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## Site-Directed Nucleases

### *'Give it a break'*

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Mutations are essential for life as we know it. Without mutations, there would be no evolution and no biodiversity. The variation created by natural mutations has also been the basis for all plant breeding since we started cultivating plants many thousand years ago. Then starting in the 1930s, scientific progress enabled us to use radiation and chemicals to induce mutations and thereby increase the genetic variation available for plant breeding. The discovery of *site-directed nucleases (SDN)* – also called ‘molecular scissors’ – are now expected to revolutionize the field of mutation breeding. Compared to the randomness of mutations induced by radiation or chemicals, the enzymatic approach of SDN presents an unprecedented level of control and efficiency in plant research and breeding.



### **Benefits**

The precise engineering of genomes now possible with SDN techniques provides a level of control never seen before in the history of plant breeding. Induced mutations through radiation or chemicals have yielded fantastic results over the years, but require time-consuming and expensive screening on large populations and takes several generations to reach the final result. With SDN in the plant breeder's toolbox, we leap from the past trial-and-error to a future knowledge-based precision breeding.

## Scientific description

Decades of basic research has greatly improved our capacity for precise engineering of DNA sequences. The common enzymatic ‘molecular scissors’ were discovered in the 1970s and are now used to break the double-stranded DNA at precise locations. The break triggers the natural DNA-repair process in the cells, which can be exploited to introduce different kinds of mutations. There are several different systems in use today. *Zinc finger nucleases (ZFN)* and *transcription activator-like effector nucleases (TALEN)* consist of two artificially connected proteins that can be delivered to the plant cells either as a stable genome integration event or on a transiently expressed plasmid vector. *Meganucleases* are naturally occurring enzymes that are highly specific with a large DNA recognition site. The recently adapted *CRISPR/Cas9* system is by far the most efficient SDN as it takes advantage of an extremely specific guide RNA to find the target gene. Many other potentially useful enzymatic systems will also likely be discovered. SDNs are versatile and may be used for editing, inserting, deleting or replacing genes.

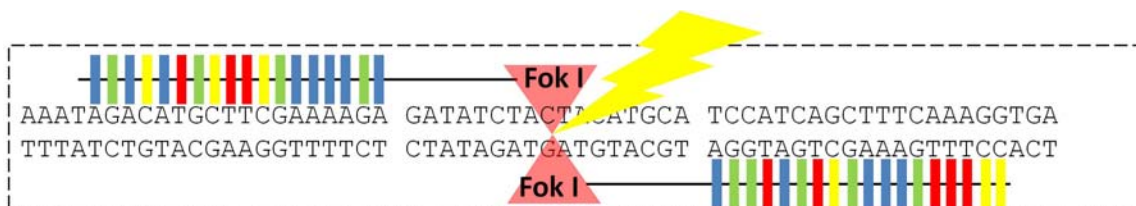


Image: Thorben Sprink, Julius Kühn-Institut

## Applications

The field of SDN has literally exploded in the last few years. Many researchers and breeders are adopting these techniques with great enthusiasm. Whereas model plants have often been used in basic research to develop SDN techniques, applications in crop plants are now increasing. ZFN technology is currently being applied in the breeding of maize, rapeseed, soybean and tomato, whereas TALENs have been used in tobacco, rice and potato. Given that SDN applications in plants are relatively recent and many more enzyme systems are expected to be discovered, the full potential of these techniques is enormous.

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## **Oligonucleotide-Directed Mutagenesis**

### ***'Matchmaking and single mismatching'***

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#### **Benefits**

ODM creates a mutation exactly where it is needed, greatly enhancing the precision, efficiency and speed of breeding. Earlier mutation breeding based on radiation or chemicals was essentially a random approach blasting thousands of seeds to induce countless mutations and then trying to cherry-pick the rare few mutations that were beneficial. A much smaller plant population is then sufficient to improve a particular trait with ODM. ODM also avoids the tedious 'cleaning up' procedure that is necessary in random mutation breeding to get rid of all the unwanted mutations through generations of backcrossing.

