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# Summary

Whenever plant biotechnology pops up in conversation, it is usually as part of a debate on genetically modified (GM) crops. Nevertheless, selective genetic modification of crops with the use of GM technology is only one of the many possibilities we have to make plants respond better to our needs. In this VIB Fact series issue, we outline how the crops we know today have evolved from nature, with particular emphasis on the role humans have played.

Since agriculture began around 10,000 years ago, humans have adapted plants to suit their purposes. To start with, only the best-performing plants that nature provided were selected and retained. In addition, useful traits that had appeared spontaneously were bred into certain crops by human selection, often by going against natural selection. Mendel's discovery of the laws of inheritance towards the end of the 19th century accelerated plant modification. With the discovery of the structure of DNA by Watson and Crick in 1953, knowledge of genetic material increased substantially. New methods directly targeting DNA saw the light of day; first in the form of mutation breeding around 1960 and later using recombinant DNA technology or GM technology in 1983. Increasing knowledge of plant genetics and above all, improved methods of DNA analysis, led to an upgrade of existing techniques (such as cross-breeding) to arrive at marker-assisted selection. Although there has been a succession of many different techniques, they have not replaced each other. They all have their own value in specific situations, and the existence of several techniques gives plant breeders the 'tools' they need to come up with new varieties.

Plant breeding is continuously on the move. And with the rise of new technologies, a debate started concerning their need, potential risks and technical aspects of how to create the appropriate legislation. In the wake of the GM debate, certain new breeding techniques—which are often referred to with the abbreviation NBTs (New Breeding Technologies)—are coming under increased scrutiny, especially from a regulatory standpoint. In this VIB Fact series issue, we explain how these techniques work, how they differ from generally accepted methods, and what advantages they have over traditional breeding techniques.

# Facts and figures

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*All the crops we are familiar with today are the result of human selection and intervention.*

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*To keep agricultural production sustainable, efforts must be made in the field of cultivation methods as well as crop breeding. The two fields complement each other.*

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*All crop breeding methods - old and new - are based on processes that occur in nature.*

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*Over the last eighty years, breeding techniques using radiation or chemical substances have resulted in over 3,000 varieties of crops that we consume almost on a daily basis.*

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*Adapting crops to our needs is nothing new. It is thus a misconception that with modern breeding techniques we would suddenly shift from “natural plants” to “laboratory plants”.*

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*New crops must be evaluated on the basis of their new traits, not on the breeding technique used to genetically modify them.*

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## Genetics and soil

The transition from a hunter-gatherer situation to agriculture was undoubtedly the most dramatic change in human history. Instead of waiting for the right conditions to collect food, humans took matters into their own hands. We selected plants and animals and crossed and bred them so that they slowly but surely became more adapted to our requirements. Natural ecosystems were ploughed, fertilized and irrigated. Many efforts were undertaken to optimize both genetic traits and conditions for growth.



## More and better food

Changing the hereditary material of plants is certainly nothing new or revolutionary. Interfering in the genetics of plants (and of animals) is something we have been doing since the dawn of agriculture, albeit largely unknowingly. The moment that humans took plants out of their natural ecosystem and planted them in a field, can be seen as the very first step in selection by man. A step that finally led to our modern agricultural crops. Of course, not the small and unhealthy plants were selected but the large and healthy ones. The plants that were picked also benefited from extra attention: they were watered when it was dry, the soil was fertilized and wherever possible, weeds and pests were combated. In other words, the crops in the field were getting human help. This is the essence of agriculture, left unchanged since then.

The common denominator between the past and present is that agriculture creates an artificial situation. In agriculture, survival of the fittest no longer applies. Human selection takes precedence over natural selection while human selection knows only one law: responding as closely as possible to human needs. We have human selection and intervention to thank for all crops we know today. Generation after generation, this was the way to achieve quality and quantity of food. The best plants in the field were selected and the seeds were kept aside to use them as planting seeds at the start of the next sowing season. Spontaneous changes in DNA—called mutations—then occurred (and they still do now). These can occur because of a “typo” when DNA is copied during cell division, or under the influence of radiation, for example from the sun. However, not every change in the DNA sequence leads to new traits.

In most cases nothing at all changes in the visible traits of an organism. But in certain situations, DNA mutations result in new—desirable or undesirable—traits. These changes were also noticed by our ancestors and, if they were beneficial, they were selected to improve crops even more. The wide variety of cruciferous vegetables we know today is a good example of this. Cauliflower, sprouts, kale and broccoli have all come about thanks to spontaneous mutations in the same cabbage-like ancestor. To obtain the appearance of a cauliflower for example, only one mutation is required in one gene. That gene has now been identified as the ‘cauliflower gene’. When this gene is silenced in other plants, the flowers get the same cauliflower-like appearance.<sup>1</sup>

A similar but more complicated process, unfolding over several thousand years, applies to maize. The crops that have filled European fields for 35 years, and that are grown on about 450 million acres of land all over the world,<sup>2</sup> come from teosinte, a plant of Mexican and Central American origin that bears little resemblance to the maize we know today.<sup>3</sup> Teosinte has side branches which makes it more bush-like than maize. The teosinte ears produce between five and twelve kernels, encapsulated within a hard protective outer covering, while modern maize consists of five-hundred or more unprotected kernels.<sup>3</sup> Teosinte sheds its seeds when it ripens so that they can spread more easily, which is an essential part of a good survival strategy. A mutation in the teosinte DNA, making the kernels stay attached to the central core of the cob, would therefore never be successful in the wild and would have disappeared quickly from the population through natural selection. For humans however, this is an advantageous feature, because if the kernels were released while the crops were still in the







field, part of the harvest would be lost. At odds with natural selection, the kernels of modern-day maize stay firmly attached to the cob. The same goes for other grain crops such as wheat, barley and rice.

Besides developing new varieties (an example being the diversity of cruciferous vegetables) and useful traits (such as kernels that stay attached to the cob), humans have also tried to increase yields. To begin with, this was done by using cultivation methods to protect the crops from pests, weed invasions and insect attacks, and later by plant breeding. It was a slow and difficult learning curve, which meant it was mid last century before a large proportion of the population could be certain of the availability of food. Before that, food shortages were the rule rather than the exception. For example, until the Second World War, eating meat more than once a week was not customary.

The most noticeable improvement to food production came about as a result of the 'Green Revolution', a period between 1960 and 1980 characterized by the emergence of fertilizers, pesticides and irrigation techniques, coinciding with the development of plant varieties that best reacted to fertilizers.<sup>4</sup> The focus here was mainly on wheat and rice, the two most important food crops. In both cases they were bred to produce shorter varieties. This seems a bit paradoxical, but dwarf varieties with shorter stalks invest more energy in producing grains and less in leaf material. With additional help from fertilizers and irrigation, the yield from both wheat and rice doubled in barely 20 years' time. This was also necessary (especially in Asia) to be able to supply food to a growing population.





## Standing still is going backwards

There is no comparison between the crops we grow today and the plants they are descended from. Thanks to the optimized genetic characteristics of plant varieties to suit the environmental conditions, but also because of the enormous knowledge on pesticides, fertilization and cultivation techniques, industrialized countries today

produce more than enough food. However, further breeding of crops is more important than ever. Why?

It is indisputable that agriculture worldwide faces great challenges. First of all, the climate is becoming more unstable. In certain agricultural regions, drought or too much rainfall are gradually making it impossible to cultivate the land efficiently. Even



minor increases in temperature can have a great impact on the yields of certain crops. For example, wheat and barley yields in Europe could be 20% lower in 2040 as a result of global warming. In other agriculturally productive regions in the world such as the USA, South America and Asia, drops in food production are also expected.<sup>5</sup> If we want to maintain food production levels in these regions, breeders must anticipate this and develop new varieties that are better adapted to higher temperatures and/or periods of drought.

Secondly, we need to reduce the impact of agriculture on the environment. This means that we need new ways to fertilize that are kinder to the environment, and to employ fewer pesticides to guarantee the safety of the farmer and consumer, while sparing useful insects. However, pesticides and fertilization form the fundamental basis of our food production today. Simply eliminating these two aspects from one day to another would inevitably result in reduced food production. This is another area where there is an important future role for plant breeding to play. Natural resistance mechanisms against mold, bacteria, insects and so on can be built into our modern, highly efficient crops, reducing their dependence on pesticides. Plants can also be genetically modified to consume water and fertilizers more efficiently so that the use of these can be reduced.

A third challenge is the improved standard of living that entails a rising demand for meat. The fact is that converting plant protein in feed to animal protein in meat is extremely inefficient. Producing a kilo of chicken meat requires double the amount of plant protein, and six kilos of feed are needed to produce one kilo of pork. As a result, the pro-

duction of plant sources of protein will intensify in certain regions.

The challenges stated above make it clear that, as a result of climate change, less land will be available for agriculture, and that a diminished use of fertilizers and pesticides will lead to less crops protection. However, because of a growth in population and wealth, there needs to be more production, especially in terms of animal proteins.

