaks can be specifically introduced at the target locus? The gene targeting method routinely used in *Drosophila* suggests a way to do this. In *Drosophila* excision of P-elements from their chromosomal location appears to involve the formation of a double-stranded break. If an ectopic copy of the sequence in which the P-element is inserted is present in the cell during P-element excision then the double-stranded gap can be repaired using the ectopic copy as a template resulting in gene replacement [16]. A similar technique can be used in the nematode *Caenorhabditis elegans* [56]. As transposon tagging systems in plants become more advanced [2, 4, 36] and there are plant species saturated with tags then it may become possible to isolate the line in which the gene of interest is tagged by pooled PCR screening of a library of the tagged plant DNAs [30]. If gene knockout is the ultimate aim of the experiment then gene replacement becomes unnecessary. However, if a more subtle mutation of the gene of interest is required then excision of the transposable element could be induced in the presence of a targeting vector resulting in a targeted mutation. However, a theoretical comparison of the models of excision of plant transposable elements and *Drosophila* elements suggests that the DSB mechanism may only play a minor role in the excision of transposable elements in plants [59] thus restricting the possible usefulness of this protocol.

Conclusion

Gene replacement techniques would be the ultimate tool in a molecular plant breeder's armoury of techniques allowing modification of the genome at extremely high resolution. So far, attempts at developing such techniques for plant cells have had limited success and it would appear that the research effort in this area is declining. However, we are hopeful that future concerted research efforts will finally achieve a gene replacement technology that can be practically applied in plant breeding research.

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