

## SYNTHETIC BIOLOGY

# Using CRISPR/Cas in three dimensions: towards synthetic plant genomes, transcriptomes and epigenomes

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## SUMMARY

It is possible to target individual sequence motives within genomes by using synthetic DNA-binding domains. This one-dimensional approach has been used successfully in plants to induce mutations or for the transcriptional regulation of single genes. When the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system was discovered, a tool became available allowing the extension of this approach from one to three dimensions and to construct at least partly synthetic entities on the genome, epigenome and transcriptome levels. The second dimension can be obtained by targeting the Cas9 protein to multiple unique genomic sites by applying multiple different single guiding (sg) RNAs, each defining a different DNA-binding site. Finally, the simultaneous use of phylogenetically different Cas9 proteins or sgRNAs that harbour different types of protein binding motives, allows for a third dimension of control. Thus, different types of enzyme activities – fused either to one type of Cas9 orthologue or to one type of RNA-binding domain specific to one type of sgRNA – can be targeted to multiple different genomic sites simultaneously. Thus, it should be possible to induce quantitatively different levels of expression of certain sets of genes and at the same time to repress other genes, redefining the nuclear transcriptome. Likewise, by the use of different types of histone-modifying and/or DNA (de)methylating activities, the epigenome of plants should be reprogrammable. On our way to synthetic plant genomes, the next steps will be to use complex genome engineering approaches within or between species borders to restructure and recombine natural or artificial chromosomes.

**Keywords:** genome engineering, synthetic DNA-binding domains, gene editing, epigenetics, gene expression.

## INTRODUCTION

Synthetic biology is a growing research field that centres on the production of artificial entities in existing living beings. In the long run even the construction of completely artificial organisms is envisaged. Synthetic biology has come into greater focus over the last decade. The greatest focus by both researchers and the general public was given to the synthesis of an artificial bacterial genome (Gibson *et al.*, 2008). The complete synthesis of an artificial chromosome in yeast was recently reported (Annaluru *et al.*, 2015). However, due to its complex genome organization and partly large genome size, a synthetic nuclear plant genome synthesised from scratch is not a realistic option for the near future. These large genome sizes contrast with the tiny organelle genomes found in mitochondria and chloroplasts in plant cells. The genomes of plastids are receiving atten-

tion from synthetic biology researchers. As synthetic organelle genomes are addressed by recent reviews in this issue and elsewhere (Scharff and Bock, 2014), the topic will not be discussed here.

In recent years, synthetic plant biology has largely been discussed in relation to the introduction of new synthetic pathways in plants. Prominent examples of these synthetic pathways covered in other reviews in this current issue are the manipulation of photosynthetic pathways or the production of new secondary metabolites. Synthetic biology also focuses on the development of synthetic tools for the manipulation of cells, such as synthetic signalling pathways and synthetic sensors (topics that are covered by other reviews in this issue; see also (Liu and Stewart, 2015)). These synthetic tools can then be used to establish

new synthetic pathways (Small and Puchta, 2014). Thus, the various levels of activity that are stimulated in synthetic biology engineering of artificial entities allow us to push the field to even higher levels. In a way it seems that synthetic biology might thus escape the 'Münchhausen trilemma'. This dilemma is named after German storyteller Baron Münchhausen, who wanted to make everyone believe that he had pulled himself and his horse out of a swamp by his own hair. The term was coined by the German philosopher Hans Albert, in unmasking the ideologies and arguments that prove some type of absolute truth. However, figuratively, it seems that developing new synthetic biological tools can help pull synthetic biology itself out of the swamp by its own hair.

During the last decade, the most widely used class of artificial tools was synthetic DNA-binding domains that consist of either proteins or protein RNA complexes that can address unique genomic sequences. These binding domains can be linked directly or indirectly to domains with different types of enzymatic activity that can induce specific changes either in the DNA sequence itself or in its transcriptional and/or epigenetic state. Indeed, a prerequisite for this type of approach is the high specificity of the respective domain to target only one unique site in a genome. Statistical calculations indicate that a unique site is defined in the range of 18–20 nucleotides (nts) in plant genomes, depending on their size. Highly specific DNA-recognition domains are found in transcription factors, recombinases and certain classes of endonucleases, and these types of enzymes were also the basis for the development of the respective synthetic tools.

Initial attempts to create synthetic tools with DNA-binding ability were made with homing endonucleases and zinc finger (ZF)-binding domains. Later, transcription activator like effectors (TALE) and, more recently, the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) system, became popular as building blocks for synthetic DNA-binding domains (Puchta and Fauser, 2014). Primarily, artificial binding domains were fused to and/or used as endonucleases that could be applied to genome engineering. The detailed development of genome engineering tools in plants is discussed in great detail in a number of excellent reviews, to which the reader is referred (Puchta and Fauser, 2013, 2014; Voytas, 2013; Mahfouz *et al.*, 2014; Baltes and Voytas, 2015; Belhaj *et al.*, 2015; Lee *et al.*, 2015; Schaeffer and Nakata, 2015; Weeks *et al.*, 2015; Zhang *et al.*, 2015).

