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Plant genome modification by homologous recombination

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The mechanisms and frequencies of various types of homologous recombination (HR) have been studied in plants for several years. However, the application of techniques involving HR for precise genome modification is still not routine. The low frequency of HR remains the major obstacle but recent progress in gene targeting in *Arabidopsis* and rice, as well as accumulating knowledge on the regulation of recombination levels, is an encouraging sign of the further development of HR-based approaches for genome engineering in plants.

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Abbreviations

DSB	double-strand breaks
ECR	extrachromosomal recombination
EGT	ectopic targeting
GT	gene-targeting
HR	homologous recombination
ICR	intrachromosomal recombination
NHEJ	non-homologous end joining
PPO	PROTOPORPHYRINOGEN OXIDASE
SDSA	synthesis-dependent strand-annealing

Introduction

During meiosis, homologous recombination (HR) guides exchanges between chromosomes that produce novel combinations of alleles and is essential for proper chromosome segregation. In somatic cells, HR allows the precise repair of DNA double-strand breaks (DSBs) by the copying of homologous templates. Consequently, HR affects genome structure in more than one way. HR not only ensures genome integrity (through DSB-repair) but also contributes to genome evolution by the creation of new alleles or new allelic combinations through meiotic and possibly somatic recombination events. Somatic recombination may be of particular importance in plants as the plant germ line, in contrast to that of animals, differentiates late in development. Thus, genetic changes that are generated in somatic cells can be transmitted to progeny. For example, a somatic rearrangement at the *zein* locus of maize reached the gametes and was transmitted meiotically to the next generation [1].

DSBs can also be repaired by non-homologous end joining (NHEJ), which requires no or very limited sequence homology. However, NHEJ often results in sequence alteration at the restored junctions [2]. Although the mechanisms and molecular components of DSB repair seem to be evolutionarily conserved, the usage of these alternative repair pathways varies between organisms. HR is prevalent in prokaryotes and yeast, whereas NHEJ is predominant in multicellular eukaryotes [3]. This difference is reflected in the dissimilar outcomes of yeast transformation experiments compared to those in plant and animal cells. In yeast, even short stretches (approximately 50 bp) of homology to chromosomal DNA within transformation vectors are able to direct the integration of transforming DNA by homologous recombination. In contrast, transforming DNA is integrated into plant and mammalian cells at random positions by the action of the NHEJ pathway, regardless of the presence of stretches in the transforming DNA that are homologous to the host chromosomes. Random DNA integration by NHEJ occurs even when the number of random DSBs is artificially increased by DNA-damaging treatments such as X-ray irradiation [4]. Such natural preference for NHEJ and the low frequency of HR are the main obstacles to the development of efficient gene-targeting (GT) technology for use in angiosperms. In contrast, GT is an established tool for engineering the mouse genome [5] and has been successful in the moss *Physcomitrella patens* [6•]. Its potential has also been demonstrated in *Drosophila* [7,8], sheep [9] and human somatic cells [10].

In this review, we briefly depict the most likely fate of foreign DNA before and after chromosomal integration in plant cells. We review how current strategies that involve HR may influence this fate by targeting DNA to a specific chromosomal position and how superfluous DNA may be eliminated by intrachromosomal recombination (ICR) events.

Fate of foreign DNA in the plant cell

Extrachromosomal phase

Transforming DNA is usually introduced into a plant cell in one of two forms, either as a purified chemical molecule (naked DNA) or as a T-DNA complex that is transferred from *Agrobacterium tumefaciens*. Interestingly, these two forms of DNA are almost equally prone to efficient extrachromosomal recombination (ECR) after uptake [11,12]. ECR has been investigated extensively during