## An integrated agricultural model

The above mentioned scenario can only be tackled by introducing an integrated agricultural model. This means combining the best of conventional agriculture with the best ecological insights from organic agriculture, together with the adoption of the latest technologies and findings. This applies both to methods of cultivation and to plant genetics. In this dossier, we discuss the different methods by which humans have been adapting the genetic traits of crops since ancient times, but we also discuss new techniques that have emerged more recently, and that will be used in the future. We focus particularly on the range of New Breeding Techniques that are getting extra political and public attention in the context of the GMO debate. By specifically focusing on plant breeding, we do not mean to detract from the value of cultivation techniques and cultivation insights. These are equally important aspects in increasing and sustaining our food production and go hand-in-hand with plant breeding.



## 2 Plant breeding until the year 2000

Until late in the 20th century, plant breeding was primarily an unintentional selection process, in which the seeds or bulbs of the most suitable crops were kept for the following growing season. Upon rediscovery of the laws of Mendelian inheritance, breeding was given a theoretical basis. Targeted cross-breeding and selection amplified the speed of the breeding process. This basic method was followed by new techniques to create additional traits and to exchange features across species boundaries.



## Selection, cross-breeding and selection

External features of plants, animals and humans are largely determined by the information stored in their DNA. Since DNA is transferred from parents to offspring, many external features are hereditary. We partly have Gregor Mendel to thank for this insight, which is common knowledge today. Mendel cross-bred pea plants with different colored flowers in a Czech monastery, and discovered that specific traits of the parent plants are not transferred randomly to the next generation, but, on the contrary, followed certain patterns. Mendel's findings opened up new horizons in plant breeding and formed the basis for the world-renowned inheritance laws that bear his name.

Cross-breeding is based on sexual reproduction. In selective breeding, pollen from one parent plant is applied to the pistil of a flower of the other parent plant. By crossing two specific plants, a specific trait from one plant (for example disease resistance) is combined with a trait of the other plant (for example high yield) in the offspring. Cross-breeding also plays a very important role in the creation of variation as each product of cross-breeding contains a unique combination of the DNA of the father and mother. Moreover, during the formation of the reproductive cells, additional rearrangements occur in the DNA, meaning that new traits can appear in the descendants. Sexual reproduction is the key to successfully maintaining a particular species. In this way, a species always has the possibility to adapt itself to changing environments over time, or more accurately phrased: over the course of generations. The wide variety of crops that we directly or indirectly use for food is primarily the

result of cross-breeding programs. Many new varieties are developed thanks to cross-breeding and combining selection with cross-breeding will continue to be a cornerstone of all breeding programs.

Despite the fact that cross-breeding is based on a natural process, namely reproduction, many products of cross-breeding would never have come about without human intervention. The strawberry is a good example of this. The modern strawberry came into existence in the plant garden of Versailles, from a cross between a small but strongly flavored strawberry from the United States and a strawberry from Chile with larger fruit.<sup>6</sup> Without the help of humans, these plants would never have met and created a new variety.

The scientific basis of the laws of inheritance thus made it possible to combine beneficial traits of different parent plants more quickly and more directly than before. However, cross-breeding has its limitations as a breeding technique as the grower does not know in advance which information is going to be passed on to the offspring. This form of plant breeding is considered to be 'trial-and-error'. Humans select parent plants with useful traits, the plants are cross-bred and the grower hopes that as many useful traits as possible will be combined in certain offspring. By cross-breeding plants, half of the maternal DNA is combined with half of the paternal DNA, but one never knows which 50% will be passed on. Moreover, certain traits are often transmitted together. Thus, when plants are crossed, an undesirable trait (for example the production of toxic substances such as alkaloids) can be transmitted to descendants along with a desirable trait, or useful traits can be unintentionally bred out. Because of this, a lot of selection is needed after cross-breed-

ing and sometimes further cross-breeding is necessary. To eliminate undesirable traits, the crossing product can be cross-bred again several times with the parent that does not have the undesirable traits. This is called 'backcrossing' (Figure 1). In other words, developing a new variety by cross-breeding is a time-consuming and demanding task. After the first cross-breeding, it takes over ten years to obtain a new variety ready for the field. For some traits and some crops, this period can easily be as much as 40 years.

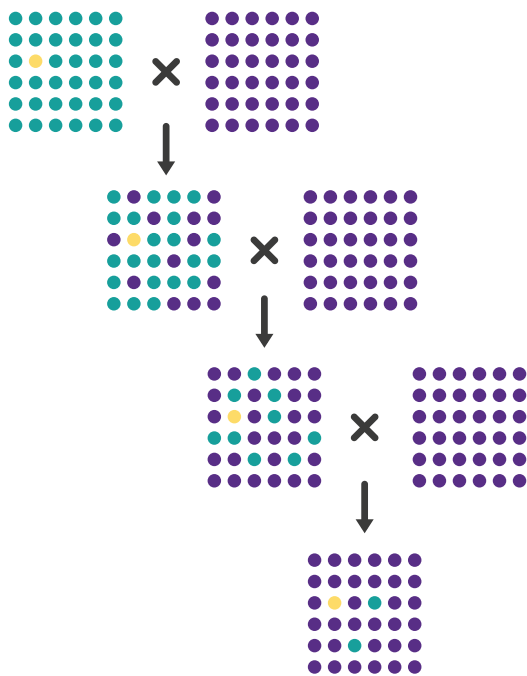


Figure 1. An illustration of a (back)crossing scheme aiming to transfer a beneficial characteristic (shown as a yellow dot) from one plant to another, while eliminating in the crossing product as many of the undesirable characteristics as possible (shown as blue dots) from one of the parents.

Another limitation of cross-breeding is that it requires sexual reproduction. This means that hereditary information can only be transferred (to obtain productive offspring) between individual plants of the same species. So a tomato (*Solanum lycopersicum*) must be crossed with a tomato. A

protection mechanism against mold available in another species such as pepper (*Capiscum annuum*) cannot be transferred to the tomato with the help of cross-breeding. This is the biological definition of a species. There is also a taxonomical definition based on external characteristics and DNA sequence. This means that individuals are sometimes divided into separate taxonomical species although they can produce fertile offspring together. Rapeseed is an example of this. This species (*Brassica napus*) is a hybrid of the species *Brassica rapa* and *Brassica oleracea*.<sup>7,8</sup> Also our modern potato (*Solanum tuberosum*) can in some cases produce fertile offspring with species of wild potato such as *Solanum demissum*.

## F1-hybrids: 1+1=3

In the ongoing quest for better yielding crops, plant breeding has taken diverse routes. In about 1930, the value of F1-hybrids was discovered. An F1-hybrid is a crossing product of two parental lines obtained through inbreeding. The hybrid technology therefore combines inbreeding with cross-breeding. The method is standard in maize breeding, and also most vegetable seeds sold nowadays are F1-hybrids.

As a first step in obtaining a F1-hybrid, inbred families are created through self-pollination: the pollen of one plant is applied to its own pistil. As a result, traits are fixed in homogenous genetic material, also called the homozygous form. Crops have two or more copies of each gene. Homozygous means that all copies are genetically identical. This is often indicated with letters of the same size, e.g. AA or aa. When a gene has two alternative forms, it is called heterozygous, indicated as Aa.



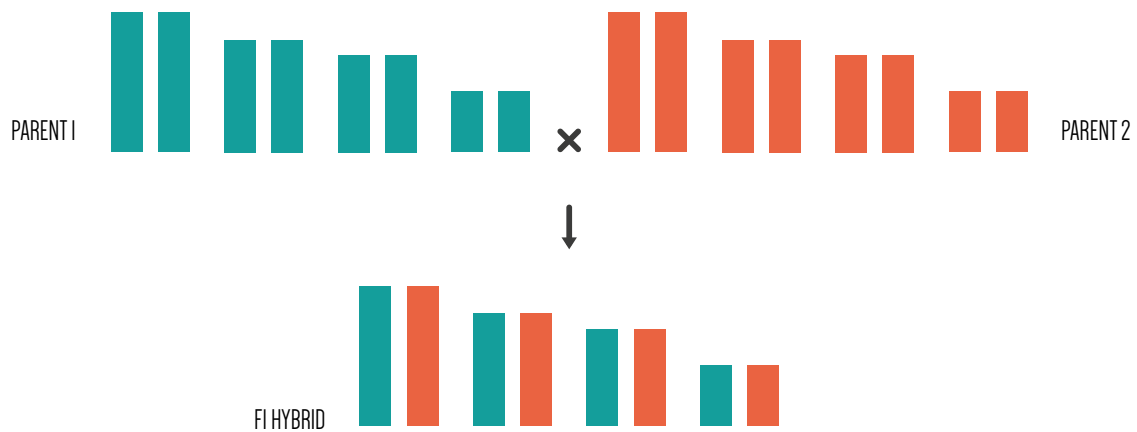


Figure 2. As a first step in obtaining a F1-hybrid 2 parents with identical copies of each chromosome are needed. In this scheme 4 chromosomes are shown. Blue illustrates the genetic material of the mother, orange that of the father. All direct offspring of these parents are genetically identical and have 2 different version of every chromosome.