Using the ZNF and TALE approaches, it was possible to target a single unique site in the genome. However, to address more than one site, a second DNA-binding domain had to be expressed in the respective cell simultaneously. The only exceptions were to construct protein–DNA-binding domains that could recognize a sequence that was present more than once in the genome or to reduce the

binding specificity so that multiple different but closely related similar sites could be recognized by one and the same DNA-binding domain. The specificity, particularly of zinc-finger nucleases (ZFNs), was often just not good enough to target only one site in a large eukaryotic genome. Other unwanted double-stranded breaks (DSBs) at similar sites were induced that often were harmful to the cells (Ramalingam *et al.*, 2011). This phenomenon is called the 'off-target' effect. Only with the recent discovery of the bacterial CRISPR/Cas system of the human pathogen *Streptococcus pyogenes* as an easy programmable nuclease (Jinek *et al.*, 2012) did it become possible to overcome these serious limitations and target more than one specific, unrelated genomic site at a time (for details, see below). Thus, for the first time, a second dimension of genome manipulation – the simultaneous addressing of multiple genomic sites – was opened by the CRISPR/Cas system-based, DNA-binding domain.

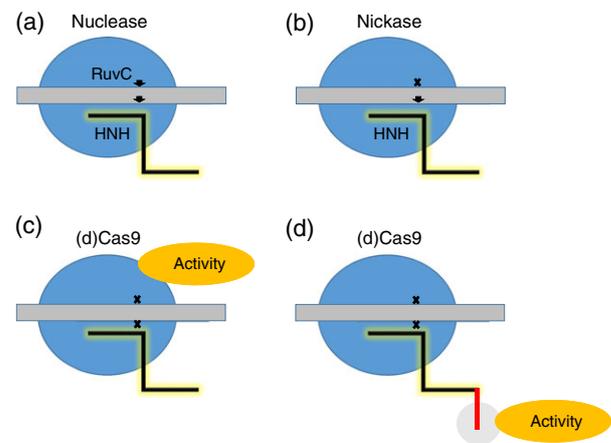
The aim of this review was to discuss which kind of potential of the CRISPR/Cas system holds for synthetic plant biology. Obviously, developments expected in the near future with regard to plants must be discussed in relation to recent breakthroughs obtained with other eukaryotes. Here especially, recent results with mammalian cells are inspiring. The one-dimensional addressing of a single activity to a single site in the genome can be achieved by the use of a single sgRNA and a single Cas9 protein species. The two-dimensional addressing of several sites can be achieved by the use of a Cas9 protein and multiple sgRNAs that define different binding sites. However, in such a way only one unique enzyme activity can be targeted. By using several Cas9 orthologues or several sgRNAs that differ in their RNA-binding domain specificity, two-dimensional approaches can be combined, resulting in a 'third' dimension in the simultaneous addressing of different sites in the genome with different enzyme activities. These developments indicate that we are getting closer to synthetic transcriptomes and epigenomes and that also steps to a synthetic plant genome are taken with accelerated speed.

#### **CRISPR/CAS: AN ALL-PURPOSE TOOL TO TARGET VARIOUS ENZYME ACTIVITIES TO GENOME SITES**

Since the development of the PCR technique, no tool has had such a tremendous influence on the development of molecular biology as the CRISPR/Cas technology. Still, not all of the consequences of CRISPR/Cas's current or putative applications of the system can be evaluated (Doudna and Charpentier, 2014; Ledford, 2015). CRISPR/Cas is of particular importance to the future of synthetic biology due to its great potential to open new avenues of manipulation and expression of genetic information. The system is part of the immune response in bacteria for fighting infecting phages or plasmids. In their genome, the respective bacteria carry unique sequence stretches of 20 nucleotides that are

derived from pathogens from previous infection flanked by short repeats. After transcription of a long RNA, short individual RNA molecules (crRNA, abbreviation for CRISPR RNA) are processed and set free. Together with a second conserved RNA (tracrRNA, abbreviation for tracing RNA), they define the specificity of the Cas9 nuclease by pairing with their non-redundant sequence to the invading pathogen. There are three classes of the bacterial CRISPR/Cas system, types I to III. Only type II is characterized by an active nuclease entity consisting of a single protein (Cas9) and the crRNA–tracrRNA complex (Louwen *et al.*, 2014). This type II system is again subdivided into types II-A to II-C (Fonfara *et al.*, 2014; Hsu *et al.*, 2014). Cas9 proteins of the type II-A are primarily being applied for genome engineering in eukaryotes at the moment (Kleinstiver *et al.*, 2015; Ran *et al.*, 2015). The type II-A Cas9 orthologue of *Streptococcus pyogenes* (SpyCas9) has been used in the field almost exclusively until now. The SpyCas9/RNA complex recognizes a target-specific sequence that is 23 bp in length. In addition to the 20 bp specified by the crRNA, a 3-bp motif ending with two guanines (GG), the PAM (protospacer adjacent motif) sequence, is recognized by the Cas9 protein. Out of the two RNAs, a single guide (sg) RNA was constructed for biotechnological applications (Jinek *et al.*, 2012).