the past decade in plant species such as tobacco, *Arabidopsis*, maize and petunia. In all of these species, it occurs with efficiencies ranging from 10^{-1} to 10^{-3}, as measured by a comparison of the transformation frequencies with two non-overlapping deletions of a selectable marker gene to transformation frequencies with the intact gene. Linear molecules are more prone to ECR than circular DNAs, suggesting the involvement of single-strand annealing (SSA) [13] or synthesis-dependent strand-annealing (SDSA) processes in ECR [14]. Both SSA and SDSA processes involve strand invasion but the latter is also supported by DNA synthesis [15]. Co-transformation by *Agrobacterium* strains harboring two different T-DNAs or by two different purified plasmids has facilitated the analysis of ECR [16,17]. Junctions between DNA molecules that are involved in recombination can be associated with deletions or insertions of filler DNA that originates from either transforming or host chromosomal sequences. Regardless of the detailed mechanism, ECR results in head-to-tail linked arrays of transgenes. Although ECR is thought to occur before chromosomal integration, it is still possible that the concatamerization also takes place during or even after integration through some kind of 'homing mechanism' [16–19]. Clearly, a certain proportion of transgenic DNA integrates without ECR, resulting in a single-copy locus or several unlinked loci that contain single copies of foreign DNA that can be separated by genetic segregation.

Intrachromosomal phase

Linked multicopy transgenes are prone to epigenetic and genetic instability governed by gene silencing or by ICR, respectively. The frequency of ICR in plants has been determined using various recombination substrates that were inserted into the genome as direct or inverted repeats [20–22]. Recombination frequencies have usually been measured by the frequency with which gene function is restored after ICR. This system only reveals precise recombination events, however, and probably overlooks inaccurate rearrangements. As in mammals, the basal frequency of ICR in plants is low, varying between 10^{-5} and 10^{-6} of cells containing a recombination cassette. This can be increased by up to 9-fold by genotoxic stress factors such as irradiation (e.g. ultraviolet light or X-rays) or radio-mimicking chemicals (e.g. methyl methanesulfonate [MMS]) [23,24].

Importantly, a transient *in vivo* induction of DSB at a predetermined position between repeats of a recombination substrate efficiently induces local ICR events. Chiurazzi *et al.* [25] introduced a DSB into an *Arabidopsis* chromosome using HO-endonuclease, and thereby stimulated the frequency of ICR approximately 10-fold. Surprisingly, a very high stimulation of ICR (1000-fold) was achieved by expressing the *Activator* (*Ac*) transposase in *Arabidopsis*. Although this stimulation was possibly attributable to DSB generated by transposon mobilization, less-defined transposon-specific effects may also contribute to these unusually high induction levels [26]. Other stress factors that are not directly linked to the formation of recombinogenic DSBs have been implicated in the stimulation of ICR. For example, pathogen attack stimulates ICR [27], providing a compelling link to the proposed involvement of HR in the rapid evolution of resistance gene clusters [28]. On the other hand, the potential instability of loci with sequence redundancies can be induced by adverse stress conditions. This potential source of instability must be considered when designing transgenic constructs, selecting reliable transgenic strains or devising strategies for further processing of transgenic loci by ICR.