## Scientific description

ODM was developed already in the 1970s and improvement over the years enabled the first application in plants in 1999. This tool for targeted mutagenesis takes advantage of hybridisation, which is the matching of complementary stretches of nucleotides, such as DNA. Short oligonucleotides are synthesized to match a corresponding sequence in the gene of interest – but with one or a few mismatches. When delivered to the plant cell, these oligonucleotides will hybridize to the gene and introduce the mutation. The induced mutation– often consisting of a single nucleotide – will be stably inherited whereas the oligonucleotides are degraded in the cells. With full control of the entire process, the result in the end is an improved trait in the plant.

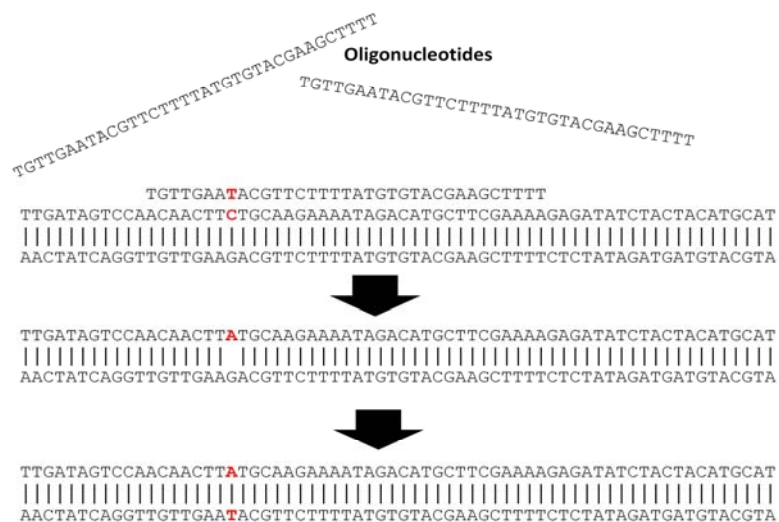


Image: Thorben Sprink, Julius Kühn-Institut

## Applications

ODM has been applied extensively in plants, both in research and in commercial applications. As a research tool, it has greatly improved the capacity for reverse genetics, the approach where genes are knocked out to analyse their functions. The most common commercial applications so far have been to engineer herbicide tolerance in maize, wheat, rice, tobacco and rapeseed. As an example, the US based company Cibus is applying ODM in their Rapid Trait Development System (RTDS) and recently put forward a herbicide-tolerant rapeseed as a test case in several EU countries.

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## RNA-directed DNA Methylation

### 'Wrap it up'

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The breeder who wants to alter a particular trait in a crop may attempt to modify the gene, or group of genes, controlling that trait. Another way to do it though, is to modify the way the genes are expressed. The result of these so-called epigenetic changes is a change in phenotype whereas the gene sequences are left untouched. This is exploited in a method called *RNA-directed DNA methylation (RdDM)*. Technically speaking a genetically modified organism, or GMO, during the initial stage of development, the resulting plants do not carry any introduced foreign DNA or new combinations of genetic material.



#### Benefits

RdDM allows highly targeted gene silencing in plants. The end result with a modified trait is similar to plants with a stable gene transformation or an induced mutation, but with the difference that the nucleotide sequence of the gene is not affected in any way. The technique is applicable to a number of crops and traits, and it is a very useful tool for both researchers and plant breeders.

### Scientific description

The expression of genes can be influenced by a number of molecular processes, referred to as epigenetics. One common example is DNA methylation, which wraps the DNA in methyl molecules to block transcription of – and effectively silencing – the gene. This process is required for normal development and plays an important role in stress responses and virus protection. Small RNA molecules can be used in the laboratory to trigger DNA methylation at specific locations. The introduced methylation pattern is meiotically stable, which means it will be inherited by the next generation. However, the transgene expressing the RNA can be removed by segregation, resulting in a non-transgenic plant with an altered trait. The methylated status is thought to continue for a number of generations before eventually fading out.



### Applications

This versatile and specific technique has been used both in basic research and in trait modification of crop plants. Biochemical pathways have been elucidated and marker genes have been silenced in model plants such as Arabidopsis and tobacco. In maize, RdDM has been used to introduce male sterility in order to facilitate hybrid breeding, and in potato to modify the starch content. Ornamental petunias have had their flower pigmentation modified, and RdDM-altered maize and rapeseed are currently waiting in the breeder's pipeline.