Up to now, most scientists use the CRISPR/Cas system as sequence-specific nuclease for genome engineering. In its most prominent application a DSB is induced in a gene of interest. As the most efficient DSB repair pathway in plants, nonhomologous end joining (NHEJ), is often imprecise, mutants can easily be obtained that way. The nuclease activity of the Cas9 protein depends on two enzymatic domains, the HNH and the RuvC domains (see Figure 1a). The exchange of single amino acids (aa) in both active centres results in a 'dead' (d)Cas9 protein that is still able to bind in a sequence-specific way to DNA by its sgRNA (Gasiunas *et al.*, 2012; Jinek *et al.*, 2012). However, if only one nuclease domain is mutated, a nickase is obtained (see Figure 1(b)). Thus, specific single-stranded breaks (SSBs) can be targeted to any position in the genome. It has been shown that in contrast with a DSB a single SSB is not mutagenic (Fauser *et al.*, 2014). With two so-called 'paired nickases', two SSBs in close proximity to each other can be induced in opposite strands, resulting in a mutagenic DSB. This is possible by expressing two different sgRNAs together with the Cas9 nickase. (Mali *et al.*, 2013; Ran *et al.*, 2013). In this way, the specificity of the induced DSB can be enhanced by orders of magnitude because it is then defined by 40 instead of 20 bps. Thus, off-target effects, due to the limited specificity of the nuclease that has been a concern for CRISPR/Cas technology (Cho *et al.*, 2014) and that has also been documented for plants (Endo *et al.*, 2015; Sun *et al.*, 2015), can be avoided. The paired nickases technology was recently applied in plants for heritable mutagenesis (Schiml *et al.*, 2014).



**Figure 1.** The multiple facets of CRISPR/Cas-based synthetic engineering tools.

(a) The classical nuclease depicted with a single guiding RNA. The HNH and RuvC domains that are responsible for inducing a SSB in one or the other DNA strand are also shown.

(b) The Cas9 nickase can induce a SSB in only one of the strands because the other SSB-inducing activity (in this case, the RuvC) is inactivated by a mutation in its active centre.

(c) The dead (d)Cas9 as DNA-binding domain, the specificity of which is defined by its sgRNA fused to another protein domain with specificity. Thus, activation or repression transcription but also epigenetic change on the DNA level or by covalent modification of histones can be achieved.

(d) A (d)Cas9 with a modified sgRNA that is enlarged by an aptamer-binding site for an RNA-binding domain (e.g., from the MS2 phage). The RNA-binding domain is then fused to the enzyme activity that should be targeted to the specific site to induce the desired change in, e.g., the transcriptional and/or epigenetic state. Blue: Cas9 protein, black: sgRNA; grey bar: DNA.

Whereas the functions of nuclease and nickase are inherent to the Cas9 protein, any other type of enzyme activity that should be targeted to a specific site in the genome must be linked physically to the Cas9/sgRNA complex. This linking can be achieved by either fusing the respective enzymatic domain directly to the Cas9 open reading frame (ORF) or by using a modified sgRNA (see Figure 1c, d). Mainly Cas9 protein fusions with domains that either activate or repress transcription were initially made [e.g., (Gilbert *et al.*, 2013)]. By adding sequence-specific aptamer motives, modified sgRNAs have recently been constructed that enable the recruitment of enzymatic functions via the RNA moiety of the complex. Aptamers are RNA sequence motives that are defined as binding sites for the sequence-specific binding of a protein domain, e.g., derived from the coat protein of an RNA phage such as MS2. The respective aptamer-binding domain is then fused to an enzymatic domain that should be targeted to the specific site. More than one aptamer can be used here, and thus several active domains can be recruited using one single Cas9/sgRNA complex (Konermann *et al.*, 2015). There are pros and cons to each approach. On the one hand, RNA binding must be as specific as the DNA of the Cas9/sgRNA binding because the complexity of the cellular transcriptome is not

much lower than that of the genome of the particular organism. Binding of the respective RNA-binding domain to cellular RNAs might otherwise perturb the cell metabolism. On the other hand, several molecules of the respective enzyme activity can be targeted by a unique Cas9/sgRNA complex to a specific site using the aptamer approach. Additionally, the use of an RNA instead of a protein linker might allow a more efficient application of the enzyme activity to the target site due to lesser steric hindrance between Cas9 and the fused protein domain.

It was recently discovered that by manipulating the sgRNA-DNA-binding site, the RNA/Cas9 complex can be changed from nuclease to a DNA-binding protein. sgRNA that have a DNA-binding site that is 17 or more nts cut DNA effectively in the presence of an active Cas9 nuclease protein (Fu *et al.*, 2014). However, reduction to 15 nts or fewer results in a complex that is able to bind to the respective sequence but no longer induces breaks (Kiani *et al.*, 2014). Based on this principle, induction of DSBs and transcriptional regulation can be achieved simultaneously by expressing two different types of sgRNAs with a single active Cas9 (Dahlman *et al.*, 2015). For DSB induction, the classical sgRNA would be used with 20 nts binding sites for transcription control, and for a specific sgRNA with a 14mer binding site and aptamers for binding of the MS2, an RNA-binding motive can be fused to the respective transcriptional activator or suppressor domain. However, it is doubtful whether such an approach will be useful for plants because the 14 nts might be insufficient in targeting unique sequences in their large genomes.

