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Breaking DNA in plants: how I almost missed my personal breakthrough

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If I had not been so lazy, I never would have realized that what turned out to be the most important experiment in my scientific career had actually went well. Indeed, for the first time in plants, I had just enzymatically induced a unique site-specific genomic double-strand break (DSB). From all we knew, this should have been the key to the controlled induction of various types of genomic rearrangements. However, there was no indication that the experiment had worked. But let us start from the beginning.

A passion for gene technology

During my study of biochemistry in the early eighties of the last millennium at the University of Tübingen in Germany, I became more and more interested in molecular biology and especially in gene technology. Although already in worldwide use for some time, it was hard to find groups in Germany that were using methods of gene technology routinely in the laboratory. For me, the opportunity to get involved came at the Max Plank Institute in Martinsried near Munich, where I earned my diploma and wrote my PhD thesis in the department of the late Heinz-Ludwig Sänger, one of the co-discoverers of viroids. Viroids are small, circular, single-stranded RNA molecules measuring 250–400 nucleotides in length that are able to infect plants and cause somewhat severe disease symptoms. During my time in laboratory, we cloned cDNA out of viroids, which turned out to be infectious when inoculated on plants. This was an amazing demonstration of the power of gene technology. During my PhD, I also discovered a new viroid that we found distributed in hops all over the globe without causing symptoms. Indeed, this was the only time in my career that my research was broadly covered by daily newspapers, but alas this was mainly because some journalist who craved sensationalism speculated that the world-wide distribution of an infectious agent in the hop plant might be a serious health threat for millions of beer consumers, especially in Bavaria.

The small size of viroids made them for me no promising objects for gene technology in a long-term perspective. Therefore, my interest in the transformation of plants grew. At that time, the Friedrich Miescher Institute (FMI) in Basel, Switzerland, was one of the leading centres of plant molecular biology in Europe. There, Barbara Hohn, who was already well known as a pioneer of molecular biology due to her invention of the cosmids, was analysing the mechanism of *Agrobacterium*-mediated transformation in plants. Although it seems crazy to me today, after I finished my PhD, I sent out only a single job application, and it was to join her laboratory. Things worked out well: I got the position and stayed in her laboratory for the next five and half years, which turned out to be the most interesting, productive and formative time of my scientific career. At that time, Barbara became interested in studying plant genome stability. A main focus of my work was to set up a scorable assay system for measuring the frequency of homologous recombination (HR) between repetitive genomic sequences. The assay was initially developed for Arabidopsis (Swoboda *et al.*, 1994) and tobacco and was based on the restoration of the ß-glucuronidase gene from overlapping nonfunctional parts. Over the years, the assay became a very valuable tool for plant biologists. It could not only be used to characterize the roles of individual proteins in genome stability in plants but also served as an assay that could define stress factors that challenge genome stability. Fortuitously, the setting up of this assay system also enabled us to prove that T-DNA is indeed transferred from Agrobacterium into plant cells as a single-stranded molecule (Tinland *et al.*, 1994).

Targeting a break to solve the targeting problem

During my stay in Basel, I became more and more interested in DNA recombination mechanisms (Figure 1). For all we knew,

Figure 1 The author looking enthusiastically into the future of plant genome engineering.

recombination reactions were initiated by DSBs in the DNA. Unfortunately, there was no way to induce a unique DSB *in vivo* at a specific position in the genome of a multicellular eukaryotic organism at that time. In studying the literature, I learned that Bernard Dujon's group at the Institute Pasteur had discovered a special type of sequence-specific endonuclease: the homing endonuclease I-SceI, which has an 18-mer recognition site. This site was long enough to be unique if transformed into smaller plant genomes. Statistically, the same sequence is not expected to occur naturally within such genomes. I-SceI exists in yeast mitochondria; its ORF is contained within a ribosomal RNA gene of mitochondrial DNA. The induction of a DSB can subsequently induce HR, allowing the endonuclease gene to spread into mitochondrial genomes that do not yet harbour its ORF (Jacquier and Dujon, 1985). I obtained a codon-optimized ORF from Bernard and performed pilot experiments with plasmid DNAs transfected into plant protoplasts. The result indicated that I-SceI can be used in plant cells to induce DSBs into plasmid DNAs. HR between different plasmid molecules could be enhanced by *in vivo* induction of such DSBs drastically (Puchta *et al.*, 1993). Indeed, ours was the first publication demonstrating this approach in any multicellular eukaryote. However, the question remained: could a mitochondrial enzyme cut nuclear DNA that is complexed with chromatin? Furthermore, would the induction of DSBs indeed enhance the integration of a specific piece of transgenic DNA within a locus that carries the same sequence information ['gene targeting' (GT)]? At that time, GT was the Holy Grail of gene technology: a way to knock out gene function or integrate DNA into a specific genomic position. With the exception of mice, GT was not yet an established technique in most multicellular eukaryotes, including plants. Years earlier, at the ETH Zürich, Jurek Paszkowski had demonstrated that GT was achievable in plants, but only at a very low frequency (Paszkowski *et al*. 1988). I set up the respective experiments by transforming parts of an artificial target locus including an I-SceI site into tobacco (Figure 2a). As a marker that should be restored by HR, Jurek provided me with an artificial kanamycin resistance gene that contained a eukaryotic intron sequence to extend the length of homology in the targeting experiment. I obtained plants that contained single copies of the target locus and then retransformed them with two *Agrobacterium* strains that contained one T-DNA each, one with an expression cassette of I-SceI and the other with the targeting vector that included a part of the kanamycin resistance gene that was homologous to the target locus. The basic idea was that if we could indeed induce a DSB at the target locus via I-SceI expression, then multiple kanamycin-resistant calli should arise. However, to my great disappointment, at the time point during which we would normally observe resistant calli following transformation, I did not see anything growing on my plates. A week later and nothing again. Under normal circumstances, enough time had elapsed to warrant taking the plates out of the incubator and throwing them out; however, as I was lazy, I instead just left them there. After shifting them to the very back of the growth chamber, I forgot about them. Barbara had a busy laboratory with little room to spare.