Once the inbred families are formed, they are crossed with each other. The families are selected so as to have a variety of positive characteristics that complement each other. To illustrate this schematically, for traits A, B, C and D, the inbred mother might be AABbCcdd and the inbred father, aabbCCDD. If these two parents are crossed, all the offspring—the F1-hybrids—will be genetically identical, i.e. AaBbCcDd (Figure 2).

This provides uniformity in the seeds and evenly growing crops, which is a beneficial characteristic for farmers because it makes mechanized ag-

riculture (for example at harvest time) easier. In addition, F1-hybrids have all the traits in a heterozygous state (AaBbCcDd). As a result, F1-hybrids usually have *hybrid vigor* or heterosis. This is the effect where offspring perform better than the average of the two parents for one or more traits (Figure 3).<sup>9</sup>



Figure 3. The effect of hybrid vigor on maize growth (left) and seed yield (right). P1 and P2 are the mother and father inbred lines, B represents the F1-hybrid.

© Plants in the field: Courtesy of Jun Cao, Schnable Lab, Iowa State University  
Ears of corn: Courtesy of Ruth Swanson-Wagner, Schnable Lab, Iowa State University

Commercial F1-hybrids display robust growth and give a higher yield. But not all inbred lines are equally suitable for creating hybrids. It must be examined, through test breeding, which combinations of inbred lines give good results. In general, the more parents genetically differ from each other, the greater the heterosis effect that can be expected. The disadvantage of F1-hybrids for growers is that their performance weakens in the following generations and the heterosis effect completely disappears after a number of generations. This is because the offspring of F1-hybrids are no longer uniform. They have differing appearances and quality. As a result growers need to buy new F1 seeds every year if they want to maintain the same level of productivity. It goes without saying that this is clearly a profitable trait for breeders and seed companies.

## Crossing the not crossable

A next step in the history of plant breeding was crossing of related but different species. According to the biological definition of a species (see above), this is spontaneously not possible. For example, certain plants cannot produce offspring together because the parents have a different number of chromosomes, the pollen grains do not reach the egg cell or the embryo formed is unable to develop to seed. However, it became possible to circumvent some of these barriers thanks to the emergence of plant tissue culture techniques midway through the 20th century.<sup>10</sup> Tissue culture is also referred to as *in vitro* tissue culture and is a method by which plants grow and reproduce under sterile conditions. The cells, tissues or entire plants grow on an artificial culture medium in dishes or in jars. By adding chemical substances to the culture medium that for example regulate the division of plant cells, the

number of plant chromosomes can be adjusted. It became also possible to rescue plant embryos by taking them from seeds that would not otherwise survive and growing them under controlled conditions in the presence of all the nutrients they need.

In the middle of last century a cross-breeding program was set up for wheat (*Triticum durum*) and rye (*Secale cereale*). The idea was that the crossing product would combine the higher yield and better quality of grain found in wheat with the reduced susceptibility to fungal infections found in rye. However, the two types of grain are genetically too far removed from each other to be able to produce offspring spontaneously. By using different types of techniques, such as doubling the chromosomes using specific chemicals and growing plant embryos in vitro, a hybrid was nevertheless successfully obtained. The crop was named triticale. Triticale is a crop that would not have existed without human intervention. Even today, it is not always known how drastically the DNA was rearranged during the breeding of triticale and which wheat-rye-DNA combinations have emerged. It is a crop that has been grown in the field since the 70s and is primarily used as animal feed. Worldwide, 15 million tons of triticale are produced on almost ten million acres of land.<sup>2</sup>

## Marker-assisted selection

The selection steps in cross-breeding programs are the most demanding part. Certain traits such as disease resistance cannot simply be evaluated on the basis of the plant's appearance. For this, the disease must first exist in the field. This is not always the case, so breeders often have to infect their hybrids on purpose to be able to select the least sensitive plants from the population. Along-



side the existence or non-existence of a particular trait—which is relatively easy to select during a breeding program—there are also quantitative traits such as yield or growth speed. These quantitative traits are often determined by multiple genes, which makes breeding a lot more difficult and above all, time-consuming.

Thanks to increasing knowledge about how plant genes work and their role in plant growth and development, it is known for a large number of traits which genes are encoding. By detecting a specific DNA fragment (also called a ‘marker’) which is linked to a trait (e.g. disease resistance) in a crossing product, it can be determined at a very early stage whether or not the plant will be disease resistant, without needing to infect the plant. Cross-breeding programs can therefore be conducted much more efficiently by selection based on DNA than on appearance. This is possible when the genes that have a direct influence on the trait are known, or the neighboring genes are known. For each offspring of a certain cross-breed, it can be thus determined, with the help of molecular DNA marker techniques, which combination of genes is present in the DNA.<sup>11</sup> The offspring with the most beneficial combination of genes can thus be quickly identified and used for further breeding. So marker-assisted selection makes use of biotechnological know-how to diagnose the availability or unavailability of specific genes (Figure 4).

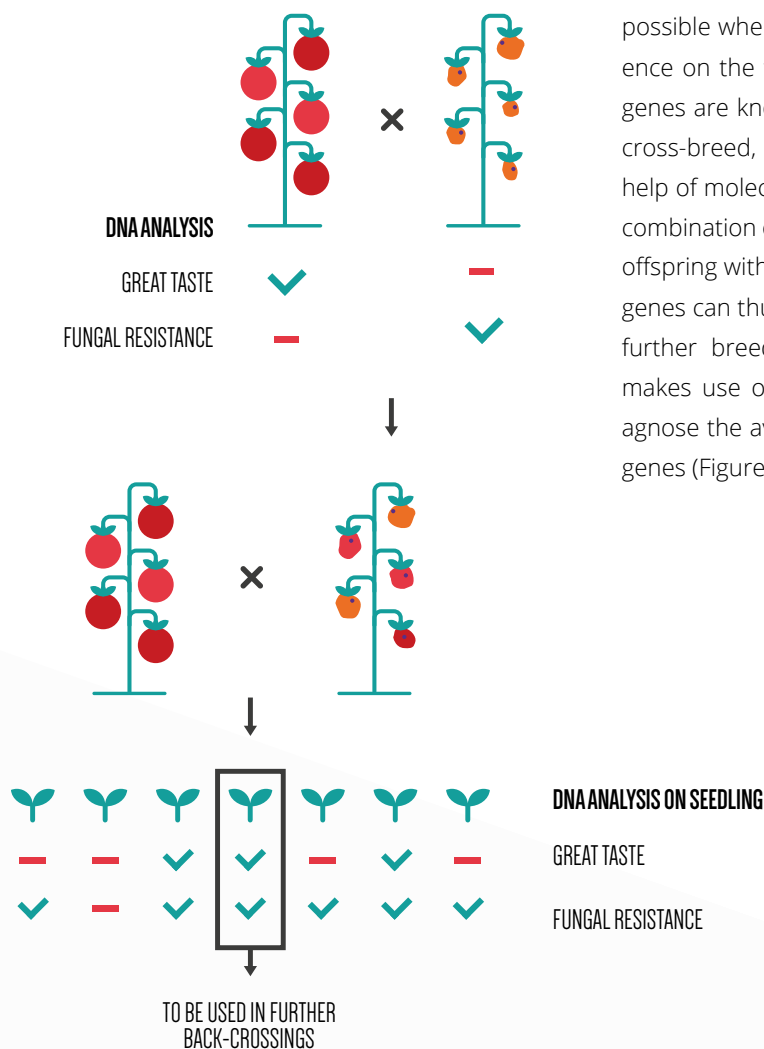


Figure 4. Schematic representation of marker-assisted breeding. A fungus susceptible but nice tasting tomato plant (illustrated by plant with big red tomatoes) is crossed with a fungus resistant tomato plant with poor tasting characteristics (illustrated by plant with small orange tomatoes). Using DNA-analysis techniques tomato seedlings can be selected that are both fungus resistant and good tasting.



Today, marker-assisted selection has almost become standard in crop improvement programs. In most cases, the DNA to be analyzed is isolated from a piece of leaf tissue from a young plant. It can even be done at earlier stages. Breeders can now use 'seed chipping': a technology that allows a small piece of the seed to be shaved off to extract the DNA without harming the embryo or its capacity to germinate.<sup>12</sup> With seed chipping, it can therefore be determined at an extremely early stage, which seeds contain which beneficial traits. The less suitable seeds can be discarded before they are even planted. This speeds up breeding programs and makes for a considerable reduction in selection costs.

