

Chapter 3

Homologous Recombination in Plants: An Antireview

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Abstract

Homologous recombination (HR) is a central cellular process involved in many aspects of genome maintenance such as DNA repair, replication, telomere maintenance, and meiotic chromosomal segregation. HR is highly conserved among eukaryotes, contributing to genome stability as well as to the generation of genetic diversity. It has been intensively studied, for almost a century, in plants and in other organisms. In this antireview, rather than reviewing existing knowledge, we wish to underline the many open questions in plant HR. We will discuss the following issues: how do we define homology and how the degree of homology affects HR? Are there any plant-specific HR qualities, how extensive is functional conservation and did HR proteins acquire new functions? How efficient is HR in plants and what are the *cis* and the *trans* factors that regulate it? Finally, we will give the prospects for enhancing the rates of gene targeting and meiotic HR for plant breeding purposes.

Key words: Homologous recombination, Meiotic recombination, Zinc-finger nuclease, Gene targeting, Chromatin remodeling, Plant breeding

1. Introduction

A number of recent reviews have covered what is known on various aspects of homologous recombination in plants (1–8). Here, we wish to write an “antireview,” i.e., to focus on what is not known. We will provide a brief update on the current knowledge in the plant HR field in order to point out what we view as the existing huge gaps in our understanding of this process.

2. What is Meant by Homologous?

Homologous recombination (HR) is a complex process whereby DNA segments that share significant sequence homology are exchanged. This definition, however, does not state what is considered to be extensive length of homology that can be used as a substrate for the HR machinery. In yeast, it is thought that as little as 30 bp are sufficient (9). In plants, we know that a few hundred base pairs can engage in HR (10), but we do not know whether there is a lower limit, nor what is the dependence on the type of partners. Sequence microhomology is often found at the borders of nonhomologous end-joining (NHEJ) events in plants (11). Such microhomologies seem to serve as sticky ends that stabilize the ligation of nonhomologous ends without involving HR proteins. Another level of complexity that will be discussed further is the degree of homology. How divergent can partners be to still be considered as homologous by the HR machinery?

3. What is Meant by Homologous Recombination?

There are many ways to catalyze strand exchange between homologous sequences and the outcomes can be quite diverse. In all HR cases there is a need for a homology search. Remarkably, the basic question of homology search, namely, how do homologous partners find each other? How do the various DNA molecules move within the nucleus until partners come into the physical proximity that is necessary to enable strand invasion? Is all the genome scanned to find a proper alignment? Does the scanning process proceed in a random or organized manner? Is strand invasion involved in the identification of homology or is there another, yet undiscovered, scanning process? This topic is very poorly understood in plants, as well as in bacterial (12) and yeast models (13), and remains one of the most puzzling and basic open questions in the field. Out of the many alternative pathways that can be used to recombine, we still understand very little on how the cell “decides” in which path to proceed. The choice can be between various mechanisms involving Holliday junctions, an interference-independent pathway, single-strand annealing (SSA), synthesis-dependent strand annealing (SDSA) (see review (14)) or some other yet undiscovered pathways.

4. Anything Special About HR in Plants?

Prior to asking whether there is something special about HR in plants, one should think whether there is anything special in plants' life cycle that might, through natural selection, affect HR (15). One aspect is that the lack of a germline and the vegetative propagation of several species, may suggest that somatic HR plays a greater role in plants than in other species, which rely solely on sexual reproduction from a germline that is differentiated early on in development. Another potentially relevant fact is that most plants are polyploids (16). Whole genome duplication confers buffering of deleterious mutations and thus might enable the polyploid plant genome to be more tolerant to genetic rearrangements than the diploid one. For example, there might be a "release" of selection or repression of HR between direct or inverted repeats. Another challenge of polyploidy is to maintain fertility, which might be reduced through HR between homoeologs (in allopolyploids) and through multivalent pairing during meiosis. Note that we do not know how polyploidy per se, might affect HR rates and patterns in an autopolyploid or allopolyploid genome.