#### GENOME ENGINEERING IN TWO DIMENSIONS

The basic principle behind modern genome engineering is the setting of site-specific DSBs that are then repaired in different ways by the repair machinery of the respective organism. A wealth of knowledge on DSB repair in plants has accumulated over the years. For reviews, see Knoll *et al.* (2014) and Puchta (2005). Indeed two decades ago, it has been demonstrated that inducing a single site-specific DSB gene targeting (GT) via homologous recombination (HR) can be enhanced for plants (Puchta *et al.*, 1996). Additionally, NHEJ can be used to knock out a functional ORF (Salomon and Puchta, 1998; Kirik *et al.*, 2000). For these experiments, a homing endonuclease was used to cut a unique site in the plant genome that was transformed before the respective experiments were performed. With the development of ZFNs that were based on fusing the nuclease domain of a restriction enzyme to a ZF binding domain, artificial and programmable nucleases could be constructed (Kim *et al.*, 1996; Smith, 2000). It took some time before these synthetic enzymes were successfully applied to the induction of genomic DSBs throughout the plant genome. Thus, the knockout (Lloyd *et al.*, 2005) or GT (Wright *et al.*, 2005; Shukla *et al.*, 2009; Townsend

*et al.*, 2009) of any natural gene by the induction of a unique DSB became a possibility for plants. Later, transcription activator-like effector nucleases (TALENs) were developed and applied to the same types of genomic changes. For a review, see (Voytas, 2013).

Using ZFNs or TALENs as well as homing endonucleases, it became possible to induce other types of genomic changes by the induction of more than one DSB. If two DSBs are induced at the same time in a genome, the putative outcome depends on their location. Apart from the repair of individual DSBs by NHEJ, it has been shown that two DSBs on the same chromosome in closer proximity can lead to deletions (Siebert and Puchta, 2002; Petolino *et al.*, 2010), whereas if they occur on different chromosomes, reciprocal translocations might be induced (Pacher *et al.*, 2007).

For the CRISPR/Cas system, the induction of multiple breaks is a system-inherent property. In the natural situation, the bacterial cell has the potential to fight multiple different pathogens at the same time due to the presence of multiple gRNAs. Due to the simplicity of this process, we can mimic it by using multiple sgRNAs to simultaneously induce a larger number of DSBs within a single plant genome. Multiple independent transcriptional units were used initially to express the individual sgRNAs. By exploiting the natural tRNA-processing system, which cleaves both ends of the tRNA precursor, an efficient tool for multiplex genome editing was recently established for plants. Here, multiple sgRNAs flanked by tRNA genes can be integrated in a single polycistronic transcriptional unit. This tool's potential can be demonstrated in rice for multiplex genome editing and chromosomal-fragment deletion (Xie *et al.*, 2015).

The simultaneous induction of multiple DSBs opens new avenues for genome engineering. By inducing four DSBs, targeted gene exchange has been induced in plants (Fauser *et al.*, 2012; Weinthal *et al.*, 2013; Schneider *et al.*, 2015). The CRISPR/Cas system is mainly applied to delete plant genomic sequences, e.g., (Zhou *et al.*, 2014; Johnson *et al.*, 2015) or to simultaneously induce mutations in several genes at the same time, e.g., (Ma *et al.*, 2015). However, the CRISPR/Cas system may soon see additional applications. The engineering of single genes is popular, whereas plant genome engineering in the true sense of the phrase has hardly been addressed yet (see discussion below).

#### REDIRECTING TRANSCRIPTION

Modifying natural DNA-binding specificities was first performed using transcription factors. This process started with the manipulation of zinc-finger binding motifs. By manipulating zinc-finger-containing DNA-binding motifs of transcription factors, new binding specificities were obtained that did not previously exist in nature (Rebar and Pabo, 1994). Later, transcription activator-like effectors (TALEs) of plant pathogenic bacteria were discovered that

can modulate gene expression in the host genome (Boch *et al.*, 2009). Fusing these DNA-binding domains to protein domains that either activate or repress gene expression then became an obvious avenue for action, and thus synthetic transcription activators or repressors could be obtained.