## Mutation breeding

Given that cross-breeding is largely limited to plants within the same species, its success depends on the number of traits within a species. The greater the genetic variation within a species, the more possibilities there are to find traits of interest and combine them. Plant breeders are continually on the lookout for new opportunities, but cross-breeding is not the appropriate method for creating new traits. With an expanding body of knowledge on DNA and its importance for plant characteristics, breeders have taken matters into their own hands. Instead of waiting for spontaneous mutations to occur in DNA (to obtain new traits), they started work on mutation breeding in the 1930s.<sup>13,14</sup> With this type of breeding, changes to plant DNA can be applied at a much higher frequency. However, one does not know what and where in the DNA changes will occur.

Between the late 19th century and early 20th century, scientists began to experiment with changing genetic material using X-rays, first in

fruit flies for scientific purposes and later in crops for more practical applications.<sup>15</sup> Where the natural mutation rate stands at around one in a 100 million nucleotides (letters in the DNA code), radiation and chemicals such as ethyl methanesulfonate (EMS) can, depending on intensity and concentration, increase this rate to one in a thousand. The mutations can be subtle changes in the DNA code—as is often the case in natural mutations—but most of the time, the mutations are even more drastic, such as major rearrangements in the DNA or the elimination of entire DNA fragments. Radiation can easily cause thousands of such changes, while only one or a few may be useful. The method can be compared with a scattergun effect. Some of the shots might end up where they are supposed to, but there is a big question mark as to where the rest goes and what their effect is. Again this is a matter of trial-and-error.

Large-scale initiatives were taken to expose seeds to radiation in radioactive fields (called gamma gardens) or to use EMS with the intention of causing changes to the DNA. Seeds have also been sent into outer space to expose them to cosmic radiation.<sup>15</sup> These seeds, with random DNA mutations, were then sown in the hope that specific new traits would appear (Figure 5). Alongside a range of new rice and banana varieties, which are cultivated and consumed in great quantities, the pink grapefruit is a well-known product of this method.<sup>16</sup> The durum wheat that is used to make spaghetti has also acquired traits that came about through mutation breeding. By using mutation breeding, over three thousand crop varieties have been created over the past eighty years.<sup>17,18</sup> At a regulatory level, no distinction is made between crops that have acquired traits through mutation breeding and classic breeding products.



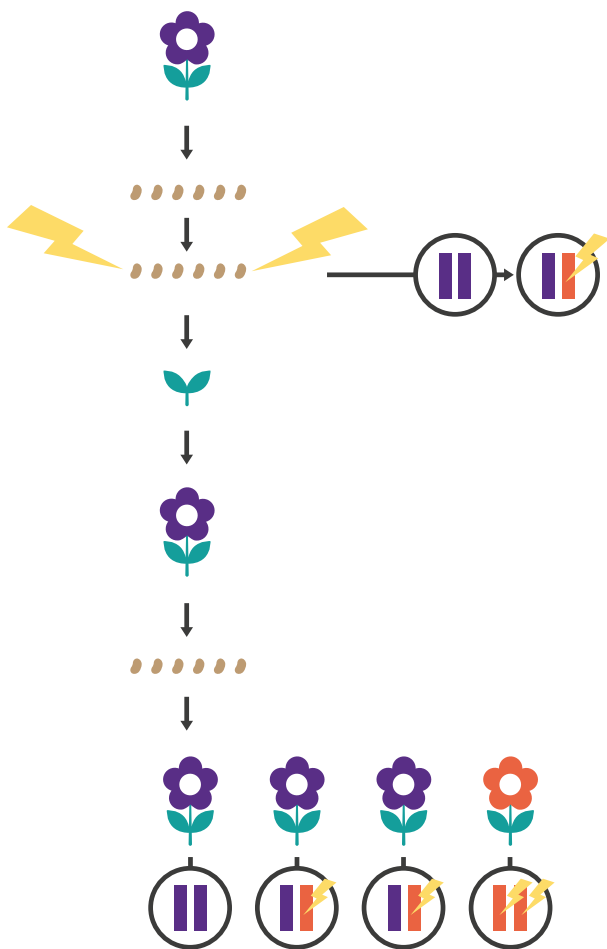


Figure 5. Schematic representation of mutation breeding. Seeds of plants with purple flowers are treated with radiation that cause mutation in the plant's DNA. In the next generation, these mutations can lead to a red flowering plant.

Products of mutation breeding do not need specific labeling either.

## Genetic modification

For hundreds of years already, man has been on the lookout for methods to obtain new traits in plants or to combine several traits of interest in a single crop. The main issue in cross-breeding remains the dependence on sexual reproduction.

Towards the end of the 1970s, a technology was developed that allowed genetic information to be inserted into a plant's DNA without the need for cross-breeding. All of a sudden, it became possible to insert genetic information from peppers or corn into a tomato. This method was dubbed genetic engineering or genetic modification and its products were called genetically modified organisms (GMO) or GM crops. The advent of GM technology opened up a whole world of possibilities, just as Mendel's laws caused a revolution in plant breeding.

Genetic modification makes it possible to insert one or more selected traits of interest into a certain plant. If you think of the plant's genetic material as a software package, GM technology is like an upgrade to the software: the genetic code for a certain trait is added to the plant. Or, if you compare a plant to a smartphone, genetic engineering is like uploading an additional application. Just as a smartphone is still the same smartphone once you have uploaded an additional app, a GM plant is still the same plant, with the GM version simply able to do more. In comparison with the breeding techniques discussed above, GM technology is more precise, predictable and controllable. Moreover, the characteristics of the variety remain the same. A GM Bintje potato remains a Bintje potato but its GM version has an extra trait. In contrast to this, with cross-breeding, the father and mother DNA are combined so intensely that a new variety is obtained. Today, there are four major applications of GM: tolerance of herbicides, resistance to pests, resistance to viruses and drought resistance (for more information see *The GMO Revolution*,<sup>19</sup> or the VIB Fact series<sup>20</sup>).

The original and still the most efficient method to genetically modify plants is based on the natural

ability of the soil bacteria *Agrobacterium* to transfer DNA. This bacteria infects certain host plants and subsequently builds a part of its own genetic material into the plant's DNA. In that piece of bacterial DNA lies the information for the production of substances which the bacteria can feed on, and for the production of plant hormones that cause the infected cells to multiply. This complicated biological process was unraveled in the late 1970s by the research groups headed by Jef Schell and Marc Van Montagu at the Ghent University.<sup>21</sup> The researchers immediately realized that *Agrobacterium* is essentially a DNA transporter. They replaced the section of bacterial DNA (that *Agrobacterium* normally inserts into plant DNA) with the genetic information of a trait useful for agriculture. After infecting the plant material with the genetically modified *Agrobacterium*, they discovered that *Agrobacterium* inserted this information into the plant's DNA in the same way (Figure 6). The first genetically modified plant was developed in Ghent in 1982. It was a tobacco plant engineered to be resistant to herbicides.

Alongside the biological method, there is also a mechanical method to insert DNA into plants. The most significant is the particle acceleration method, also sometimes called particle bombardment, the gene gun or biolistics. For this, miniscule particles of gold are coated with the DNA that is to be inserted into the plant. These gold particles are then "shot" under high pressure into the plant tissue. In some cases, the DNA penetrates the nucleus where it is sometimes spontaneously incorporated into the plant's DNA. In comparison with *Agrobacterium*, this method leaves more to

chance and is less efficient. Often only parts of the desired DNA are incorporated into the plant DNA and often only after many tries. However, this remains the most successful way to genetically modify plants that are difficult to infect with *Agrobacterium*.

How do we get from a genetically modified cell to a genetically modified plant? In contrast to humans and animals, plants have the unique property of being able to make a new plant out of a single plant cell. This means that with a modification to a single plant cell (with *Agrobacterium* or with a gene gun), an entire genetically modified plant can be developed. Sometimes this occurs spontaneously, but in most cases the process is controlled by adding plant hormones enabling it to produce shoots and roots.

The breeding methods covered in this chapter show that adjusting the genetics of crops to our wishes is nothing new. In other words, it is a fallacy to think that with GM technology we have suddenly shifted from "natural plants" to "laboratory plants". Since the beginning of agriculture, humans have adapted plants to suit their needs, and since 1900, plant varieties have been developed that would never have existed without human intervention. New breeding techniques are largely an improvement to existing methods through which plant genetic material is adapted in a more controlled and almost surgical manner.