Finally, due to their sessile nature, plants are constantly exposed to DNA-damaging agents, such as UV and, in some areas, heavy metals (17). Did HR evolve differently in plants than in other kingdoms due to this chronic exposure? Would such a difference be due to quantitative (regulation, level of expression) or qualitative (the machinery itself) dissimilarities? Does HR play an important role in the adaptation of plants to different habitats (e.g., high altitude, high UV, or contaminated soils)? Plants provide a wonderful system to address such questions due to their sessility and the wide ecological distribution of model species such as *Arabidopsis*. Interestingly, plants grown in the Chernobyl area were found to exhibit higher mutation and HR rates than plants grown in noncontaminated areas (18, 19). We might assume that the HR machinery is necessary for survival under such conditions, although this has not been proven. Testing such hypothesis is not an easy task. It would require performing "evolution" experiments, namely, growing HR mutants and WT in genotoxic and in normal habitat for a number of years and comparing their fitness. In bacteria that grow under chronic exposure to irradiation, e.g., *Deinococcus radiodurans*, HR plays an important role in DNA repair (20). However, it is still debated whether this is the main cause for radiation resistance or whether other pathways (e.g., NHEJ) are responsible for this phenomenon (12).

The HR machinery is, together with other DNA maintenance functions such as replication and repair, one of the most conserved in the cell. Protein homologies run as deep as between

prokaryotic proteins, e.g., RecA, vs. their eukaryotic homologs Rad51 (21). So is there anything special about the plant HR machinery? There are undoubtedly many differences that have been pointed out (see above reviews). These differences concern mostly the different number of homologs, or the lack thereof for certain key genes when comparing yeast and plants or plants and mammals. Are these differences the result of random drift between species, or were these differences selected to serve some plant-specific HR needs?

There are plant-specific processes, not related to HR per se, that have “adopted” HR proteins, assigning them with new functions. The Rad51D protein, for instance, is an AtRad51 paralog which plays a role in HR (1, 22). It was found, in a genetic screen in Arabidopsis, that Rad51D is a repressor of Sni1, by itself a repressor of pathogenesis-related proteins, linking Rad51D with the plant’s defense response (23). Hence, it seems that Rad51D has acquired a new, plant-specific function following gene duplication (neofunctionalization). Interestingly, a mutation in *RAD51D* was also shown to enhance the *tebichi* (*teb-1*) developmental phenotype, using a *teb-1 rad51d* double mutant (24). Mutation in another *AtRAD51* paralog, *XRCC2*, also enhances the *tebichi* phenotype (24). These data point to novel plant-specific functions of DNA repair genes related to the pathogenesis response or to development.

Similarly, in a genetic screen for hyper-recombinogenic plants, Molinier et al. found that *CENTRIN2* (*CEN2*) modulates both HR and nucleotide excision repair in Arabidopsis (25). Later on, interactions were found between *CEN2* and the *CULA-DDB1A-DDB2* E3 ligase, suggesting that these proteins are involved in the same pathway (26). The tomato *DDB1* homolog was previously characterized as the gene responsible for the *high pigment-1* mutant phenotype (27). Altogether, these data are linking DNA repair, HR, protein degradation, photomorphogenesis and secondary metabolites production, again pointing to neofunctionalization of HR genes in new plant-specific traits.

Other examples where HR is linked with plant-specific cellular mechanisms include the *abo4* mutant, which was isolated based on its sensitivity to abscisic acid (ABA) and shows a 60-fold increase in somatic HR (28) or the chromatin-remodeling gene, *AtBRM*, that controls shoot and flower development (29) and is involved in intrachromosomal recombination (ICR) (30).

What is the “physiological” basis – if any – for these diverse types of neofunctionalization? Has nature simply tinkered with HR proteins, borrowing domains such as helicases, DNA binding, ATPases, etc., for non-HR functions? Or is there a more rational explanation, such as a connection to DNA replication, or – who knows – programmed DNA damage may serve as a signal for certain types of biological processes not related to HR.