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## Cisgenesis

### *'Flowers from the same garden'*

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Does it matter where a gene comes from? Some may argue it is an irrelevant question, since all genetic material is made up by the same basic building blocks - the four different nucleotides in DNA. However, the origin of a particular gene may sometimes require our attention. Transgenic plants are familiar examples of genetically modified organisms, or GMOs, carrying one or more genes from any other organism. Another category is *cisgenic* plants, designated for those carrying artificially inserted genes only from the same, or from a cross-compatible, plant species. Mostly applied in potato and apple so far, the final cisgenic products are difficult to distinguish from those developed by conventional breeding despite being developed by a GM technique.

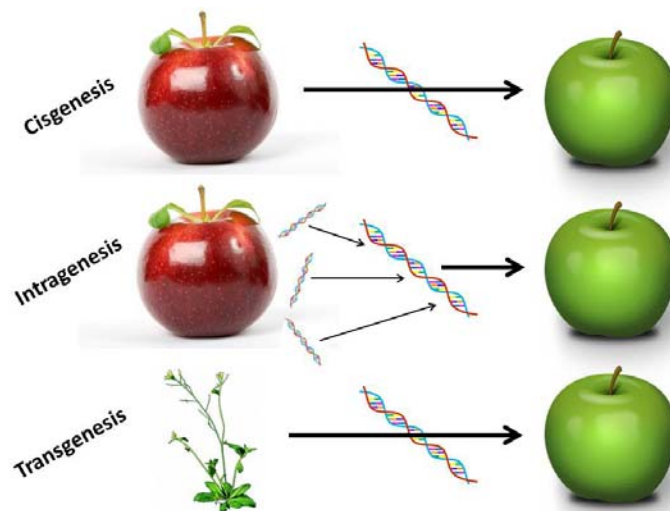


#### **Benefits**

Compared to conventional breeding, cisgenesis enjoys the advantages of GM techniques such as specificity and efficiency. One major advantage is to avoid the tedious and time-consuming linkage drag, which is when a conventional cross between two plants carries along many unwanted genes that have to be 'cleaned up' by backcrossings. Another advantage is that for clonally propagated crops, such as potato, apple, strawberry and grapevine, it is feasible to keep the original cultivar and add for example disease resistance from a crossable plant. That is not possible for these crops by conventional breeding.

## Scientific description

A GMO, as discussed back in the 1980s and outlined in the EU Directives, carries genetic material – often from a completely different species – that has been prepared outside of the organism and in a way that normally does not occur in nature. In the early 2000s though, another category within plant breeding techniques was proposed, called cisgenic plants. This category resembles transgenic plants but with the difference that only genes from a sexually cross-compatible plant have been inserted. A variation called *intragenesis* is sometimes also proposed, where rearrangements of the genetic material are allowed. The methods of gene transfer are the same, such as the commonly used *Agrobacterium* or biolistics. Since the available gene pool in cisgenic plant breeding is limited to cross-compatible species, the end products are difficult to distinguish from those achieved by conventional breeding.



## Applications

Cisgenesis has been widely applied both in research and in breeding. The most investigated traits and crops have been disease resistance in potato and apple, however it has also been applied for fungal disease resistance in grapevine, strawberry and melon, higher feed quality in barley, baking quality in wheat, drought tolerance in perennial ryegrass and to limit acrylamide in french fries. In 2010, an intragenic potato with industrial starch quality, on field trial since 1994, was approved for commercial cultivation in the EU and grown in Sweden, Germany and Czech Republic. A cisgenic potato with strong late blight-resistance has also been developed and is waiting in the pipeline.

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## Grafting using GM plants

### *'The better half'*

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Plant cells have a great capacity for regeneration and growth, as any gardener multiplying plants through cuttings is well aware of. Even more remarkable is the possibility to fuse the parts from two separate plants to create a new, chimeric plant. Commonly used in fruit orchards, the technique called *grafting* exploits this phenomena to join the scion, or above-ground part, of one plant and attach it to the rootstock of another plant. The resulting chimeric plant takes advantage of the best of two different cultivars. It is thereby possible to use for instance a transgenic rootstock with improved rooting ability but leaving the edible fruits without the slightest trace of a transgene.