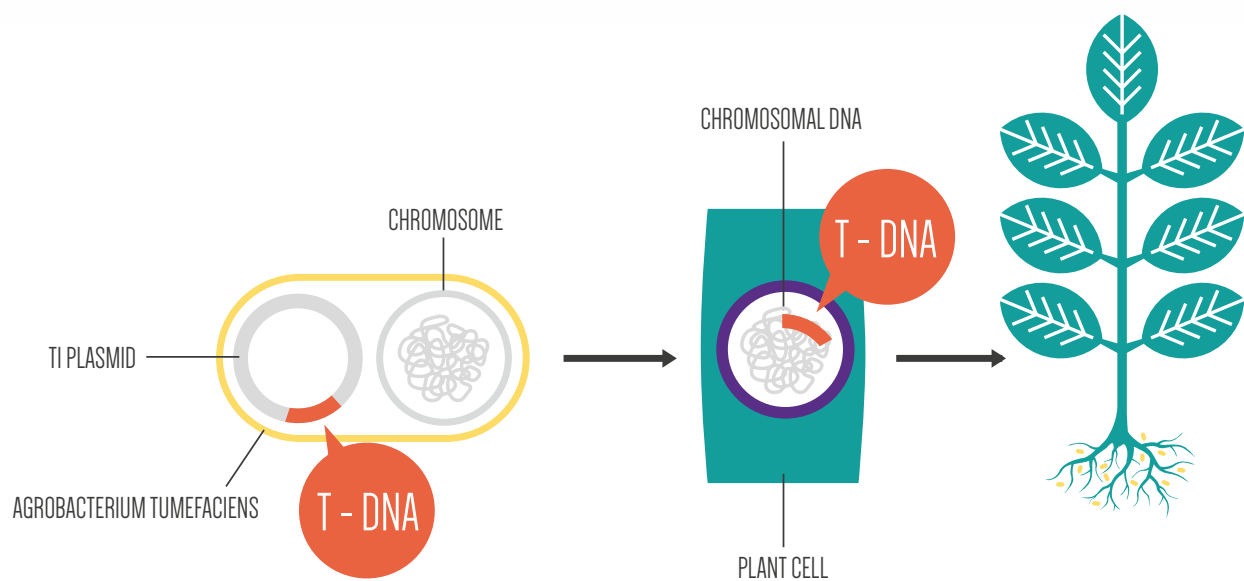


Figure 6. Schematic representation of *Agrobacterium tumefaciens*' gene transfer mechanism. Alongside its chromosomal DNA the bacterium (present in the rhizosphere of the plant and illustrated as yellow dots) possesses a Ti-plasmid (represented by the grey circle). The genetic information in the T-DNA (red piece) is transferred by *Agrobacterium* to a plant cell where it is incorporated in the latter's chromosomal DNA. From this genetically modified plant cell, a full plant can be regenerated. This plant is identical to the original plant but the GM version has an additional piece of genetic information.





# 3 New breeding methods

GM technology is not the end of the story when it comes to adapting crops to our needs. New methods are under development to be able to intervene in an even more targeted way in the DNA of plants. Given the commotion around GMO, the fundamental public and political concern is whether or not these techniques deliver GMO products. The new technologies are often, and in fact incorrectly, grouped together under the common denominator “New Breeding Technologies”. In reality they differ greatly in terms of mechanisms and results. Therefore, we will divide them into four groups here.

# No adjustment to plant DNA

## Grafting

Grafting is a frequently-used technique in which the stem of one plant species or variety is grafted onto the root of another species or variety (Figure 7). This technique is almost standard in horticulture and tree nursery. Almost all roses for example are grafted. Equally, all European grape vines are grafted. Equally, all European grape vines are grafts of a *Vitis vinifera* scion on a *Vitis labrusca* rootstock. *V. vinifera* is sensitive to grape phylloxera, an insect that lives in the soil and was responsible for virtually wiping out all European vineyards around 1870.<sup>22</sup> In America, another species was found, more specifically *V. labrusca*, which was naturally resistant to grape phylloxera. Given that the insect is only to be found in the soil, it sufficed to have a resistant root system. As a result all *V. vinifera* grape varieties—from Pinot Noir to Sauvignon Blanc—are grafted onto a *V. labrusca* rootstock. Fully intact *V. vinifera* plants only exist now in specific parts of the world, such as Chile. Natural barriers such as the Pacific Ocean, the Andes, the Atacama Desert and Antarctica have protected Chile against the spread of grape phylloxera.

As such, grafting is anything but a new technique. However, this technique has come under scrutiny again as part of the GMO debate. This has to do with the question of what should be done at a regulatory level if a non-GM scion is grafted onto a GM rootstock? Do the fruits of the non-GM scion come under the GMO regulations? The scientific answer is simple: the DNA of the scion that includes the fruits is not modified, therefore there are no scientific arguments to regulate those fruits according to the GMO legislation. This does not mean that there is no exchange between the scion and the rootstock. Alongside water, sugars and other metabolites, small molecules (such as RNA molecules) derived from the GM rootstock can be transferred.<sup>23</sup>

A GM plant that is used as a rootstock can be developed to silence the expression of one or more genes. This often occurs through the production of RNA molecules. These molecules can be transported to the scion where they can influence the expression of specific genes in an identical manner. So even though the DNA of the scion is unchanged by the rootstock, the production of certain proteins in the scion can still be adjusted by the rootstock.

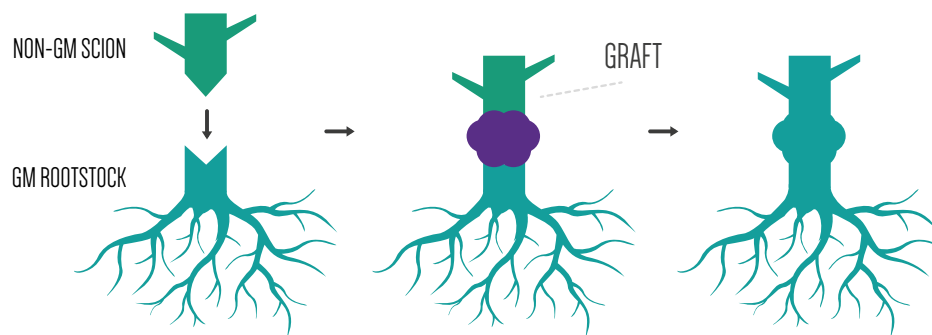


Figure 7. A non-GM scion is grafted on a GM-rootstock.

## Reverse breeding

Reverse breeding is the reverse of the F1 hybrid making process (see pages 12-13). Where parental lines are used to make a better-performing hybrid in classic breeding, the parental lines are re-created from a hybrid in reverse breeding. These newly created parental lines can then produce hybrids in other combinations. This technique makes use of GM technology during the breeding process, but in the DNA of the end product no extra DNA is inserted or included.

Reverse breeding makes advantage of a disturbed reduction division during the formation of reproductive cells (gametes). In many organisms chromosomes come in pairs—the homologous chromosomes—one of which comes from the mother and the other from the father. During the reduction division, the number of chromosomes is halved. This halving is necessary to ensure that once the egg cell and sperm cell combine, the original number of chromosomes is obtained again. Just before the homologous chromosomes are pulled apart, the chromosomes often exchange DNA fragments. This exchange (which is also referred to as ‘crossing over’) helps to ensure that the gametes from the same parent differ genetically and thus that offspring from the same parents are different. Hence, crossing over creates a source of extra genetic variation. To perform reverse breeding, the exchange between homologous chromosomes must be prevented. A number of genes appear to be essential to this exchange. With the help of GM technology, one of these genes can be silenced in the selected hybrid (by building in an RNAi-construct). This GM plant will consequently produce pollen grains in which the number of chromosomes is halved without