Early experiments to manipulate gene expression in plants were performed by fusing 'natural' DNA-binding domains of classical transcription factors to a shorter peptide sequence that inhibits transcription (Hiratsu *et al.*, 2003; Matsui *et al.*, 2005). Thus, genes regulated by specific classes of transcription factors could be repressed. The use of synthetic DNA-binding motives had a tremendous effect on widening the field of application. After its successful implementation in mammals (Liu *et al.*, 2001), the technology was applied to plants to control gene expression (Stege *et al.*, 2002). It was demonstrated that the lignin content could be changed by regulating the 4-coumarate:coenzyme-A ligase-1 gene in *Arabidopsis thaliana* by an artificial zinc-finger chimaera fused to either an activator or a repressor domain. Transgenic activator lines produced an increase in lignin content, whereas lines with the repressor fusion resulted in reduced lignin content (Sánchez *et al.*, 2006). This strategy has also been applied to trait development in crop plants (Gupta *et al.*, 2012). Zinc-finger transcriptional activators were designed to bind DNA sequences common to two canola  $\beta$ -ketoacyl-ACP synthase II promoters. Transgenic canola plants contained significant decreases in palmitic acid and increased total C18 fatty acids. Thus, agronomically relevant traits in crop species can be improved not only through genome editing using synthetic nucleases, e.g., (Haun *et al.*, 2014) but also by artificial transcription factors. The CRISPR/Cas system was recently adopted to switch single genes on and off in *Arabidopsis* (Lowder *et al.*, 2015; Piatek *et al.*, 2015).

In addition to the targeting of single genes with artificial transcription factors, it is also possible to influence the transcriptome on a more complex level. The basic idea behind this approach is to use a DNA-binding domain that targets either activators or repressors to multiple sites in the genome due to reduced binding specificity (Park *et al.*, 2003). Whereas in the case of synthetic nucleases such an approach would usually result in a lethal phenotype due to the induction of multiple DSBs, a significant change of the transcriptome might lead to the production of new phenotypes. Such an approach, which is also called 'genome interrogation', was set up in plants by Bert van der Zaal to produce *Arabidopsis* plants with increased frequencies in HR (Lindhout *et al.*, 2006; Jia *et al.*, 2013, van Tol and van der Zaal, 2014). However, although sets of genes can be stochastically targeted using genome interrogation, it is impossible to define most individual genes that will be addressed beforehand. Although new phenotypes might be selected for, it is not feasible to target predefined genes

that are part of a common metabolic pathway using such an approach.

## EPIGENETIC EDITING

In general there is a large overlap in activities that might be fused to DNA-binding domains to achieve transcriptional and epigenetic change. Often, the domains are themselves mediators that recruit further proteins to the site of action. The epigenetic landscape of the genome is defined on one side by the state of methylation of DNA sequences. On the other side, the chromatin that is involved in the packing of the respective DNA is of utmost importance. This applies not only to the presence of individual subspecies of classical histones but also to the covalent posttranslational modifications of individual histone molecules by, e.g., methyl or acetyl groups. For a set of recent reviews in plants, see (Gutierrez and Puchta, 2015).

From early on, different approaches have been taken to change the epigenetic state of nuclear genomes. In the beginning chemicals such as Azacytidin that influence the state of methylation of DNA on a global level, or Trichostatin A, which influences histone acetylation on a genome-wide level were used. Epigenetic change can also be achieved by manipulating the enzyme machinery involved in the process. However, no site-directed changes can be achieved in this way. Site-directed changes can only be made by fusing DNA-binding domains to enzymes that are able to directly influence histone modifications or DNA methylation. Such approaches have been successfully applied in mammals. Overexpressed oncogenes can be silenced by using specific ZF domains fused to the DNA methyltransferase 3a to methylate cytosine residues within the promoters of the respective genes. (Rivenbark *et al.*, 2012; Stolzenburg *et al.*, 2012). Using fusions of engineered TALE repeat arrays and the TET1 hydroxylase catalytic domain, it was possible to modify the state of methylation of CpG positions of the promoter of the endogenous human genes (Maeder *et al.*, 2013). Using a programmable CRISPR-Cas9-based acetyltransferase based on the catalytic core of the human acetyltransferase p300, it is now possible to specifically acetylate histone H3 lysine 27 at its target sites. Histone H3 lysine 27 acetylation is enhancing gene expression by enhancing the recruitment of transcriptional activators as well as the transition of RNA polymerase II from initiation to elongation. (Stasevich *et al.*, 2014). Thus, transcriptional activation of target genes from promoters and enhancers can be achieved in mammals (Hilton *et al.*, 2015).

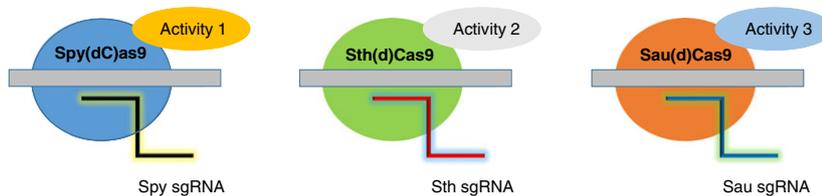
The field of synthetic epigenetics is still in its infancy, although its potential is now becoming more apparent. For recent reviews, see (Jurkowski *et al.*, 2015; Laufer and Singh, 2015). No work on the epigenetic editing of plant genes has yet been published, but it is only a matter of time until they become available.

