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Homologous recombination in plants

H. Puchta, P. Swoboda and B. Hohn

Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel (Switzerland)

Abstract. In plants three different approaches have been used to study homologous DNA recombination; extrachromosomal recombination (ECR) between transfected DNA molecules, intrachromosomal recombination (ICR) between repeated genes integrated into and resident at the genome and recombination between introduced DNA and homologous sequences in the genome (gene targeting). ECR is efficient (10^{-1} to 10^{-3}) and occurs mainly during a limited time period early after transfection. It proceeds predominantly via nonconservative single-strand annealing. ICR, which in most cases is described best by the double-strand break repair model of recombination, occurs at frequencies of one event in 10^5 to 10^7 cells. ICR takes place throughout the whole life-cycle of a plant, in all organs and at different developmental stages. As there exists no predetermined germline in plants, somatic recombination events can be transferred to the next generation. Recombination frequencies are enhanced by DNA damage. Gene targeting, like ICR, occurs at low rates in plant cells. Almost nothing is known about the enzymes involved in homologous recombination in plants.

Key words. Extrachromosomal recombination; intrachromosomal recombination; gene targeting; single-strand annealing; double-strand break repair.

Introduction

Whereas multiple studies have been performed on the topic in mammalian cells, homologous recombination in the plant cell nucleus has been analyzed only recently. This review will concentrate on recent experiments performed with model substrates to learn more about ECR, ICR and gene targeting in plants.

Extrachromosomal recombination

Extrachromosomal homologous recombination occurs naturally in plant cells between DNA viruses[18,37] and between T-DNAs of *Agrobacterium tumefaciens*[25,41]. However, ECR is most conveniently analyzed by transfecting plant protoplasts with plasmid molecules carrying overlapping, nonfunctional deletion mutants of a specific gene (for review see 35). Restoration of the functional gene by homologous recombination is either measured by transient expression[22,32] or as stable transformation[3]. In general, ECR is efficient, recombination frequencies of 10^{-1} to 10^{-3} being found. The recombination frequency increases with increasing length of the overlapping region of the plasmid substrates[3,32].

The most interesting but also the most complicated aspect of homologous DNA recombination is the pathway by which it proceeds. For eukaryotes two different working models are frequently used to describe homologous DNA recombination (for additional alternative models see Nicolas and Petes in this issue). One is the double-strand break repair (DSBR) model proposed by Szostak et al.[40], the other the single-strand annealing (SSA) model as proposed by Lin et al.[21] (fig. 1). Both models explain the general finding that double-strand (ds) breaks in certain locations of a gene enhance recombination rates. In the DSBR model a single ds break is the prerequisite of the recombination event. The ds break is enlarged by cellular nucleases and then repaired by the homologous sequence of the partner DNA. This process leads to the formation of a double Holliday structure as an intermediate. The structure can be resolved in two ways: either the flanking markers remain on the same DNA molecule or they are exchanged. The stochastic probability should be the same for both events. The process is a conservative one since both molecules taking part in the recombination reaction are restored. The predictions of the SSA model are different. According to this model a ds break is required in each DNA molecule. Ds breaks are the entry sites for single-strand exonucleases, which digest away one of the DNA strands. The resulting overhanging ss ends of the recombination partners are complementary. The two strands basepair and the intermediate structure is repaired using the corresponding ends of ds DNAs as primers. As both recombination partners suffer degradation of the DNA ends not involved in the exchange, this recombination process is nonconservative. Since only chimeric DNA molecules survive this process, flanking markers are exchanged in every case.