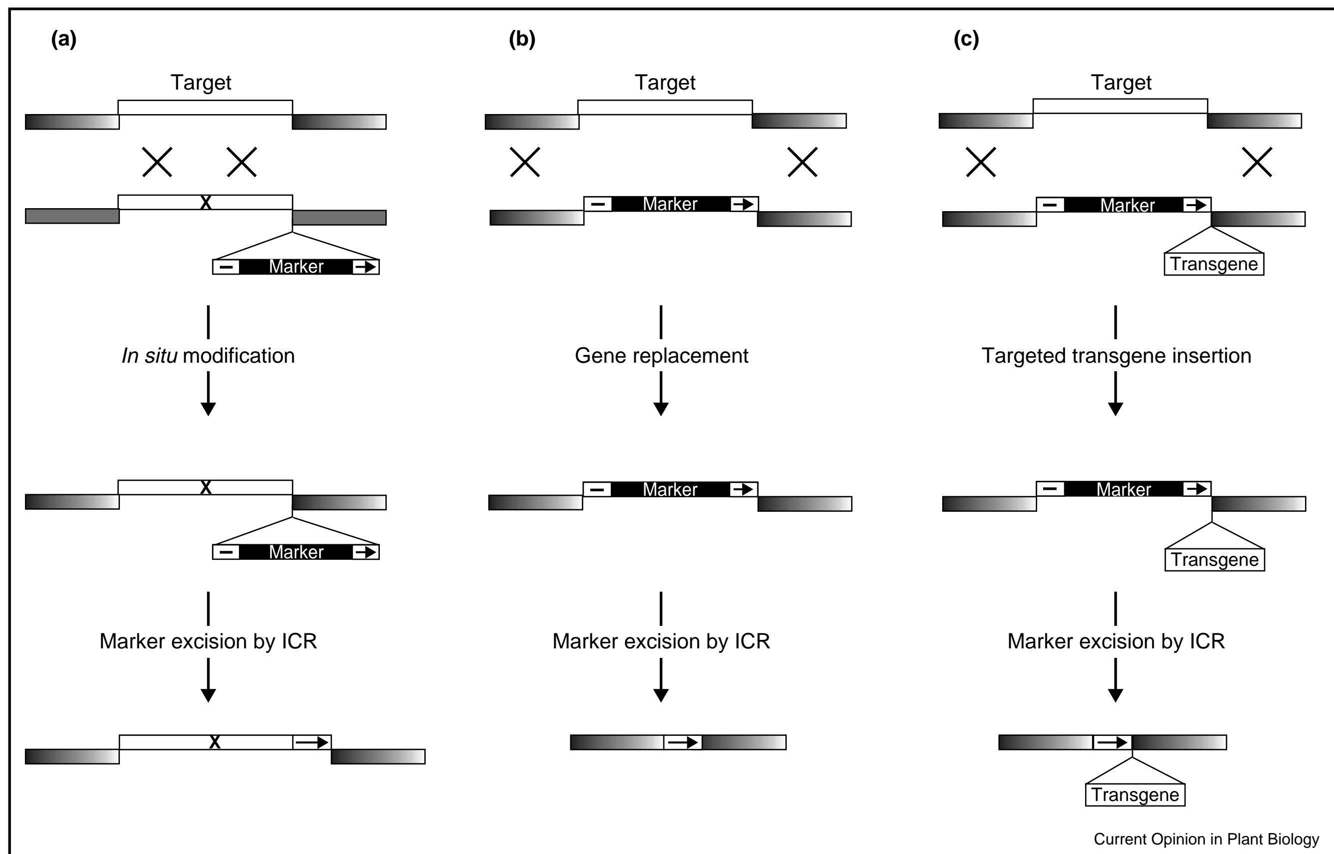
Altering the fate of foreign DNA by homologous recombination

The most straightforward and widely explored way to reduce or prevent the disadvantageous integration of multicopy DNAs is to select single copy inserts, assuming that single-copy DNAs have an enhanced genetic (physical) and epigenetic (functional) stability. In general, transformation followed by the selection of single copy inserts has been successful. As the transformed DNAs are integrated into random positions, however, this approach requires that several transgenic lines are characterized in detail to identify a line with a single functional copy of the inserted DNA. Site-specific recombination systems for the integration of DNA into a predetermined chromosomal position would appear to be an attractive way to eliminate position effects. Surprisingly, however, this is not the case; the integration of identical transgenic inserts into the same genomic location by site-specific integration resulted in variable levels of expression among the transgenic events studied [29]. The ultimate goal of plant genome modification is not only the controlled addition of foreign genes with regard to position, orientation and copy number, but also the *in situ* modification of endogenous genes. Here, the technologies of choice are certainly the various GT strategies (Figure 1).

Gene targeting

The first reports of successful gene targeting in mouse and plant cells appeared within the space of three years [30,31]. By 2001, the functions of more than 7000 chromosomal loci had been analyzed by targeting in mouse embryonic stem cells [5] but only a single targeting event of an endogenous gene was reported for flowering plants [32]. Using *Agrobacterium*-mediated transformation, Kempin *et al.* [32] reported knockout of the *AGAMOUS-LIKE5* (*AGL5*) MADS-box gene of *Arabidopsis* by HR. As these researchers obtained only a single targeting event among 750 transformants, they were unable to evaluate the reproducibility of GT or its frequency. There have been several attempts to determine the parameters that are crucial for efficient GT. Unfortunately, parameters that are known to be important for GT in mammals — for

Figure 1



Selected homologous recombination (HR)-based strategies. **(a)** *In situ* modification, for example the introduction of a point mutation (X). **(b)** Gene replacement, in which the target gene is deleted and replaced by a marker gene. **(c)** Targeted-transgene integration, in which the targene is inserted precisely into the genome by HR. In all cases, the marker gene used for transformation can be removed by ICR between direct repeats flanking the marker (represented by boxes containing an arrow).

example, the use of vectors that have long stretches of isogenic DNA, the DNA delivery method, the target cells or the species — seem to be of little or no consequence in plants [2].