5. How Efficient is HR in Plants?

It is often mentioned that HR is not efficient in plants, possibly owing to the repetitive nature of the plant genome. This common view should be refined. Gene targeting (GT) rates are indeed very low in higher plants, with reports ranging from 10^{-3} to 10^{-6} (31), but other types of HR are not fundamentally different than in other species. Moreover, the correlation between the repetitiveness of the plant genome and HR is not clear-cut. GT rates are relatively similarly low in Arabidopsis, rice and tobacco which have a genome with few, average, and abundant amount of repeats, respectively. More significantly, the genome of the moss *Physcomitrella patens* is proficient at gene targeting (32) even though it is approximately threefold larger than that of Arabidopsis and it is rich in repetitive DNA (33). Is this due to peculiar recombination machinery, or owing to an organization and structure of *Physcomitrella*'s chromatin that is different from that of higher plants?

5.1. Meiotic Recombination

Regarding meiotic HR, plants are not different from other eukaryotic species with only a few crossovers per chromosome arm (except for very short arms). There is no linear dependence between chromosome size and the number of crossovers it experiences. Plant chromosomes, as those of most eukaryotic species, must have their "obligatory chiasma" to ensure proper segregation, and are subject to genetic interference. As the size of chromosomes is highly variable in plants, the kb/cM ratio greatly varies, from a few hundred Kbs per cM (e.g., Arabidopsis) to values that are two orders of magnitude higher in plant species with a very large genome. Increasing meiotic crossovers would be of immense value to breeders. Unraveling the pathways that control meiotic crossovers could help achieve this goal. One pathway that controls meiotic crossovers is interference. A MSH4-independent meiotic recombination pathway, that is not sensitive to interference was discovered in plants (34). This pathway, however, seems to promote only up to ~15% of the crossovers and it is an open question at this point whether it is possible to boost it and whether there are other major bottlenecks. One may also enhance the frequency of DNA double-strand breaks (DSBs), which are initiating the crossover events. However, there seems to be a great excess of breaks compared to crossovers, with these breaks being repaired by a pathway (probably SDSA) that leads to gene conversion and does not promote crossovers (35). Therefore, there is no guarantee that such an approach would be successful.

5.2. Somatic Recombination

The early studies on the molecular control of HR were done using plasmids transformed into plant cells. Extrachromosomal recombination (intra- or interplasmids) was then monitored

using reporter genes (36). In these experiments, the rates that were reported were relatively high compared to intramolecular HR (see below), suggesting that the structure of the DNA in a plasmid is more prone to HR than that of a chromosome, which is packaged in chromatin. Alternatively, it is plausible that the large amount of DNA molecules introduced into the cell (which was not controlled in these early experiments) has enhanced the chances for HR. These experiments may suggest that the cell machinery is not strongly limiting, provided the substrate has the proper quantity or quality.

Another type of HR that has often been used to quantify the effect of various mutants, as well as biotic and abiotic factors, is the assay of somatic recombination between repeats, in direct or inverse orientation, namely ICR. With these assays, recombination can be scored either through selectable markers (36) or through reporter genes (37). ICR between such repeats in *cis* is in the 10^{-3} to 10^{-5} range. However, it can be enhanced by 2–3 orders of magnitude upon DSB induction (38). In an experiment that enabled the determination of the outcome of I-SceI-induced DSB repair, namely homologous vs. nonhomologous, it turned out that up to one-third of the DSBs were repaired via ICR (39). Similarly, when repeats are present from both sides of a break induced by a transposon excision, as in the P locus of maize, a large proportion of the repair events occurs through HR rather than via the typical end-joining and formation of transposon excision footprints (40). This suggests that while spontaneous HR between nearby repeats in *cis* is not very frequent, ICR is an efficient DSB-repair pathway. How is ICR frequency affected by the distance between the repeats is not well established. We can assume that the frequency decreases with an increase in the inter-repeat distance, maybe as a function of the extent of DSB end resection that enables annealing of homologous segments, but according to what function? Do repeats found on the same chromosome, i.e., physically linked, always have a higher chance to engage in HR than repeats located on different chromosomes (ectopic HR)? Indeed spontaneous HR between ectopic repeats is extremely low (below the 10^{-7} range). It can also be induced by a DSB, but even then remains in the 10^{-5} range (41, 42).