### THE THIRD DIMENSION: TARGETING DIFFERENT ENZYMATIC ACTIVITIES TO DIFFERENT SITES IN A SINGLE CELL

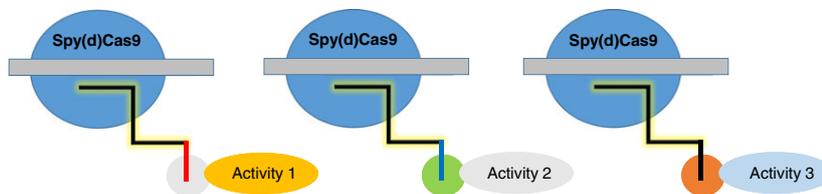
Due to the possibility of multiplexing with the CRISPR/Cas system, sets of genes can be either switched on or off, or sets of genomic loci can be epigenetically modified by using a set of sgRNA with different DNA-binding sequences. Likewise, multiple DSBs or multiple SSBs can be induced at different positions in the genome. However, for the build-up of synthetic entities, more than one type of activity in a cell must be controlled, particularly for gene expression. A strategy for the redirection of the transcriptome of a cell to switch on specific metabolic pathways will not only enhance the expression of certain key regulators of the pathway of interest, it will also be necessary to shut down the expression of other factors that are involved in pathways that compete for the respective substrates. Only under such conditions can the optimal product yield be obtained. To give an easy example from the field of genome engineering for a putative application: NHEJ and HR are competing pathways for DSB repair (Puchta, 2005). Using HR predefined changes like the exchange of single amino acids can be introduced into the plant genome by the use of a homologous repair template ('gene targeting'). However, in somatic plant cells NHEJ is the prevailing pathway by which most DSB are repaired. Thus, by transcriptional activating factors involved in HR and at the same time repressing the expression of factors involved in NHEJ the equilibrium might be shifted to HR and GT might become much more efficient.

There are two ways how simultaneous but independent targeting can be achieved. First, one can use different orthologous CRISPR/Cas9 systems, and each Cas9 orthologue would be fused to a unique enzyme activity. The Cas9 orthologue of *Streptococcus pyogenes* (SpyCas9) is almost exclusively used in the field. A prerequisite for the use of several different orthologues is that if expressed in the same cells, the respective sgRNAs of each orthologue should only interact with their species-specific Cas9 protein but not with the proteins from other species. It can be demonstrated in plants that the Cas9 orthologues from *Streptococcus thermophilus* (SthCas9) and *Staphylococcus aureus* (SarCas9) can both be used for efficient heritable genome editing in *Arabidopsis thaliana* at comparable frequencies as those of the SpyCas9 system (Steinert *et al.*, 2015). Because both SthCas9 and SaCas9 differ from SpyCas9 in their PAM sequences, they are also valuable alternatives to genome editing because the restrictions in site selection differ between all three orthologues. Importantly, it can be demonstrated that Sar- and SpyCas9 proteins enhance HR by DSB induction only in the presence of their species-specific single guide (sg) RNAs but not with their orthologous counterparts (Steinert *et al.*, 2015). Thus, at least the CRISPR/Cas systems of *S. pyogenes* and *S. aureus* can indeed be used to simultaneously address different sequence motives with different enzyme activities in the same plant cell (Figure 2).

In addition to using two different orthologues directly fused to transcriptional enhancers and repressors, specific sets of genes can be switched on and others off at the



**Figure 2.** Cas9 orthologues can be used to achieve targeting of different types of enzymatic activities to different genomic sites simultaneously. The specificity of the different orthologues is specified by their respective species-specific sgRNAs. If the respective orthologues are fused to different enzymatic domains, different types of change on the transcriptional, genetic or epigenetic level can be induced at the same time at different locations in the cell. Grey bar: DNA



**Figure 3.** Modified sgRNAs can be used to achieve targeting of different types of enzymatic activities to different genomic site simultaneously. Using a single species of (d)Cas9 and multiple sgRNAs, it is possible to target different enzyme activities to different sites in the genome. Prerequisite is that these RNA not only differ in their DNA-recognition sequences but in their aptamer structure for binding different types of RNA-binding domain. Thus, different types of enzymatic activities can be fused to the respective RNA-binding domain. Grey bar: DNA.

same time by fusing different enzymatic activities to specific sgRNA-interacting proteins. With this approach, one specific type of (d)Cas9 protein but different classes of sgRNA can be used in one cell (Figure 3). Each sgRNA class would target one specific type of activity to a set of different sites in the genome. Thus, members of each class would differ in their sequence defining the DNA-binding domains. They would be identical in sequence in the part of the sgRNA where, due to the inclusion of aptamer sequences, specific protein binding can occur. Here, the RNA-binding domain fused to the enzyme activity that should be targeted will bind. For each class of sgRNA, independent multiplexing would be possible. The classic case would be to use to one type of RNA for transcriptional enhancement and another type of RNA for transcriptional repression. There are some limitations to this approach, such as that one cannot induce DSBs and SSBs in the same cell because the (d)Cas9 protein is used for the sgRNA targeting at the same time.

**SYNTHETIC PLANT TRANSCRIPTOME AND EPIGENOME ARE COMING CLOSER**

By the combined use of different orthologues and different types of modified sgRNAs, one can theoretically target

more than two different enzymatic activities (e.g., general repressors or general activators) to two different sets of genes (Figure 4). It should be possible to target multiple types of enzymatic activities to multiple different genomic locations with current tools. Thus, quantitative differences in the amount of induction and repression can be defined differently for different sets of genes (Figure 5). Thus, particularly in cases where the expression of transcriptional regulators is addressed, the transcriptome of a cell can be changed dramatically.