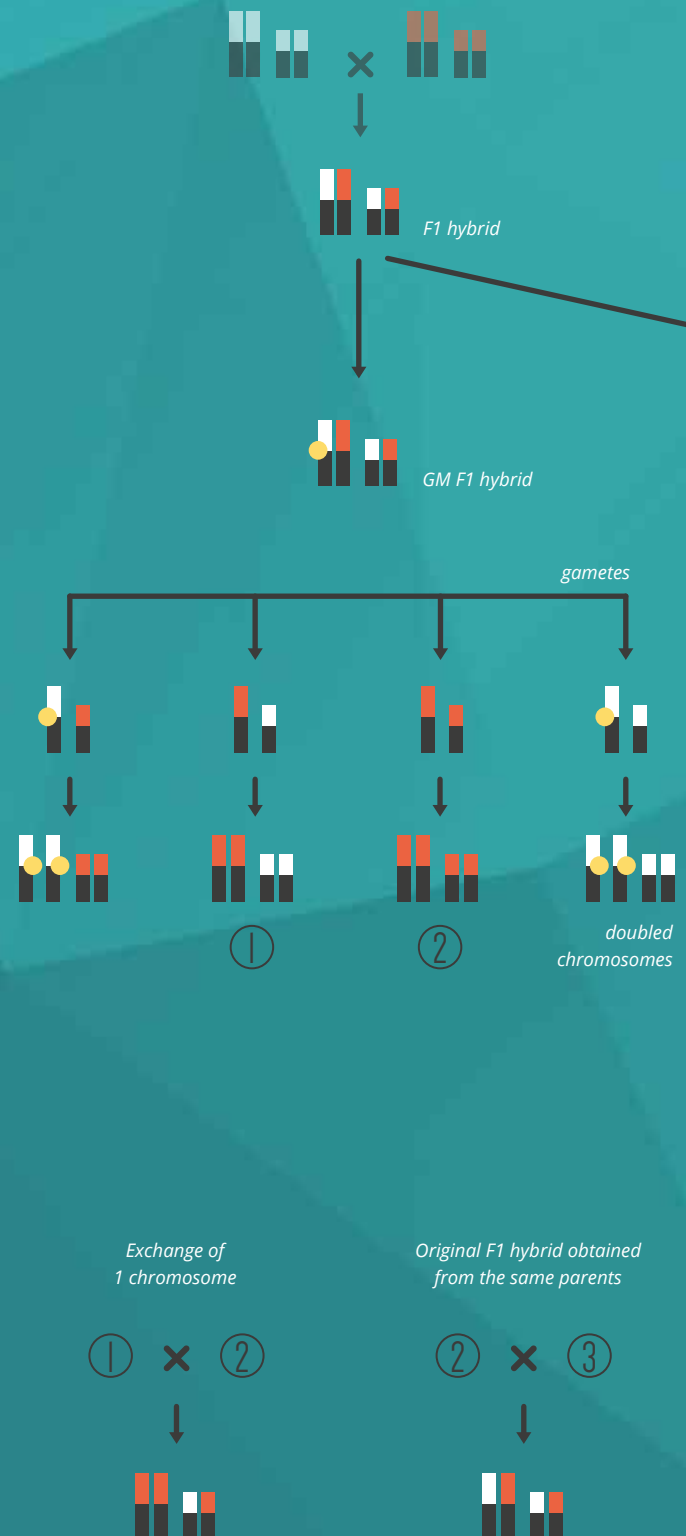
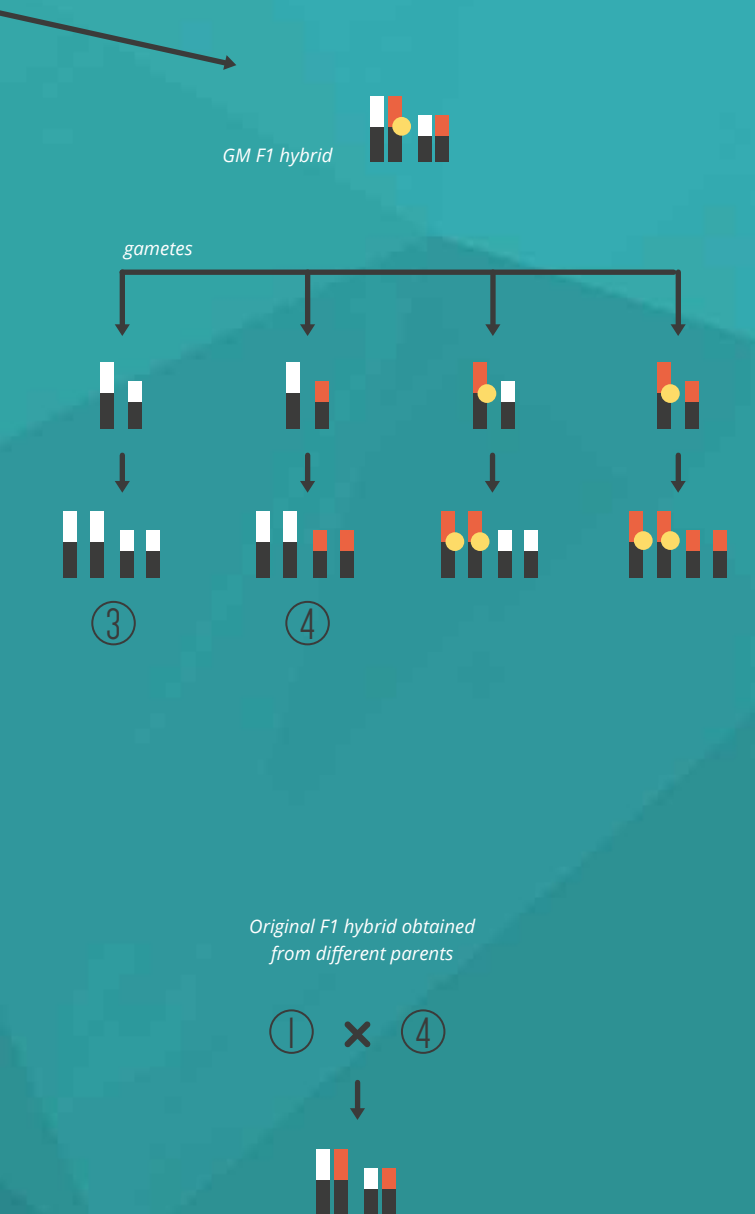


Figure 8. Schematic representation of reverse breeding. Using GM technology an extra DNA fragment that will prevent crossing over of homologous chromosomes is incorporated in the DNA of a F1 hybrid (yellow dot). During the formation of gametes (e.g. pollen) the number of chromosomes is halved so that there is only 1 copy of every chromosome. The DNA of those gametes is subsequently doubled so that every chromosome gets an identical copy. Finally new plants are regenerated out of the doubled gametes. Some of the





resulting plants are genetically identical to the original parents of the F1 hybrid (represented by number 2 and 3), other plants (1 and 4) differ from the original parent but can be used to produce the same F1 hybrid. Reverse breeding also allows the production of new combinations (e.g. between 1 and 2). In this case the F1-hybrid is genetically identical to 1 of the parents except for 1 chromosome. Important to note is that only those plants having yellow dots in the above scheme are GM plants.

having undergone any crossing over. The pollen grains (only half of which will contain the RNAi construct) are isolated, after which the DNA of the pollen grains is doubled. With in vitro cultivation, a full plant can be regenerated from these pollen grains. As a result, half of the plants are non-GM plants because they have not received the RNAi construct built into their DNA. These non-GM plants will be selected and can subsequently be used to re-construct the original elite line or to produce new hybrids (Figure 8). The technique can also be used to replace one chromosome from a pair of homologous chromosomes with a copy of the other chromosome (Figure 8). This can be useful if there are for example too many undesirable traits on one chromosome.

## Fast-track breeding for trees and shrubs

If breeding annual agricultural crops takes time, imagine the patience required to breed trees or other perennials. For trees that bear fruit (e.g. nuts, plums or avocados), the breeding cycle can easily span 10 years.<sup>24</sup> This means that after planting the first hybrid, a breeder has to wait up to 10 years for the hybrid to flower and therefore to use the plant in a breeding-program. The reason for this is the long juvenile phase. This is the phase after germination during which the tree does not yet flower or bear fruit. The juvenile phase is a type of protection mechanism. It means that the tree can only bear fruit once it is big and strong enough to compete with other plants. After all, flowering takes a lot of energy. Flowering and bearing fruit too early can affect the tree's growth. Because several consecutive crosses are

necessary to obtain a new variety, breeding a new fruit tree can easily take 30 years.<sup>24</sup> To shorten the juvenile phase and subsequently be able to cross-breed more quickly, seedlings are often treated with plant growth hormones, or the environmental conditions, such as temperature and daylight hours, are adjusted. These tricks only meet with sporadic success, however. The juvenile phase can also be shortened by means of DNA technology. The method is based on the discovery of certain genes that determine when a

plant flowers. Under the influence of specific internal and external signals, these flowering genes are activated, after which the plant prepares itself to flower and bear fruit. Overexpression of these flowering genes can be induced in a tree through genetic engineering, meaning that the seedlings will not wait 5 to 10 years to flower for the first time but rather do so in the first year. The total breeding period of a fruit tree can be shortened in this way from 30 years to 5 years.<sup>24</sup> Similarly to the method explained previously (reverse breed-