6. The Regulation of Homologous Recombination

In a recent review, Li et al. (2007) conveniently distinguished between the *cis* and *trans* factors that affect HR (8). The *cis* factors are related to the DNA itself or to the chromatin and the *trans* factors correspond to the machinery that regulates or catalyzes the HR reaction.

6.1. The *cis* Regulators

The *cis* factors related to the DNA include the degree of sequence similarity and the modification of the DNA such as DNA methylation.

6.1.1. Sequence Divergence

It is now well established that even minor sequence divergence between HR partners can cause a strong reduction in HR, somatic or meiotic (43). The antirecombination effect of sequence divergence can be abolished in some mismatch repair mutants (44, 45). In plants, most reported studies do not separate all the products of a HR event, as can be done through tetrad analysis. It is thus difficult to estimate whether HR is restricted to purely identical sequence segments, or whether recombination intermediates, such as double Holliday junctions (DHJs) that contain mismatches are processed into crossovers. All that can be said is that crossovers occurred between two polymorphic markers. We cannot say whether these markers are included in the intermediate. In maize, the intergenic region, which is usually repetitive and divergent, is a cold (or even frozen) spot of recombination (46). Genes are highly conserved in sequence between maize varieties. Therefore, this supports the possibility that crossovers are restricted to regions of very high, and possibly full, identity. In some allopolyploid organisms, mechanisms have evolved to prevent pairing and crossover between homoeologous chromosomes. Such pairing might indeed promote the formation of multivalents and affect fertility. In wheat, the *Ph1* locus, which prevents homoeologous pairing, has been the subject of intensive studies. It co-segregates with a cluster of repeats of cyclin-dependant kinase genes (47). Mismatch repair genes, which were candidates for *Ph*-like activity did not map to the *Ph1* locus (48); however, one mismatch repair member mapped to *Ph2*, another suppressor of homoeologous pairing (49). In *Brassica napus*, *PrBn*, a gene that controls the pairing between homoeologs has also been characterized (50). Here, as well as in the above cases, the mode of action on homoeologous pairing suppression remains unclear.

6.1.2. Chromatin Structure and Methylation

Intuitively, it is expected that accessibility to the DNA would facilitate the physical interaction between homologous partners and thus promote HR. Indeed, as mentioned above, plasmids introduced into plant cells seem to recombine with each other at relatively high rates. In addition, plant mutants in CAF1, a chromatin assembly factor, have a loose chromatin structure and show an increase in ICR by 2 orders of magnitude (51, 52). Along the same line, the upregulation of *RAD54*, a chromatin-remodeling gene, enhances the rate of GT (53), while the downregulation of other *SWI2/SNF2* chromatin remodelers *At2g46020* and *At5g44800* (30) reduces the rate of ICR. Similarly, altering the activity of *MIM* (54) or *BRU1* (55) through overexpression or mutation affects the rates of ICR. Finally, regions of heterochromatin, like knobs,

or regions around centromeres are cold spots of recombination. Altogether, this points to a tight connection between DNA packaging and HR. Interestingly, heterochromatic regions tend also to be hypermethylated. It would be of interest to test the effect of cytosine methylation on HR. Small RNAs, which also affect chromatin structure and methylation could thus have a direct or indirect effect on HR. Can we manipulate chromatin structure to enhance GT and meiotic HR, which are both of interest to the breeder? This might be tricky, as the *caf1* mutant, which shows the most dramatic increase in HR, has a very stunted phenotype. Strategies for transient and localized remodeling of chromatin are therefore attractive directions for future efforts. Another holy grail in the field of plant HR is to understand the basis for high GT rates in *Physcomitrella*. Chromatin packaging may be a key to this puzzle.