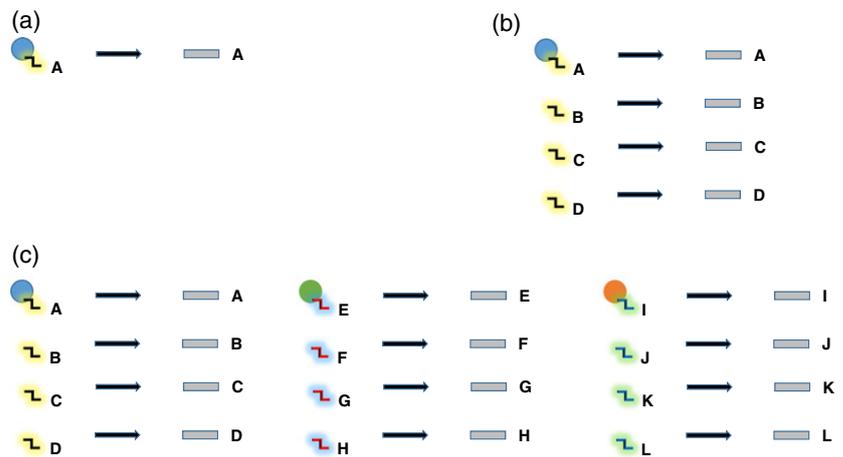
The same holds true for the epigenetic state of a cell. Here, DNA methylation and various types of covalent histone modification can be changed in a specific region of the genome at the same time. As euchromatic and heterochromatic regions in the genome differ in several epigenetic marks, it might not be too overoptimistic to speculate that by the concerted application of enzyme activities that introduce or remove such marks, we will be able to transform heterochromatic regions in euchromatic regions and vice versa. Interestingly, it has recently been shown that a change of DNA methylation can silence meiotic crossover hotspots in Arabidopsis (Yelina *et al.*, 2015). Obviously, massive epigenetic reprogramming of a cell would also be accompanied by massive change of the

**Figure 4.** CRISPR/Cas mediated change in three dimensions.

(a) The one-dimensional addressing of a single activity to a single site in the genome can be achieved by the use of a single sgRNA and a single Cas9 protein species.

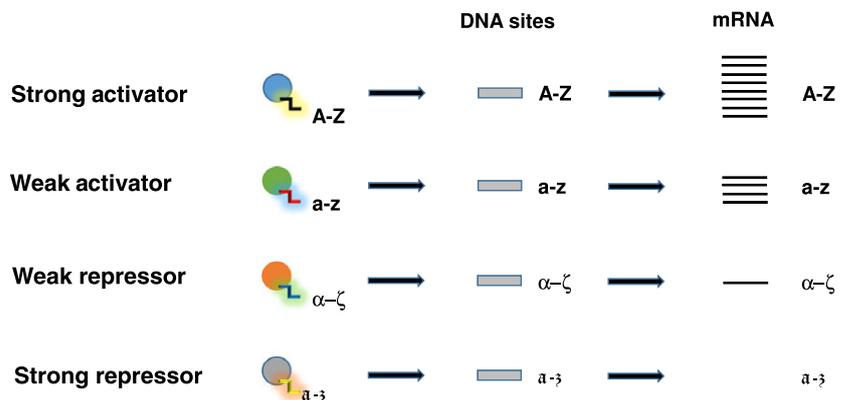
(b) The two-dimensional addressing of several sites by a unique enzyme activity can be achieved by the use of a Cas9 protein and multiple sgRNAs that define different binding sites.

(c) By the use of several Cas9 orthologues or several sgRNA that differ in their RNA-binding domain specificity, two-dimensional approaches can be combined in a third dimension, resulting in the simultaneous addressing of different sites in the genome with different enzyme activities. Grey bars: DNA sites.



**Figure 5.** Putative three-dimensional change of the cellular transcriptome by application of CRISPR/Cas system.

By using sets of different Cas9 proteins, a class of strong and a second class of weaker activators of transcription can be applied at the same time as a class of stronger and a class of weaker repressors in all the same cells. Each class would address a larger set of genes individually (each gene class depicted here as a grey bar with members defined by alphabetic order, for each class in a different style, mRNAs are depicted as black lines). Particularly if genes of transcriptional regulators are addressed in this way, the transcriptome of a cell may be changed so drastically that it can be regarded as synthetic.



cellular transcriptome. Thus, synthetic transcriptomes and epigenomes might be two sides of the same coin.