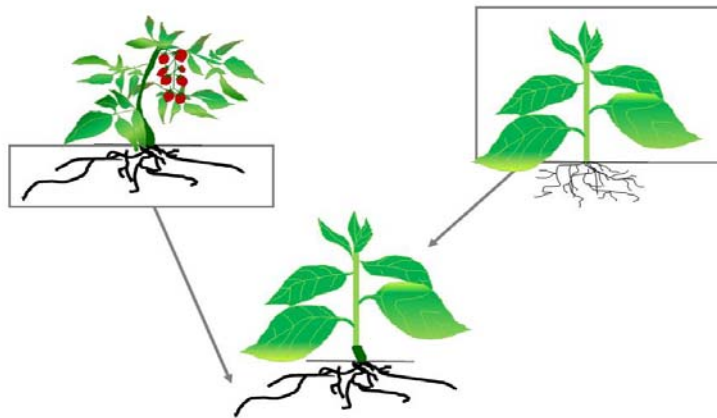


#### **Benefits**

Grafting makes it possible to combine the excellent rooting properties of one cultivar with the desired above-ground traits of another. This will greatly improve the vegetative propagation of elite cultivars as many scions may be difficult and slow to root by themselves. Many fruit trees also have a painstakingly long juvenile phase, but grafting can shorten the time to reproduction and harvest. Similarly, new hybrids from breeding programmes can be raised more rapidly this way. An alternative to breeding for disease resistance and stress tolerance is also to combine selected rootstocks and scions for a resistant and hardy plant. To increase the harvest, the desired scions are often grafted onto the rootstocks of dwarf cultivars that yield more fruit per unit of area.

## Scientific description

Grafting is a horticultural technique that has been used for millenia. Common in fruit trees, parts from one plant are attached to those of another plant so that the two sets of vascular tissues join to produce a chimeric plant. In most cases one plant is selected for its rooting ability – providing the rootstock – whereas the other plant will have desirable traits for the above-ground parts – providing the scion. The scion is most often a branch or a twig, but in the case of for example roses and peach, buds can also be used. The most common method is the so called cleft grafting, where a small cut is made in the stalk of the rootstock and the pointed end of the scion is inserted. Tape is used to hold the scion in place and grafting wax is applied to prevent the cambium layers from drying as well as a protection against infections. Whip grafting is also common, with a “z” shape of the cut to add strength.



*Image: Helga Schinkel, Fraunhofer Institute*

## Applications

Most commonly used in woody fruit trees such as apple, cherry, plum and orange tree, and also grapevine, grafting is also applied in non-woody, herbaceous plants such as tomato, potato, cucumber, eggplant and watermelon. Field trials in the EU have been carried out with GM rootstocks of apple and pear for improved rooting ability, grapevine with virus resistance, as well as orange tree with dwarf phenotype and fungal disease resistance. In the breeding pipeline are also GM rootstocks of walnut resistant to bacteria, fungi, virus and nematode.

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## Reverse breeding

### *'Meet the parents'*

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Hybrid plants are often superior in size, growth and yield compared to the parent plants. This phenomenon, called heterosis, has been exploited in plant breeding since the early 20<sup>th</sup> century. However, hybrid breeding has always been more or less based on a trial-and-error approach, since it is difficult to predict which parental lines will give the best possible progeny. Many pairs of parents then need to be crossed and their progeny tested. *Reverse breeding* puts this century-long endeavour up-side-down by starting with superior hybrid selection followed by recovery of the parental lines. This is an excellent tool in the plant breeder's toolbox, as it allows for a much more efficient and targeted hybrid plant production.