6.1.3. Hot Spots

As discussed above, the HR coldness of heterochromatin regions has probably a simple physical cause, namely, accessibility. Understanding the nature of hotspots is more challenging. Mapping of recombination breakpoints on chromosome 4 of Arabidopsis has confirmed the lack of homogeneity in the rate of HR along the chromosomes, with nearby segments varying in HR frequency by orders of magnitude. The causes for this variability could not be predicted by simple sequence features (6). Interestingly, the number of Rad51/Dmc1 foci exceed by far the amount of crossovers (35) and are more homogenous in distribution than crossovers (56). This suggests that what determines the variability in crossover events along the chromosome is not the occurrence of DSBs, but rather the way these DSBs resolve, as crossover or noncrossover events. This choice of pathway (COs vs non-COs) is poorly understood. It might determine both hotspots localization and genetic interference.

6.2. The trans Regulators

In addition to the *cis* factors, related to the DNA sequence itself, *trans*-acting factors, namely, the plant proteins that are part of the HR machinery, have been extensively studied and recent reviews have covered these proteins (see above reviews). Here, we chose to focus on the core components of the HR machinery, namely, the homologs of the yeast Rad51, Rad52, and Rad54 proteins (14). Although HR is well conserved among eukaryotes, the differences that were observed raise many questions. The yeast Rad52 protein is central in HR (57) and its expression in human cells increases GT rates (58). The plant homolog is still elusive. It has been speculated that there is no Rad52 homolog in plants. Alternatively, a distant homolog remains undiscovered, and/or the Rad52 function, namely Rad51 loading on single-stranded DNA, is fulfilled by a different protein such as BRCA2, as suggested for mammalian cells (57). A *BRCA2* ortholog was found

in plants (59) and was suggested to have a similar function. Rad51 foci are formed in plants (35), suggesting that loading of Rad51 on nuclear filaments takes place. It is still unclear which proteins carry out this loading function in plants.

Considering the physical interaction between Rad52 and Rad51, as shown in yeast (60) and in human (61, 62), it would be interesting to screen the plant proteome for Rad51 “interactors” in search of new players of HR, including the elusive plant Rad52 (or a functional equivalent). It can be done by screening a yeast-two-hybrid library, or by a pull down assay and analysis of the whole complex, as was done recently for the mammalian Rad52 (63). Similarly, it might be instructive to search for new “interactors” of the plant Rad54, as well.

In *Arabidopsis*, like in vertebrates, five *RAD51* paralogs were identified, yet their exact role is not clear. As reviewed in Bleuyard et al. 2006, the five paralogs were shown to be involved in HR and DNA repair, as well as in meiotic HR. The *RAD51* paralogs form the same complexes as in vertebrates, implying functional conservation of these proteins. However, while in vertebrates (and also in *Drosophila* and *Caenorhabditis elegans*) inactivation of *RAD51* leads to embryonic lethality, *atrad51* *Arabidopsis* mutant plants are viable (64). The molecular basis for this significant difference is still unknown.

The SWI2/SNF2 chromatin-remodeling factor Rad54 enables, by nucleosome positioning, the core HR events: strand invasion and branch migration (polymerization). Thus, it can be considered a major regulator of HR. As such a central player, it might not be so surprising that the yeast Rad54 can facilitate GT in *Arabidopsis* (53) – a cross-kingdoms function. Still, many questions arise; how does Rad54 function in *Arabidopsis* without its yeast counterparts? Does it function alone, as suggested by its *in vitro* activities (65), or through interaction with its plant counterparts? Indeed, it has been shown that Rad54 interacts with AtRad51 (and AtRad54 with Rad51) in a yeast-two-hybrid assay (66). Despite similarities in function with its yeast ortholog, AtRad54 complements the yeast mutant phenotype only partially (66). This maybe underlies the fact that the plant protein is less active than its yeast ortholog in promoting GT, possibly as a result of natural selection.