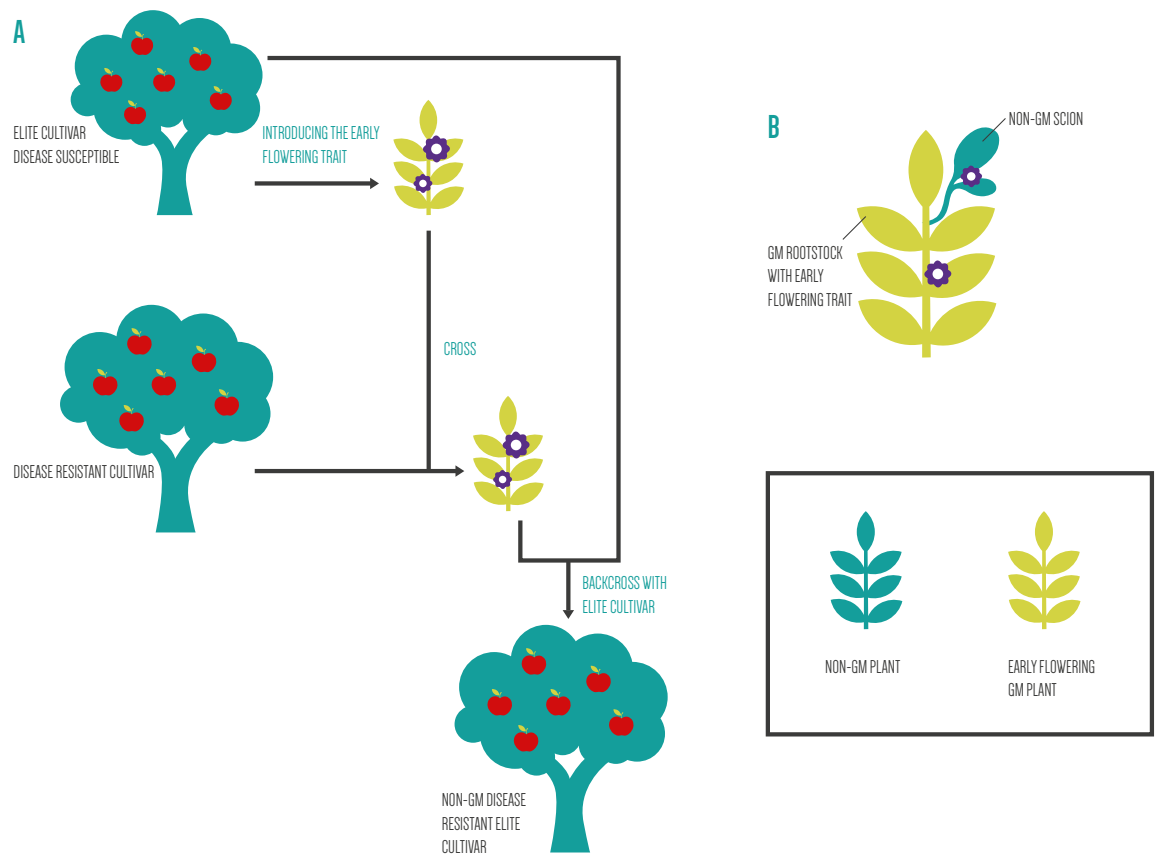


Figure 9. A) A disease susceptible elite cultivar is genetically engineered to flower early (represented by light green plants). This young GM tree will be crossed with a non-GM disease resistant cultivar (non-GM plants are represented in dark green). The GM offspring (light green) can subsequently be back-crossed with the original elite cultivar to combine as many as possible desirable traits and to remove again the early flowering gene. The final product is thus a non-GM tree that combines all the desired traits of the elite cultivar together with the disease resistance trait. B) Instead of developing a GM variant of the elite cultivar, a branch of the elite cultivar can be grafted on a GM tree expressing the early flowering gene. The flowering inducing proteins from the GM rootstock will be transported throughout the scion resulting in early flowering of the grafted branch. The obtained flowers can then be used as pollen donors for further crossings.

ing), the extra flowering genes added can be removed again in the final breeding step leading to an end product that does not differ at all from a classic hybrid (Figure 9. A). This technique is called fast-track breeding and is used nowadays in combination with marker-assisted breeding (see previously) to introduce disease resistance to apple trees and plum trees.<sup>24</sup>

A variant of the fast-track breeding method uses grafting (see page 21). The signals from outside or inside the plant that lead to flowering are detected by the leaf. The flowering genes are activated in the leaf, after which specific proteins are formed. These flowering proteins are transported to the meristems of the plant to induce the flowering process. In grafted plants, likewise, these flowering proteins are transported from the rootstock to the scion.<sup>24</sup> To prevent the additional crossings steps needed to remove the initially added DNA, a shoot from a young seedling can be grafted onto a GM tree, which overexpresses the flowering genes. The corresponding flowering proteins will then be transported from the GM rootstock to the graft where they will initiate flowering (Figure 9. B). In this way, the flowering graft can be used within a year as a parent plant (provider of pollen) for the next cross. A disadvantage of this method is that the transport of flowering proteins from the rootstock to the scion is not 100% efficient.<sup>25</sup>

## Agroinfiltration

Agroinfiltration uses *Agrobacterium tumefaciens*, the soil bacteria that transfers DNA to plant cells during the development of a genetically modified plant. *Agrobacterium* can also be used to temporarily produce certain proteins. *Agrobacterium* infiltration or agroinfiltration is a technique by which *Agrobacterium* is injected into plant tissue (usually into one or more leaves) so that the bac-

teria can spread between the plant cells. Then *Agrobacterium* introduces the gene of interest to one or more plant cells, leading to transient expression and to the production of the protein concerned. The gene can be built into the DNA of some plant cells but this is not needed for the production of the protein.

Agroinfiltration can be used for the production of complex molecules such as antibodies (see text box) but is primarily used as a selection method during a breeding program for disease-resistant plants. Plants use a range of defense mechanisms against bacteria and fungi. The most crucial part in this process is that the plant must be able to recognize the pathogens. Such a recognition mechanism is based on a lock-and-key principle: a plant is resistant when certain proteins of the pathogen (called effectors) are recognized by the plant's disease-resistance proteins. If this is not the case, the plant becomes sick. To find out whether the plant's disease-resistance proteins are able to recognize specific effectors, the effectors can be produced in the plant through agroinfiltration.<sup>26</sup> To achieve this, the genetic information of the different effectors is first introduced in the individual *Agrobacterium* strains. For every effector, there is a different *Agrobacterium* strain. The leaves of the plants to be tested are then infected with the different *Agrobacterium* strains through agroinfiltration (Figure 10). Upon recognition, a hypersensitive reaction will be seen at the spot where the *Agrobacterium* strain was injected. This reaction is visible to the naked eye as an area of dead plant cells. This specific method of analysis allows the functionality and spectrum of disease resistance genes to be investigated in a relatively simple way.



From a regulatory point-of-view, the question often arises as to what should then happen with the tested plant. Should this plant be seen as a GMO if only a limited amount of somatic cells have received DNA from *Agrobacterium* in a specific tissue? Should the entire plant be seen as a GMO if only one leaf contains a couple of genetically modified cells? And will this plant become

non-GM again if the leaf with modified cells is cut off? Do all seeds and offspring of a mother plant fall under the GMO legislation if one of the leaves of the mother plant was infected with *Agrobacterium*? The only statement that can be done so far is that the genetic material of the seeds and offspring are entirely unchanged.

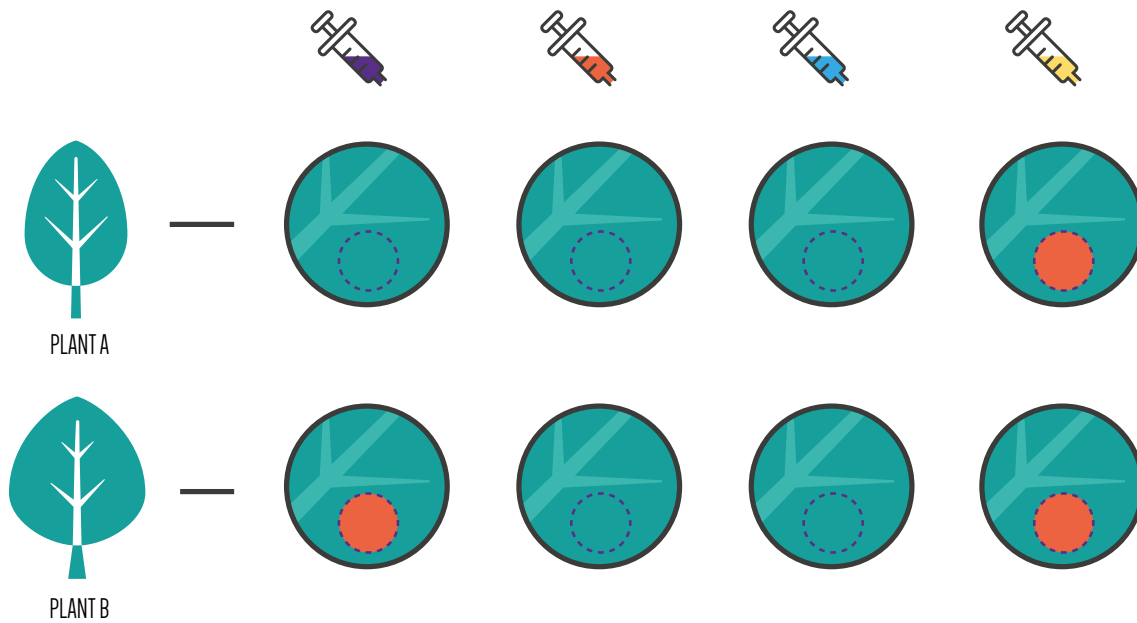


Figure 10. Schematic representation of Agroinfiltration. Different *Agrobacterium* strains (purple, orange, blue, yellow) containing the genetic information for the production of different effectors, are injected in leaves. Locally, *Agrobacterium* transfers this genetic information to some plant cells that subsequently produce the corresponding effectors. If those effectors are recognized by the plant's resistance proteins, a hypersensitive reaction is observed (red circle).