There are in principle two different ways by which recombination pathways can be analyzed; either the transfected recombination substrates are manipulated in a way that they mimic recombination intermediates, or the resulting recombination products are analyzed and compared with the predictions of the recombination models. The easiest way to mimic recombination intermediates is to introduce ds breaks into them in vitro. Linearization of both recombination substrates adjacent to overlapping sequences (the prototype situation of

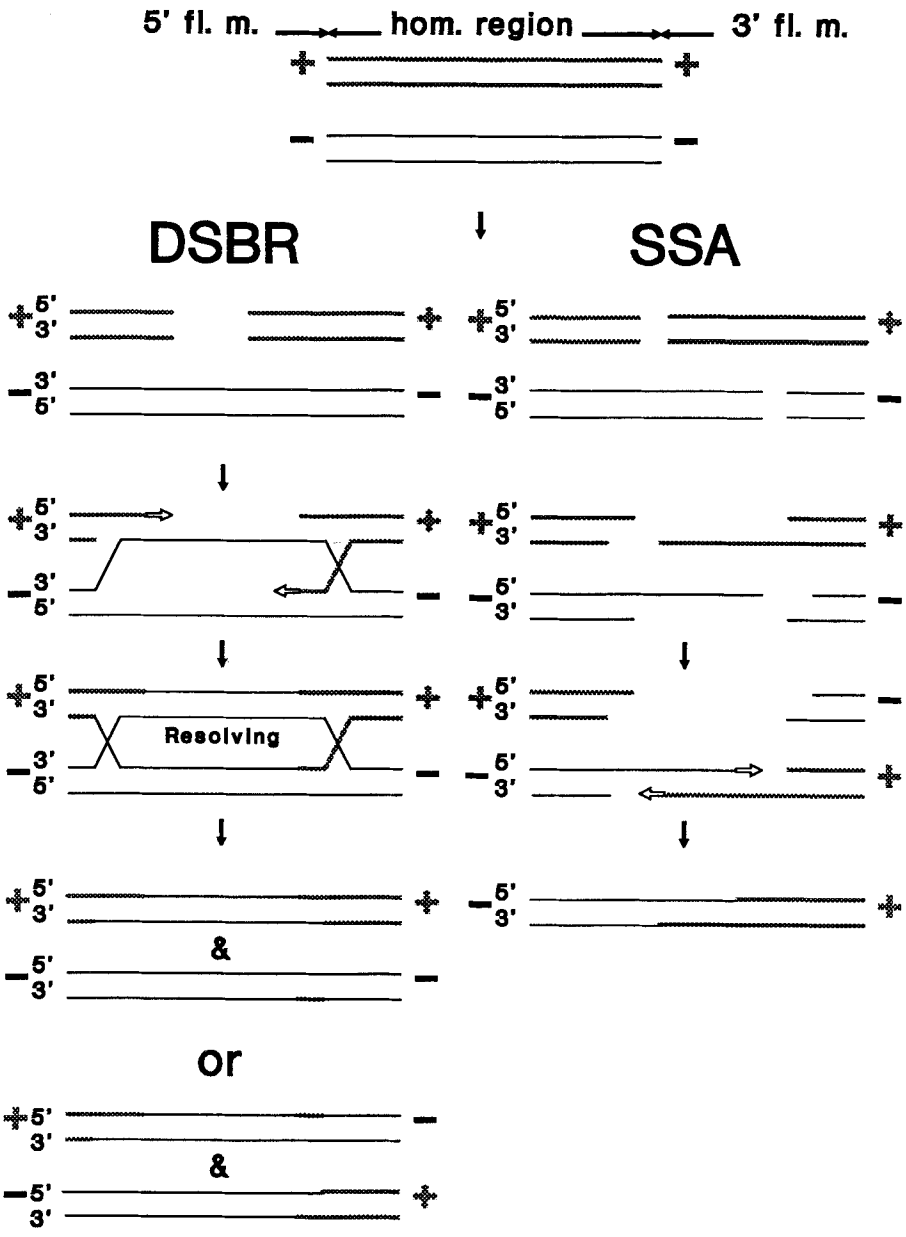

Figure 1. Two models proposed to describe homologous DNA recombination in eukaryotic cells. The two thin and thick lines represent homologous ds DNAs; '+' and '−' alleles of flanking markers; 5′ fl. m.: 5′-flanking marker; 3′ fl. m.: 3′-flanking marker; hom. region: homologous region; DSBR: double-strand break repair model; SSA: single-strand annealing model. Note that according to the SSA model one of the two molecules participating in the reaction is degraded. The flanking sequences are always exchanged. In addition to the molecule shown in the figure, in the other half of the recombination events a molecule should arise which has exchanged the flanking sequences the other way around. According to the DSBR model the number of molecules is not decreased during the recombination process. A double Holliday structure as intermediate is resolved in such a way that in half of the cases the flanking markers are conserved, in the other half they are exchanged. If both joints are resolved in the same way the original noncrossover molecules will be released. If the two joints are resolved in opposite ways a genetic crossover results.

incoming molecules for the SSA pathway) drastically enhanced recombination efficiencies[3,11,33]. Inducing the ds breaks in vivo by expression of a site-specific endonuclease in protoplasts confirmed these results[31]. As the SSA model predicts that single strands interact with each other further down in the pathway (fig. 1) single-stranded DNA molecules were used to mimic these recombination intermediates[4]. The study revealed that the highest recombination rates were obtained with ss overlapping DNA molecules cut in the vicinity of the overlap, so that direct annealing of the recombination partners was possible. On the other hand recombination models could be evaluated due to their predictions on the outcome of the recombination reaction. For this purpose two different aspects were analyzed: the exchange of flanking sequences between the recombination substrates and the conservative or nonconservative nature of this process. Puchta and Hohn[33] were able to