#### **TOWARDS THE SYNTHETIC PLANT GENOME: CHROMOSOME RESTRUCTURING WITHIN AND BETWEEN SPECIES BARRIERS AND ARTIFICIAL CHROMOSOMES**

Although the CRISPR/Cas system is largely used for genome engineering, it seems that by its application we are much closer to obtaining synthetic transcriptomes and epigenomes than genomes. A first step towards synthetic genomes would be to change the order but not the content of the genetic information of an individual. This can be achieved by the induction of more than one genomic DSB in somatic cells. If two breaks are induced on the same chromosome, besides deletions also inversions might occur (Qi *et al.*, 2013). This phenomenon has already been used to correct large factor VIII gene chromosomal inversions in cells of a Haemophilia A patient (Park *et al.*, 2015). By the induction of one DSB per chromosome, the reciprocal exchange of chromosome arms can be achieved (Pacher *et al.*, 2007). Both types of rearrangements may be interesting for plant breeders: Using chromosomal translocation, new linkage groups can be constructed or an adverse linkage of traits broken. A chromosomal inversion guarantees that within the inverted segment, no meiotic exchange will occur with the other parental homologue that does not harbour the inversion. Crossover formation between cultivars and wild relatives is often hindered by the presence of chromosomal inversions (Wolters *et al.*, 2015). Thus, inversions could be reversed to enhance beneficial trait transfer.

Alternatively, meiotic DSBs might be directly targeted to specific sites to enable the controlled exchange of traits between parental genomes. In a ground breaking study, the group of Alain Nicolas fused Spo11, the factor that enzymatically induces DSBs in meiosis, to a DNA-binding domain of a transcription factor in yeast. Thus, meiotic recombination can be targeted to a novel site in the genome (Peciña *et al.*, 2002). Using direct or RNA-based (d) Cas9 fusions to Spo11 or even the direct induction of DSBs via the Cas9 nuclease, it may be possible to break undesired linkages of traits at will. In the long run, we may be able to control inheritance on a broader level in plants.

Genomic rearrangement does not have to stop at species borders. Somatic hybrids have been achieved in plants by directly fusing the protoplasts of different species. Thus, genetic cross-barriers can be overcome (Xia, 2009). Either complete chromosome sets can be fused together or single chromosomes can be added to a complete set by microprotoplast-mediated chromosome transfer (Ramulu *et al.*, 1996). In such a situation, chunks of chromosomal DNA could be exchanged between species by inducing complementary DSBs in fused hybrid cells. For such a purpose, the use of Cpf1, a putative type 2 CRISPR effector

with a single RNA-guided endonuclease lacking the tracrRNA that was recently characterized, is particularly attractive. Interestingly, Cpf1, which was shown to work efficiently in genome engineering in mammalian cells, cleaves DNA via a staggered DNA double-stranded break. (Zetsche *et al.*, 2015). Because complementary sticky ends can be relegated, it should be possible to plan genome rearrangements on the nucleotide level beforehand. Using the recently developed haploid inducer technique [for review, see (Comai, 2014)], such engineered chromosomes might be obtained in their homozygous form much more efficiently.

Another important step towards artificial plant genomes is the setup of individual plant artificial chromosomes (PAC). The reader is referred to excellent recent reviews on this topic (Birchler, 2015; Yu *et al.*, 2015). In principle, PACs could either be assembled *in vitro* by combining centromeres, telomeres and replication origins or by truncation of existing chromosomes *in vivo*. The former approach has not resulted in reproducible success until now. In contrast, the breakage of plant chromosomes by the transformation of telomeric repeats has been successfully applied for a row of plant species since its first application in maize (e.g. Gaeta *et al.*, 2013; Kapusi *et al.*, 2012; Nelson *et al.*, 2011; Teo *et al.*, 2011; Xu *et al.*, 2012; Yu *et al.*, 2006). Thus, PACs that have been produced are not 'synthetic' in the strict sense of the word. PACs have many advantages and are a promising tool for synthetic biology. Thus, complete new metabolic pathways can be introduced into crop plants in a single entity. PACs can be used best for gene stacking. Here, depending on demand, new traits can be added later and linked to traits that are already present on the PAC. Site-specific recombination systems have mainly been used for stacking (Srivastava and Thomson, 2015). The use of the CRISPR/Cas system is an attractive alternative to gene stacking in PACs. It should be possible to use either GT techniques that are based on HR (Fauser *et al.*, 2012; Baltés *et al.*, 2014; Schiml *et al.*, 2014; Svitashv *et al.*, 2015) or NHEJ (Salomon and Puchta, 1998; Chilton and Que, 2003; Tzfira *et al.*, 2003; Weinthal *et al.*, 2013).

Although the construction of a truly synthetic plant genome is out of reach in the near future, many avenues for restructuring chromosomes and the introduction of synthetic entities into natural genomes are becoming possible due to the availability of the CRISPR/Cas system as a tool for genome engineering. Ironically, the same tool also enables us to manipulate plant genomes in a way such that the treated plants cannot be differentiated by any scientific means from a natural variant. Without the application of any transgenic DNA and solely by the transfection of preassembled CRISPR/Cas complexes, unique defined mutations were recently obtained for different plant species (Woo *et al.*, 2015). Hopefully, these plants help to

alleviate GMO regulatory concerns (Hartung and Schiemann, 2014) and, in the long run, to pave the way for the use of synthetic approaches in agriculture.

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