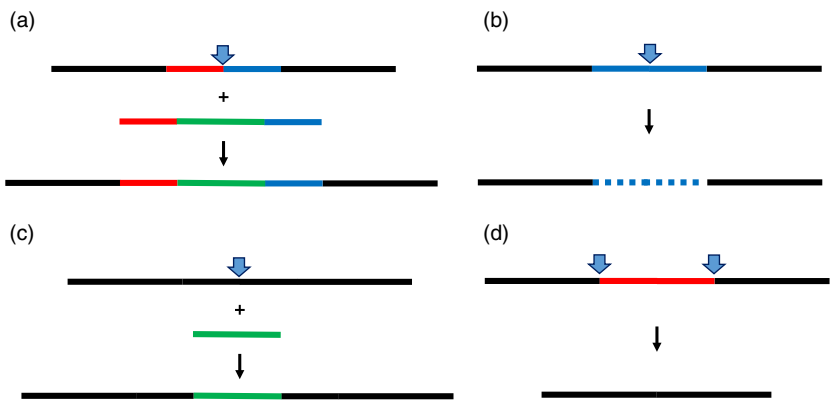

Figure 2 Schematic representation of different types of genome engineering that can be achieved by double-strand break (DSB) induction. (a) Gene targeting by homologous recombination (HR) (Puchta *et al.*, 1996), red and blue: regions that are homologous between vector and genome, green: foreign sequence to be integrated into the genome; (b) mutation induction by NHEJ (Kirik *et al.*, 2000; Salomon *et al.*, 1998), blue: gene to be knocked out; (c) DNA integration by NHEJ (Salomon *et al.*, 1998), green: foreign sequence to be integrated into the genome; (d) induction of controlled DNA deletions (Siebert *et al.*, 2002), red: genomic sequence to be deleted. Blue arrows represent in all cases I-SceI sites.

Thus, 2 weeks later, my colleagues came to tell me that space was needed and that I should remove my old plates. I took them out, and there they were as follows: green calli on all of the plates! The experiment had worked. We were able to enhance targeting via DSB induction by at least two orders of magnitude! We subjected the recombinants to molecular analysis, and most of them turned out to result from HR of the target vector with both ends of the genomic DSB. The change proved to be heritable and segregated in a Mendelian manner (Puchta *et al.*, 1996). So, why was I unable to obtain resistant calli earlier? The intron-containing kanamycin gene was not as efficient at conferring resistance as the intronless gene that we normally used, which increased the length of time that was required to grow the resistant calli. Thus, if I had thrown the plates away 'in time', I would have thrown away my future.

Inducing DSB is the key to various types of controlled genome modifications

The time came to leave Barbara's group. Of the upmost importance to my future career was the fact that Barbara generously agreed that I could take all of my work related to DSB induction with me. I began looking for grant money to set up a junior research group. Of course, included in this search was the question of where to go. I applied for funding in both Switzerland and Germany and was fortunate enough to obtain grants that would allow me to work in either country. It is notable that my salary in Switzerland would have been about twice as high as in Germany. Nevertheless, I decided to leave the country, because with Barbara's and Jurek's groups, two worldwide leaders in the field of DNA recombination were already situated in Switzerland, whereas no group with such an expertise existed in Germany. I decided to give up the money and go for the better perspective by returning to Germany. Actually, I never 'returned' in the true sense of the word but instead came to a country that I had never been in before. When I had originally left West Germany, there were two independent states and the Berlin wall was still standing. When I came back, there was one unified Germany, and I ended up in the eastern part. This was a possibility that would not have entered my wildest dreams before I left. The institute in Gatersleben was 'the' premium place for plant genetics in the former GDR. After the unification, it became the Leibnitz Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK). I joined the department of cytogenetics, which was headed by Ingo Schubert, one of the leading European scientists in the field.