A few years ago, the use of DNA–RNA hybrid molecules as targeting vectors was reported to be effective for the introduction of subtle, site-directed changes in chromosomal DNA [33–35]. Unfortunately, this approach has not become routine and probably needs further refinement.

Two important recent advances allowing more accurate estimation of GT frequency at chromosomal loci of *Arabidopsis* [36•] and rice [37•] have been reported. The first system is based on the modification of a gene encoding PROTOPORPHYRINOGEN OXIDASE (PPO). The acquisition of two specific mutations in *PPO* renders the gene product highly resistant to the herbicide butafenacil. Two simultaneous mutations are required for high herbicide tolerance and so spontaneous resistance has not been observed so far. GT by HR at the *PPO* locus is reproducible at a basal frequency of 2.4×10^{-3}. Moreover, this system distinguishes two types of targeting events, true gene targeting (TGT), which defines the predicted chromosomal modification, and ectopic targeting (EGT). EGT events result in ectopic integration of the incoming DNA after its extension using a homologous chromosomal template. The observed prevalence of EGT stems from the SDSA process, which is one of the main mechanisms of recombination in the somatic cells of plants. Accordingly, the ends of the T-DNA can act independently, with one of them interacting by HR before being integrated by illegitimate recombination.

Targeted disruption of the *Waxy* gene of rice was reproducible in several independent experiments [37•]. The observed GT frequency at this locus is approximately 0.65×10^{-3} (i.e. in the range reported for the *PPO* locus of *Arabidopsis*) [36•]. However, the design of this experiment included a positive/negative selection strategy coupled to a PCR-based screen for predicted insertion

into the *Waxy* gene. The TGT events detected by PCR were enriched to frequencies in the 10^{-2} range. Significantly, two copies of a negative marker that flanked the targeting homologous sequences were used in the targeting vector. Thus, effective negative selection should, in principle, eliminate random integration events. Indeed, the targeted lines showed neither ectopic targeting events nor random integration of additional copies of the targeting vector. Although targeting experiments of this sort in mouse have successfully employed positive-negative selection [5], such experiments were unable to detect the targeted disruption of the *Arabidopsis* chalcone synthase gene [38]. Thus, the efficiency of this procedure appears to vary in different species or with divergent chromosomal targets. Alternatively, it may simply rely on an optimal experimental design such as that used to disrupt the *Waxy* gene in rice.

The successful rice experiments provide an important guideline for future progress. Reproducible GT in rice may be attributable to the use of cells from embryogenic cultures. Terada *et al.* [37•] propose that these cells may be more proficient in HR or have reduced levels of NHEJ. It has been well documented that the important limiting factor for gene targeting in plants is the availability of DSBs at the target locus. The creation of a DSB using a restriction enzyme boosts targeting frequencies to the 10^{-1}–10^{-2} range in tobacco [39]. The DSB at the target can be repaired by HR or by NHEJ, and the mutual activities of these processes may determine the competence of plant tissues for GT. It has not yet been possible to identify 'GT-competent' plant cells. The current availability of reproducible GT systems in model dicotyledonous and monocotyledonous species should, however, facilitate the search for cell types and/or the molecular components that are crucial for GT competence.