show that during the recombination reaction in *Nicotiana plumbaginifolia* protoplasts the flanking sequences are exchanged in over 90% of the cases. Furthermore, they found that the remaining cases could be explained by two consecutive rounds of marker exchange. De Groot et al.[10] addressed the question whether ECR is conservative or nonconservative, i.e. whether two or only one DNA molecule survive the recombination reaction (see fig. 1). The reciprocal recombination product as predicted by the DSBR model could only be found in about 20% of the cases.

Nevertheless, not all extrachromosomal recombination events seem to be properly described by the SSA model. In half of the recombination events following cotransfection of protoplasts with a ds and an ss DNA molecule, the reciprocal molecule could be detected, apart from the recombination product with exchanged flanking markers[10]. Furthermore, in *Zea mays*[22] as well as in *Petunia hybrida*[11] indications were found for the existence of other recombination pathways. Therefore it seems that at lower rates and under certain circumstances, events probably described best by the conservative DSBR model can also take place. This conclusion is not surprising as in bacteria and yeast[15] different recombination pathways also occur within the same organism. However, taking the results of all published studies together, the nonconservative SSA model seems to be the appropriate one to describe the vast majority of ECR events in plant cells. Similar conclusions have been drawn for ECR in mammalian cells[21] and *Xenopus* oocytes[23].

The finding that ECR proceeds mainly via single-strand annealing was used to finally solve the question whether a single- or a double-stranded DNA molecule is transferred from *Agrobacterium tumefaciens* to the plant cell nucleus. Tinland et al.[41] constructed T-DNA molecules that contained β-glucuronidase (GUS) recombination substrates carrying the overlap in direct or opposite orientation. Both classes of substrates should recombine with similar efficiencies if the T-DNA is transferred as double strand. In contrast, if T-DNA is transferred as a single-stranded molecule a higher frequency of recombination is expected for the molecules carrying the sequence overlap in opposite orientation. Transfected single-stranded molecules behaved indeed according to this expectation[4]. The recombination efficiencies of T-DNAs with sequence overlap in opposite orientation were, in *cis* and in *trans*, one order of magnitude higher than the T-DNAs with sequence overlap in the same orientation. It was therefore concluded that *Agrobacterium* transfers a single-stranded T-DNA to the plant cell nucleus.

The kinetics of the recombination reaction was also analyzed. ECR takes place mainly in a thirty minute period, early after transfection[34]. Apparently the incoming 'naked' DNA is easily accessible for the recombination machinery of the plant cell. The kinetics of ECR in plant cells is similar to ECR in other eukaryotes.