At Gatersleben, I was in a very comfortable position as a young group leader. I had the unique tool of *in vivo* DSB induction in hand. At that time, nobody else in the world was using such an approach in plants. My tool could not only elucidate mechanisms of DSB repair but could also facilitate the development of new techniques for genome engineering. Indeed, our very first study turned out to already be very rewarding. We analysed DSB repair via nonhomologous end joining (NHEJ) using transgenic tobacco that contained a negative selectable marker gene with an I-SceI site and showed that the induction of DSBs enabled the ORF to be destroyed by NHEJ repair (Figure 2b). We found that some instances of NHEJ led to microhomologies at the newly formed junctions, whereas others did not, indicating that there are two different mechanisms of NHEJ. In addition to deletions, we also found a number of alleles bearing insertions. We found sequences copied from elsewhere in the genome into the DSB, as well as the integration of T-DNAs into the DSB (Figure 2c). Thus, we were able to show that DSB repair is a mechanism of T-DNA integration (Salomon and Puchta, 1998). Later, we performed similar experiments in *Arabidopsis* and found that species-specific differences exist during NHEJ repair. We suggested that the deletions associated with NHEJ might be a mechanism for the shrinking of plant genomes (Kirik *et al.*, 2000), a hypothesis which has since been supported by bioinformatics analysis.

We started a series of experiments that showed that DSB-induced HR is most efficient with sequences that are close to each other on the same chromosome and very inefficient if the sequences are in an allelic or ectopic position. Furthermore, we also developed tools for DSB-induced genome engineering. Thus, we were able to show that by inducing two DSBs in close proximity to each other, the intervening sequences can be removed from the genome (Figure 2d, Siebert and Puchta, 2002). We were later able to demonstrate that the reciprocal exchange of chromosomal arms can be achieved by inducing DSBs in the respective chromosomes.

DSB-induced genome engineering: a bright future ahead

In addition to studying DSB repair itself, I developed a second target for our group, which was to characterize the proteins involved in DSB repair and genome stability in Arabidopsis. This proved to be a rewarding decision, as only a few groups in the world were working on what was quite a wide topic. As my space in this article is limited, I will focus on only the most important discoveries. We found a unique type of topoisomerase in plants, Top6, which descended from archaea and is involved in endoreduplication (Hartung *et al.*, 2002). We also discovered a new type of mechanism for how meiotic recombination intermediates are dissolved by topoisomerase 3-alpha (Hartung *et al.*, 2008), which might be general to all eukaryotes. Indeed, over the years, this work became the major focus of our group, particularly after I left Gatersleben to become chair of the department of plant molecular biology and biochemistry at the University of Karlsruhe, which is one of the leading technical universities of Germany and after merging with a federal research institute later became the Karlsruhe Institute of Technology (KIT).

During this time, a new development began that transformed molecular biology: the construction of artificial nucleases. It all started with the development of zinc finger nucleases (ZNFs). It could be shown that by manipulation of the zinc finger containing DNA binding motives of transcription factors, new binding specificities could be obtained that did not exist in nature before (Rebar and Pabo, 1994). By fusing the nuclease domain of a restriction enzyme to such a DNA-binding domain artificial, programmable nucleases could be constructed (Kim *et al.*, 1996; Smith *et al.*, 2000). Later on, transcription activator-like effector nucleases (TALENS) were developed according to the same principle relaying on a different kind of DNA binding motive originating from a plant pathogenic bacterium (Boch *et al.*, 2009). These synthetic enzymes were applied for the induction of genomic DSBs throughout the plant genome. Thus, the knockout (Lloyd *et al.*, 2005) or GT (Shukla *et al.*, 2009; Townsend *et al.*, 2009; Wright *et al.*, 2005) of any natural gene became a possibility for plants. The biggest leap forward, however, happened just 3 years ago when the CRISPR/Cas system was discovered as an efficient tool for genome engineering (Jinek *et al.*, 2012). This technology made it possible to create a

nuclease in an extremely easy way by simply cloning new guide RNAs to define the specificity of the Cas9 nuclease. Using multiple guide RNAs, we are now also able to simultaneously induce a larger number of DSBs within the plant genome.

To me, the field of plant genome engineering has never been more exciting than it is now. At the current moment, thousands of scientists induce DSBs using artificial nucleases in various plant species to produce mutants that they would hardly be able to obtain by any other means. We recently developed a new type of GT strategy, '*in planta* gene targeting', that makes large-scale transformation and tissue culture efforts obsolete (Fauser *et al.*, 2012) because the GT reaction can take place not only shortly after transformation but also throughout the complete life cycle of a plant. Using CRISPR/Cas, we demonstrated that *in planta*, GT could be applied to natural genes in Arabidopsis (Schiml *et al.*, 2014). We were also able to show that we can efficiently induce single-strand breaks (SSBs) in the plant genome using a modified Cas9 nuclease. For the first time, we are now able to analyse in detail the different mechanisms behind SSB repair in plants, and we can use the paired induction of two SSBs for mutant generation (to avoid the off-target effects of the classical CRISPR/Cas system). Because detailed knowledge of the factors involved in DSB repair is now available, we will be able to develop more sophisticated genome engineering techniques by combining DSB induction with specific DNA repair phenotypes. At the moment, we are just now becoming able to restructure genomes within a species; however, in the long run, we may indeed be able to create synthetic plant genomes.

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