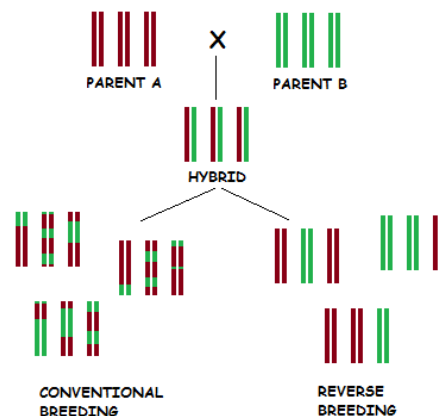


#### **Benefits**

The main advantage of reverse breeding is that it facilitates selection of superior hybrid plants. Large populations of plants can be generated and screened, and well performing plants can be regenerated indefinitely without prior knowledge of their genetic constitution. This essentially removes the elements of randomness in earlier hybrid breeding. The technique will be particularly important for crops lacking large existing collections of breeding lines. However, reverse breeding is currently limited to crops with a relatively small, diploid genome and is still difficult to carry out in crops with large, or polyploid, genomes such as wheat and oat.

## Scientific description

To multiply a highly heterozygous hybrid plant, you need homozygous parental lines. The reason is that all the gene varieties – called alleles – and their corresponding traits get shuffled around randomly in the progeny seeds if you multiply a hybrid plant by itself. You will then lose the particular allele combination that made the hybrid plant successful. Reverse breeding starts by selecting a superior hybrid plant. This plant is then transformed with a gene that will prevent recombination – or shuffling – of genes during meiosis, thus leaving the individual chromosomes exactly as they were in the homozygous parental lines, rather than reshuffled. There will still be many new combinations of entire chromosomes though, but it is relatively easy to select for the “parental” combinations as long as the number of chromosomes in that species is not too large. The transgene is also removed by selection in these reconstituted “parental” lines, which are then maintained for indeterminate hybrid production.



## Applications

Proposed in 2009, reverse breeding by engineered meiosis has not yet reached commercial application, though it has already been adopted at a research stage by many breeders. The potential is enormous – and so is also the growing interest in this technique – as breeders for many decades have sought after a reliable method to maintain superior hybrid crop plants independent of access to the parental lines. It is expected to benefit breeding of important agricultural crops such as maize, rice, pea, sorghum and sugar beet as well as horticultural crops such as cucumber, onion, broccoli, cauliflower, watermelon, tomato, eggplant and many others.

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## Agroinfiltration

### *'No strings attached'*

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To express a gene in a plant, it does not have to be stably integrated into the genome. The expression and protein translation machinery of the cells will do their work also on temporary visitors, such as genes harboured on introduced vectors. This virtue is exploited in *agroinfiltration*, a highly versatile technique that speeds up progress in the research laboratories as well as provides an excellent platform for large-scale production of valuable products in plants. Using recombinant DNA techniques, agroinfiltration leaves the genome of the plant untouched. In most cases, a next generation of plants is also not going to be generated as the plant residuals will be discarded after use.



### **Benefits**

Agroinfiltration is an excellent research tool and can be used for rapid screening and evaluation of genes under investigation. Transgenic plants with stable gene integration often take several months to develop, whereas agroinfiltration allows large-scale screening within a few weeks. The technique can also be used to screen for plants with valuable phenotypes such as disease resistance, since pathogen-specific genes can easily be applied. As a platform for production of high-value, pharmaceutical or other, recombinant proteins, it has the advantage to accumulate proteins very rapidly and in high amounts.

## Scientific description

Transient expression through agroinfiltration in plants is a relatively simple and straight-forward procedure. The most time-consuming step is the cloning of a transgene construct into a vector for transient delivery into the plant cells. The vector is commonly harboured in *Agrobacterium* cells and infiltration can be carried out either directly with a syringe or in a vacuum chamber. Rather than integrating any DNA-string into the genome, the delivered gene will only be transiently expressed in the cells. Two methods can be differentiated: '*sensu stricto*', where leaves or other non-germline tissues are infected followed by local expression; and *agro-infection*, where similar tissues are infected with a virus-based vector causing the gene to spread in the entire plant.



Image: Chandres

## Applications

Developed as a simple tool to study plant-pathogen interactions, protein localisation, protein-protein interactions and biosynthetic pathways, agroinfiltration has been applied extensively in research and breeding for screening and evaluation of genes and phenotypes. Notable examples include screening for disease resistance in potato, tomato, rice, bean, lettuce and rapeseed. As a production platform of vaccines - for hepatitis B and SARS amongst others - tomato, tobacco, white clover and lettuce have been used.

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