Particular genomic sequences have been described which stimulate frequencies of homologous recombination (see also Nicolas and Petes as well as Smith in this issue). A 'transformation booster sequence' (TBS) was shown to stimulate the frequency of ECR; frequencies of homologous recombination in protoplasts of the petunia line R101 were two- to five-fold higher when circular recombination substrates carried the TBS sequence[11]. It seems likely that the TBS sequence provides a suitable substrate for recombination due to certain structural characteristics, e.g. a topoisomerase II cleavage site.

ECR is precise; no mutations are introduced into the recombination substrates during the reaction. In a recent study J. Paszkowski's group (pers. comm.) used recombination substrates in which the homologous sequences consisted of an intron that would tolerate mutations during the recombination reaction. Analysis of the intron sequence of several recombinants, altogether almost 4 kb, revealed only 1 bp change, which was most probably due to an error in the PCR amplification step preceding the sequence analysis.

In general ECR in plant cells seems not to differ drastically from ECR in other higher eukaryotes. Extrachromosomal recombination takes place between 'naked' DNA molecules; it differs drastically from intrachromosomal recombination (see below). Transfected DNA is not packed in a highly ordered chromatin structure; it is somehow 'activated' and easily accessible to nucleases. This might explain why ECR is much more efficient than ICR (see below).

Intrachromosomal recombination

Until model recombination substrates were used only very little was known about intrachromosomal homologous recombination (as defined in ref. 30) in somatic plant cells. The studies were restricted to the use of visible marker genes such as the Sulfur locus in *Nicotiana tabacum*[7] and similar loci in other plants[12]. However, since the genes in question were not known at the molecular level, mechanistic experiments were not possible. This changed when natural repeats arranged in tandem arrays were analyzed. As an example, concerted evolution has been postulated to lead to similarity of repeats within the tandem arrays of wheat rRNA genes[2]. Interestingly, frequent somatic ICR within the locus of a duplicated zein gene of maize was shown to be responsible for the loss of one or the other copy[9].

Experimental setups

Experimental designs to study ICR in plants consist of three principally different setups, all of them using selectable or scorable marker-transgenes. Firstly, several studies describe the use of a kanamycin resistance marker gene that can be selected for upon generation of protoplasts or seed[1,29,42]. Analysis of recombination in these systems in planta, in *Arabidopsis* as well as in

tobacco, is limited to the cotyledon stage of the plant. Secondly, a system was established, which avoids selection for the recombination events and therefore allows analysis of recombination in plants at all developmental stages. The system relies upon two truncated β-glucuronidase (GUS) genes, arranged as direct repeats in transgenic tobacco or *Arabidopsis* plants. ICR leads to restoration of the functional GUS gene which can be assayed histochemically[38] (figs 2 and 3). The third system is principally different. Transgenic *Brassica napus* plants carrying partially duplicated sequences of cauliflower mosaic virus (CaMV) exhibit viral symptoms only upon recombination, which restores the viral genome[14]. This allows nondestructive visual scoring of independent events (see fig. 2).

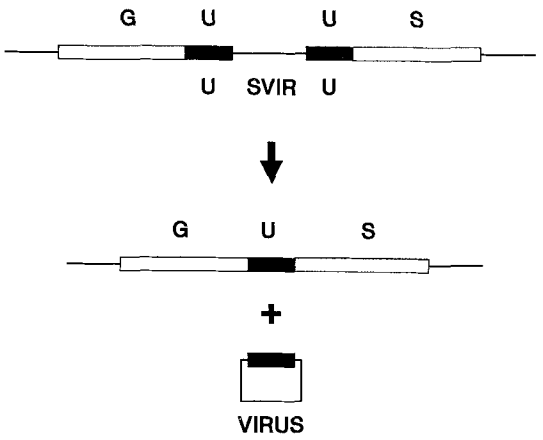

Figure 2. Simplified scheme showing two alternative ways to detect conservative intrachromosomal recombination between direct repeats. The overlapping regions are depicted by black boxes and marked by U, the intervening sequence by a thin line (SVIR). In one class of systems the reconstituted transgene (marked as GUS) can be selected for (in case of the kanamycin resistance gene) or assayed for in the whole plant (in case of the β-glucuronidase gene). Using this approach the fate of the intervening sequence cannot be determined. Alternatively, the 'popped out' intervening sequence could be marked by a replication origin and thus be visualized as VIRUS. In this case the genomic transgene and thus the cell in which the recombination event took place cannot be characterized.