Intrachromosomal recombination for sequence excision

An important challenge in transgene technology is the elimination of the superfluous foreign DNA that is introduced during transformation to allow the selection of transformed clones. Two main approaches have been developed to achieve this. The first exploits bacterial or yeast site-specific recombination systems; the second is based on plant transposable elements [40]. Although both methods are efficient, they are multi-step procedures and rather laborious. Moreover, these methods may not completely remove the undesirable sequences, the recognition site for the recombinase or the transposon footprint is usually left behind. ICR is therefore an alternative for the further trimming of transgenic loci to remove residual footprints. Low frequencies of ICR (discussed above) may restrict the broad application of this method; however, it is important to consider that the site-specific induction of DSB between repeats designed for ICR may increase the frequency of the predicted ICR events [41•]. It has been shown that up to 30% of such genomic DSB are repaired by predicted HR events, and thus the precise removal of superfluous transgenic DNA could be achieved efficiently [41•].

Recently, it was reported that efficient ICR-mediated excision between repeats of 352-bp-long attachment sites of the bacteriophage λ (*attP*) occurs in tobacco without DSB induction [42]. Although bacterial proteins that recognize *attP* were not present, a 5.9-kb fragment that contained a marker was efficiently excised by ICR in 2 out of 11 lines tested. It is possible that the high AT content of the *attP* region or the presence of a transformation booster sequence (TBS) next to an *attP* site enhances ICR [43]. Although the mechanism of this excision remains obscure and one *attP* sequence is left behind in the process, it would be interesting to test the generality of this approach in other plant species.

Towards regulation of HR

The site-specific introduction of a DSB in the vicinity of chromosomal duplications that are involved in ICR or in the neighborhood of an homologous landing path for GT vectors substantially increases the efficiency of predicted HR events [39]. It seems likely therefore that the availability of free DNA ends is an important limitation of HR. As free DNA ends in plant chromosomes are rapidly joined by the NHEJ reaction, NHEJ could successfully compete with HR and reduce its contribution to the repair of DSBs.

Attempts to improve the competitiveness of the HR machinery by overexpressing heterologous factors that are involved in recombination in other organisms, such as the *RecA* protein or the *RuvC* resolvase from *Escherichia coli*, produced some improvements in ICR frequency [44,45]. However, at least in the RecA-overproducing line, this strategy did not change the frequency of GT [45]. Given the evolutionary conservation of recombination mechanisms, the availability of the complete sequences of *Arabidopsis* and rice has intensified efforts to characterize endogenous regulatory components of NHEJ/HR [46]. Forward genetic screens for mutants that are affected in their response to genotoxic treatments have revealed novel components that influence levels of HR [47]. The continuation of these efforts should lead to a better understanding of plant-specific regulatory circuits that are involved in DSB repair. It is already obvious that chromatin structure contributes significantly to DSB repair [2]. The involvement of chromatin has been clearly illustrated by work on the MIM protein, one of a group of factors responsible for the structural maintenance of chromosomes (and therefore known as SMCs) [48]. Mutation or overexpression of the *MIM* gene reduces or stimulates ICR, respectively [47,49]. In addition, mutation of the RAD50 protein results in a 10-fold increase in ICR in *Arabidopsis* [50•]. RAD50 resembles the SMCs and is a part of a complex that includes the MRE11 and XRS2

proteins, which have been implicated in HR and NHEJ in yeast and mouse [51]. It would be of interest to isolate and characterize a novel set of mutants that have elevated levels of HR, similar to that described in tobacco [52]. Further, it would be interesting to examine the ICR and GT frequencies in plants that have mutations in genes that are known to be involved in NHEJ, for example the *ku80* mutant [53]. Existing tools such as the strains carrying well-characterized ICR substrates or the GT assays that have been developed for *Arabidopsis* and rice should speed up this process considerably.

Conclusions

The further improvement of targeting vectors and an increase of the HR competence of recipient cells seem to be crucial for more efficient GT technology in plants [54]. Vectors should not only contain negative selectable markers but also features of *in vivo* linearization that seem to be beneficial for GT in *Drosophila* [7]. Finally, the efficient HR and GT observed in the moss *Physcomitrella patens* [6•] may serve as a reference for further studies of the recombination mechanisms in seed plants.

It is clear that the ultimate goal of the routine use of GT technology for basic studies of plant gene functions and for plant biotechnology is still somewhere ahead. Nevertheless, given recent advances, it may be reached in the near future.

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