7. Gene Targeting

The homologous integration of an extrachromosomal DNA molecule into a homologous chromosomal target site is a powerful tool for the precise engineering of plant genomes. Therefore, it is not surprising that it has been a major goal of the plant community

since the first report on GT in plants (67). After years of frustrating attempts to develop efficient gene targeting in plants (31) there may be light at the end of the tunnel. We discuss below the challenges of understanding the mechanism of GT and the future prospects. In fact, it is easy to understand why GT is inefficient in plants: A small vector must scan a huge genome, which is nicely packaged in chromatin, identify the target, get into physical contact with the target, invade it, and engage in strand exchange. This seems like mission impossible, and indeed, two recent reviews (12, 13) describe the biophysical constraints of such homology search and open many questions on how it can work at all? Therefore, by addressing the question of why it does work in certain systems, such as yeast or *Physcomitrella*, one might get insight into the true bottlenecks of the system. So far, most biologists interested in GT have not tried to understand how it really works, but rather have tried to make it work. This has been achieved in mouse as in plants, by the development of clever selection systems, such as positive–negative selection (68) or by artificially creating a recombination hotspot through DSB induction. The first work in plants showed that transformed DNA could be captured at a DSB site induced via cleavage by the I-SceI meganuclease at an I-SceI recognition site (69). Then, a new generation of chimeric meganucleases, zinc-finger nucleases (ZFNs), was developed (70), in which a modular DNA-binding domain fused to a nuclease can be designed to bind any genomic target at relatively high specificity. Recently, this new technology was implemented for the targeting of endogenous plant genes (71, 72). At the mechanistic level, the broken chromosome is probably an accessible entry point that can be recognized by the vector during its scan of the genome. Indeed, the broken chromosome is probably virtually immobile (73) compared to the small vector that can more freely roam the nucleus. So, it makes more sense that the vector goes to the break rather than the break to the vector. Once the physical contact is established, it is still unclear how gene replacement occurs, namely, through an SDSA-like mechanism (74, 75) or through the classical DSB-repair pathway with formation of DHJs (76). Meganuclease technologies have brought new hopes to the field of GT and gene therapy. Nevertheless, there are still topics that must be perfected to turn it into a routine application. ZF design must be improved to really achieve the binding of any sequence. Possibly, it might be necessary to predict or test chromatin structure in the target area for optimal design of the target. Indeed, the findings that chromatin remodeling affects GT in plants (53) supports the need to include the chromatin component in any GT strategy. Ideally, one would combine both efficient design algorithms and evolution methods to develop the perfect nuclease – specific and efficient – an “intelligent evolution” approach. Finding methods to estimate

the frequency of off-site breaks induction is critical for gene therapy applications. In plants, off-site mutations might be less critical as backcrosses can be made to get rid of unplanned mutations. However, an excess of ZFNs-induced breaks can also be toxic to the cell and create large deletions and chromosomal rearrangements that may cause gamete sterility, if not cell death. Preferably, the DSB-causing agent should be expressed both temporally and spatially in such a way that induces the break specifically at the desired time and place. Transient expression of highly sequence-specific ZFNs should be used for this purpose as was done in maize and tobacco (71, 72).

8. Conclusions

Plants have contributed their share to the field of HR and, hopefully, will continue to do so in the future. We have tried to point to the many open questions that must be addressed in order to better understand the mechanisms of HR, the control of genome dynamics and stability, and to enhance the rates of HR in plants. The latter is of importance for plant breeding. Increasing meiotic crossover rates is necessary for accelerating breeding processes and for breaking undesirable linkages. It will probably require a better understanding of bottlenecks such as DSB induction and genetic interference. It is also important to better understand HR between homoeologous chromosomes in order to transfer genes between related species. Broadening the gene pool that can be used to improve our crops is essential to face the challenge of plant yield improvement in a changing environment and to find new sources of resistance to biotic and abiotic stresses. Finally, there are some reasons for optimism on the GT front, and we might be at the beginning of a new exciting journey that might make GT routine in plant biology. Nevertheless, careful optimization of the method will be required for each plant species and possibly for each cell type where GT experiments will be carried out. Finally, the emergence of new technologies of genotyping, sequencing, protein design and imaging are opening opportunities for making new discoveries on the HR mechanism and for building new HR-based tools.

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