Mechanism of ICR

Intrachromosomal recombination events in plants are in the majority of the cases described best by the conservative double-strand break repair (DSBR) model of recombination, although events described by the nonconservative SSA model cannot be excluded (fig. 1). The different systems described above were used to

Figure 3. Visualization by histochemical staining of recombination events in whole plants: An *Arabidopsis* plant transgenic for a GUS recombination substrate reveals many independent recombination events (blue sectors) in different plant organs[38].

different extents for the analysis of the mechanism of ICR. Due to the different experimental setups not all conceivable recombination products could be analyzed. Peterhans et al.[29], using a tobacco system with direct repeats of truncated segments of the kanamycin resistance gene flanking a hygromycin resistance marker, recovered reciprocal exchange and concomitant loss of the center fragment. Similar results were obtained with the GUS system, where again in reciprocal recombination events the intervening marker gene was lost, as evidenced by hybridization and resistance[38].

Interestingly, Peterhans et al.[29] were able to recover an event in which the central, hygromycin resistance gene-containing fragment was reinserted at a new chromosomal position. This can only be explained with conservative intramolecular recombination as described by the DSBR model, which gives rise to a circular molecule containing the hygromycin resistance gene (fig. 2). Further evidence for conservative reciprocal exchange came from the CaMV system[14], in which the virus sequences were arranged in such a way, that only the 'popped out' recombination product could be detected as spreading virus (fig. 2).

Assaad and Signer[1], used a similar setup as Peterhans et al.[29] in transgenic *Arabidopsis thaliana* plants. They selected for kanamycin resistant green or chimeric seedlings. On the molecular level they were able to describe, in addition to simple reciprocal events, gene conversion events in both directions. In the latter cases the intervening hygromycin resistance gene was retained. Tovar and Lichtenstein[42] used inverted repeats of the truncated kanamycin resistance gene, in transgenic *Nicotiana tabacum*. Interestingly, no reciprocal recombination events were recovered. Due to an elegant experimental setup, gene conversion events could easily be distinguished from one another. In homozygous plants there was a strong bias in favor of correction of the gene carrying a small mutation (20 bp linker), as compared to the gene which carried a deletion of 180 bp. Interestingly, in plants hemizygous for the recombination substrate both conversion events could be recovered with about equal frequency. The reason for the difference in recombination behavior of homozygous and hemizygous plants is not clear. In addition, in about 15% of all cases, both defective genes were converted. Models for a mechanism of conversions of both genes are offered in Lichtenstein et al.[20].

Concerted recombination

The probability that two recombination events take place within closely spaced repeats is higher than expected from statistical calculations. This so called negative interference (see ref. 24 for a review on bacteriophage recombination) points to an unknown relationship between two recombination events. Also in plants recombination events seem to induce rearrangements in the genomic neighborhood; recombination substrates, arranged as trimers, tended to yield trimeric recombination products with a surprisingly high frequency[29]. Concerted recombination events have also been noted by Assaad and Signer[1].

Recombination hotspots and mismatch repair

In order to analyze stretches on the DNA that may be preferred by the recombination machinery a system had to be used in which crossover points could be mapped. In the viral system described above this was possible since the repeated segments contained multiple base-changes[14]. They resulted from the use of different isolates of viable virus. In addition, out of the 34 base differences which were distributed over a range of 1033 bp only two led to an altered amino acid at the respective position. Crossover points were found distributed over the entire region, with one hotspot. This region may have been a spot preferred by the recombination enzymes since it contained the longest stretch of perfect homology. It is known from other systems that mismatches within the homologous region lead to drastically reduced recombination frequencies[44]. An interesting alternative explanation makes a viral promoter located in the sequence overlap responsible for the recombination hotspot. Indeed, transcription has also been documented to increase recombination frequencies in other systems. (Gangloff et al. in this issue).

Due to the many mismatches in the homologous region of the viral system the involvement of mismatch repair in resolving the heteroduplex intermediate of the homologous recombination event could also be studied. Two cases were identified in which mismatch repair was likely to have been involved. Heteroduplexes of 200 or 300 bp, respectively, may have been repaired, although multiple crossover events cannot be excluded[14]. In one case a single mismatch was corrected, namely from G/T or C/A to G/C. This is also the preferred repair of mismatches introduced into turnip plants with artificial CaMV heteroduplex DNAs[36].

Frequency of ICR

In the individual recombination systems described above, the frequencies of homologous somatic recombination between genomic repeats ranged in the order of 10^{-5} to 10^{-7} (with 10^{-4} in exceptional cases[29]). This is similar to frequencies found in animal systems, or to the frequency found for a molecularly non-characterized visible marker in plants[12]. The fact that the provirus system gives efficiencies which are similar to the ones of the non-viral systems is interesting because of the many mismatches which it contains[14,39].

A direct comparison between frequencies of mitotic and meiotic recombination proved to be difficult in most systems analyzed. Rough estimates, however, point in the direction of a relatively small frequency difference of the two events[1,42]. These results are in sharp contrast to

data obtained for fungi where meiotic recombination is several orders of magnitude more frequent than mitotic one (discussed in 1).

Comparison of recombination frequencies in cells hemizygous or homozygous for the same recombination substrate revealed a simple additive effect (refs 17, 39; Puchta et al., unpublished) or a slightly more than additive effect in favor of the homozygous case[42]. Plants resulting from crosses of two lines transgenic for the same recombination substrate did not exhibit recombination frequencies strongly differing from an additive one[39].

The use of different transgenic plant lines for the study of ICR in plants allowed comparison of genomic positions and their influences on the respective recombination frequencies. Differences between two transgenic lines have indeed been observed, both for mitotic and for meiotic recombination[1]. Similar results were obtained with the CaMV and GUS recombination systems (ref. 39; Puchta et al., unpublished). Several explanations can be offered, which may, in a mutually not exclusive way, account for influences on recombination behavior; e.g. transcriptional and/or replicational activities may enhance recombination activity, possibly in a way similar to genomic position dependent effects on gene expression in plants. Such influences may be exerted directly, by supply of single- or double-strand breaks in the DNA, or indirectly, via display of the recombination substrate in a form accessible to the recombination machinery.

ICR in plant development

The setup of the GUS system allowed the detection of ICR events through the whole life cycle of a higher eukaryote via histochemical staining (fig. 3). Thereby cells in which recombination events occurred, and their progeny, could be precisely localized. Recombination events in meristematic tissues resulted in large sectors. Recombination events that most probably took place in the L2 and L3 layer of the shoot apical meristem during *Arabidopsis* embryo development could be detected as well as early and late events in roots. Recombination events were found during flower development and in the seed coat. In summary, recombination was observed in all examined plant organs.

Relative recombination frequencies for the organs leaf, cotyledon and root were determined in three transgenic lines. It turned out that recombination can vary between different organs within the same transgenic line. In two lines recombination frequencies in roots were found to be twice as high as in leaves. This might be explained by the fact that the recombination substrate is integrated in a genomic position that is very 'active' in roots. Moreover, in three lines tested the recombination frequency was slightly higher in cotyledons than in leaves. As cotyledons are developmentally older than leaves it is tempting to speculate that recombination events accumulate with increasing age[38].

The impact of environment and development on ICR

The sizes of genomes vary drastically from plant to plant, ranging from 0.1 to 100 pg DNA/haploid nucleus[13]. Also between closely related species a relatively large variation in genome size has been described. Plants with larger genomes contain more repetitive DNA. This implies a certain flexibility in the genome composition, which is also evident in a certain tolerance of plant genomes to chromosomal abnormalities. Since much of the variation is manifested in repeated sequences, homologous recombination could be involved.

Plants in their normal lives are exposed to all sorts of environmental stresses. An especially interesting case is flax which answers nutritional deprivation with changes in plant size, total nuclear DNA content and copy number of several repeated seqences[8]. The mechanisms underlying these changes are not known but regulation of ICR surely must be involved. Another kind of stress can be exerted by agents interfering with DNA metabolism, such as UV, X-ray or gamma radiation. It was shown, that radiation indeed increased the rate of recombination (refs 7, 17, 42; Puchta et al., unpublished). Also the result that treatment with mitomycin C or exposure to elevated temperatures lead to increased recombination activity supports the notion of the high capacity of the plant genome for stress induced rearrangements[17].

One important property which distinguishes plants from animals is the lack of a predetermined cell-line responsible for reproduction. Only late in development is the germline differentiated from somatic tissue. Thus, genomic changes that may have accumulated during somatic development, can be transmitted to the germline and the progeny. The plants thus possess the unique capacity to pass on to their progeny not only mutated but also *selected* genetic material, since in somatic development selective pressure may have been exerted at cell lineages before germline differentiation (as discussed in 43). Further, a single plant is, in a sense, a collection of 'competing' individual branches, each of which may transmit acquired genetic changes to the next generation, so adding to the variability and adaptiveness of the plant.

It is of great importance, therefore, to analyze homologous recombination at the molecular level both in somatic tissue and following meiosis. The finding noted above, that the frequency of somatic recombination is in the same order of magnitude as of germinal recombination contrasts sharply to the situation in yeast in which the frequency of meiotic recombination is several hundred-fold higher than of the mitotic one. This is at least consistent with the possibility that mitotic recombination events are passed on to the offspring, as has been demonstrated in maize[9]. This mechanism thus allows transmission of somatic changes to the next generation and thereby contributes to variation and evolution.

Gene targeting

So far four different groups reported on homologous recombination between delivered DNA and a chromosomally located target (for review see 27). In all cases restoration of selectable marker genes was used as assay system. In two approaches delivered DNA was put into plant protoplasts via direct gene transfer[16,28], in the two others via *Agrobacterium* mediated transfer[19,25]. The frequencies of homologous to illegitimate recombination reported in these cases which were performed with selectable marker genes were around 10^{-4} to 10^{-5}. Thus, in contrast to mouse embryonic stem cells[5,6] gene targeting is far from being a routine process in plant cells. As gene targeting is precise and does not induce mutations in plant cells (finding of J. Paszkowskis' group with use of an intron sequence as homologous region in gene targeting experiments), the reason for the low efficiency might be that the length of homology, which varied between 0.4 and 3.6kb was below a critical value necessary for targeting. Using longer pieces of DNA is limited by technical problems. On one hand, it is hard to insert very long pieces of DNA into binary vectors of *Agrobacterium*, which is used for plant transformation. On the other hand, direct delivery of long pieces of DNA into plant cells via PEG, electroporation or particle gun leads to very efficient scrambling of DNA molecules.

Recombination between the extrachromosomal copy and the genomic target might occur in a nonreciprocal way in plant cells. Offringa et al.[26] found that an incoming T-DNA was accurately corrected by the target locus. The corrected copy was then inserted somewhere else in the genome.

Proteins involved in homologous recombination in plant nuclei

Enzymes involved in homologous recombination of nuclear DNA of plants have not been characterized yet. However, it is only a question of time until plant analogs of the recently characterized *RAD* genes from yeast – especially the members of the *RAD 52* family (see Heyer in this issue) – will be characterized in a similar way as in vertebrates (Bezzubova and Buerstedde in this issue).

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