## PRODUCTION OF MEDICINES IN PLANTS USING AGROINFILTRATION

People mainly use plants for the production of food and textiles, but plants can also be used to produce medicines. ZMapp, one of the most promising treatments so far for Ebola, has been developed with the help of plant biotechnology.<sup>27</sup> ZMapp is not a vaccine, but rather a blend of three different antibodies. Antibodies are our bodies' fighters and neutralize specific pathogens that they have been developed to fight. In contrast, a vaccine depends on the patient's immune system.

The ZMapp antibodies were originally obtained from mice that had been brought into contact with a specific protein from the Ebola virus. As a reaction to this the mice produced antibodies. These antibodies were purified and adapted to look more like human antibodies. The genetic information for the production of these adapted antibodies was introduced to tobacco plants (with the help of agroinfiltration), which then produced the desired antibodies. The experimental treatment with the plant-based antibodies was tested in monkeys. Of the 21 animals infected with Ebola, 18 received the antibodies produced by plants. All 18 animals recovered while the 3 untreated animals in the control group died.<sup>27</sup> In the meantime some people have also been treated with this medicine.<sup>28</sup> To be clear, these plants are not intended for consumption and are only used as "production plants" for medicines. There is less of a debate around this application of agroinfiltration given that the plants are only used as a production tool and the product is purified before use. No DNA or plant material remains in the end product. This can be compared with the production of insulin using GM bacteria. The final medicine is free of DNA and bacteria.

## Mutation breeding 2.0

### RNA-dependent DNA methylation

RNA-dependent DNA methylation is a form of gene silencing, or in other words: shutting down the expression of a gene.<sup>29</sup> The technique doesn't cause any mutations at DNA level and the plants developed in this manner did not incorporate any extra DNA in their genome. We are however including this technique because hereditary changes are induced, more specifically at an epigenetic level.<sup>30</sup> Epi means 'outside of' or 'over' the genetic information that is coded in the DNA. Epigenetics is the area of genetics that studies hereditary changes that do not originate in the sequences of DNA letters.

The genetic information in higher organisms such as plants and animals is encoded in the DNA. This information only comes to the fore when the DNA code is first transcribed to messenger RNA, in a process called transcription. Subsequently the RNA code is translated into the production of proteins. To prevent a certain protein from being produced and therefore to silence the activity of the corresponding gene (gene silencing) the aforementioned mechanism can be disrupted in two different ways: either by stimulating the destruction of the intermediate RNA molecules so that no protein can be formed, or by ensuring that the DNA code is not transcribed to messenger RNA.<sup>31</sup> The first method (also referred to as

post-transcriptional gene silencing) is a technique frequently used to silence a plant trait but also to develop resistance to viruses (see the VIB Fact Series issue 'A virus-resistant papaya for Hawaii'<sup>20</sup>). The second method is called transcriptional gene silencing. RNA-dependent DNA methylation is an example of this.<sup>31</sup>

To adjust the function of a gene without changing the DNA itself, the promoter of that gene can be methylated. A promoter of a gene can be compared to a switch that determines when a gene is on or off. DNA methylation means the binding of methyl groups to DNA. Through the presence of methyl groups on the DNA of a promoter, the expression of the corresponding gene can be fully silenced because an RNA messenger is prevented to be formed. In plants, such methylations can be inherited.<sup>32</sup> The traits induced by DNA methylation are then transferred from the parents to the offspring. The commercial application of this technique is slightly undermined by the fact that methylation—and therefore the useful trait or traits—can weaken or even disappear over several generations.<sup>30</sup>

The power of the RNA-dependent DNA methylation technique is that methyl groups are not added at random places. This means that, unlike in conventional breeding techniques, it can be determined in advance for which gene or genes expression will be influenced. To make the technique specific, RNA molecules are used; not the messenger RNA molecules mentioned above (see previous paragraph) but non-coding RNA molecules. These RNA molecules do not lead to the production of proteins but regulate gene expression. This is also where the name comes from: RNA-dependent DNA methylation. To silence the expression of a specific gene, a non-coding RNA

molecule that can bind with a specific part of DNA in the desired promoter is inserted into the plant cell or tissue.<sup>32</sup> The RNA molecule leads the methylation machinery which is available naturally in the plant cell to the specific promoter, after which the latter is methylated.<sup>30</sup> The RNA molecules that determine the specifics of this technique can be inserted into the plant using a variety of techniques, for example through plant viruses or through GM technology.<sup>32,33</sup> If GM technology is opted for, extra DNA will be inserted (enabling the production of the non-coding RNA molecule) and the plant will fall under GMO legislation. However, given that methylations are hereditary and the DNA fragment inserted can again be removed from a plant through cross-breeding, a plant can be obtained in which a specific promoter is methylated but without possessing the foreign DNA-fragment that was encoding the methylation step. In such a case, the GM technology is used to adjust a trait of a plant but the final plant that ends up in the field does not include any changes in the DNA sequence.

## Oligonucleotide-directed mutagenesis

This breeding method and the techniques covered hereafter are improved versions of the mutation breeding described earlier (see pages 16-17). Where the earlier form of mutation breeding causes random changes in the DNA, the effects of which are impossible to predict, the new mutation breeding techniques can bring about a specific number of DNA changes in predetermined places with a view to a specific effect (Figure 11).

It is first determined in which part of the plant's DNA a change must be brought about. Subsequently a piece of single-stranded DNA is made



(or a combination of DNA and RNA) containing 20 to 100 building blocks in which the desired change is available. That piece of DNA will then bind with the DNA fragment that must be adjusted in the plant.<sup>34</sup> This desired change can be a deletion (one or several DNA building blocks are eliminated from the original plant DNA), a change

of a DNA building block or an addition of one or several building blocks. The piece of DNA that is designed and that includes the desired change is called an oligonucleotide. Subsequently, the oligonucleotide is inserted in the plant cell (often mechanically, see earlier) where it will bind with the plant DNA that has to be changed. At the location of the mutation there is no correspondence with the plant DNA and therefore no binding occurs (Figure 11). The natural DNA repair mechanism of the plant recognizes this error. The cell re-

pairs the DNA by adopting the mutation of the oligonucleotide into the plant's DNA (Figure 11).<sup>34</sup> This is a stable way of building the mutation into the plant's DNA, after which the oligonucleotide is spontaneously degraded. The plant cells are then grown, through tissue culture, into plants which can transfer the added mutation to elite lines with the help of cross-breeding.

The oligonucleotide-directed mutagenesis technique has already been successfully applied for over 10 years, both for experimental purposes and for crop breeding. Using this technique several crops were developed to tolerate herbicides and for instance rice with a higher nutritional value (more tryptophan) was developed.<sup>35-39</sup> Rapeseed

varieties tolerant of the herbicide sulphonylurea and developed through oligonucleotide-directed mutagenesis are now commercially available in the United States.<sup>40</sup>

Through the controlled and precise mode of action of oligonucleotide-directed mutagenesis, random or excessive mutations are prevented. The technique does not incorporate extra genes: the oligonucleotide functions only as a template of how the plant has to change

its own DNA. The final product is therefore indistinguishable from a plant that is obtained through cross-breeding or mutation breeding. Oligonucleotide-directed mutagenesis is, in other words, an improved version of the generally accepted mutation breeding, with far fewer risks of unintended mutations. Scientifically speaking, therefore, there is not a single argument for treating plants bred through oligonucleotide-directed mutagenesis

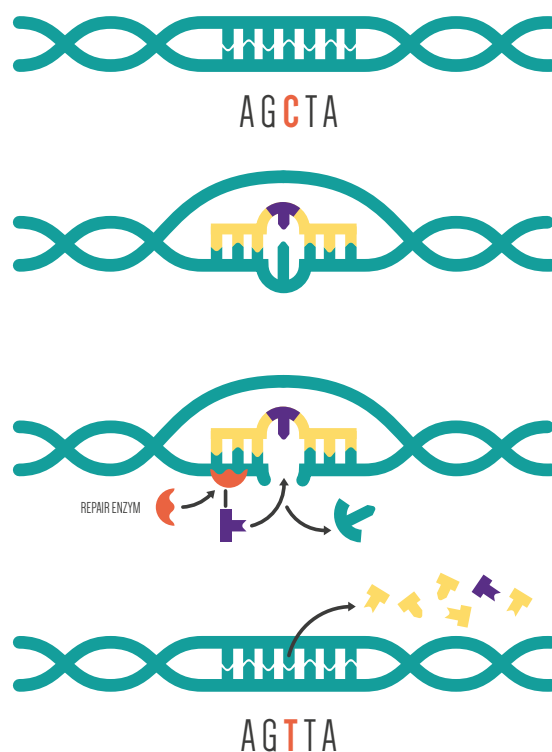


Figure 11. An oligonucleotide with the desired sequence binds to the DNA fragment that should get mutated. The natural repair machinery of the plant recognizes the mismatch and adjusts the original DNA fragment of the plant.

differently than plants obtained through conventional mutation breeding.

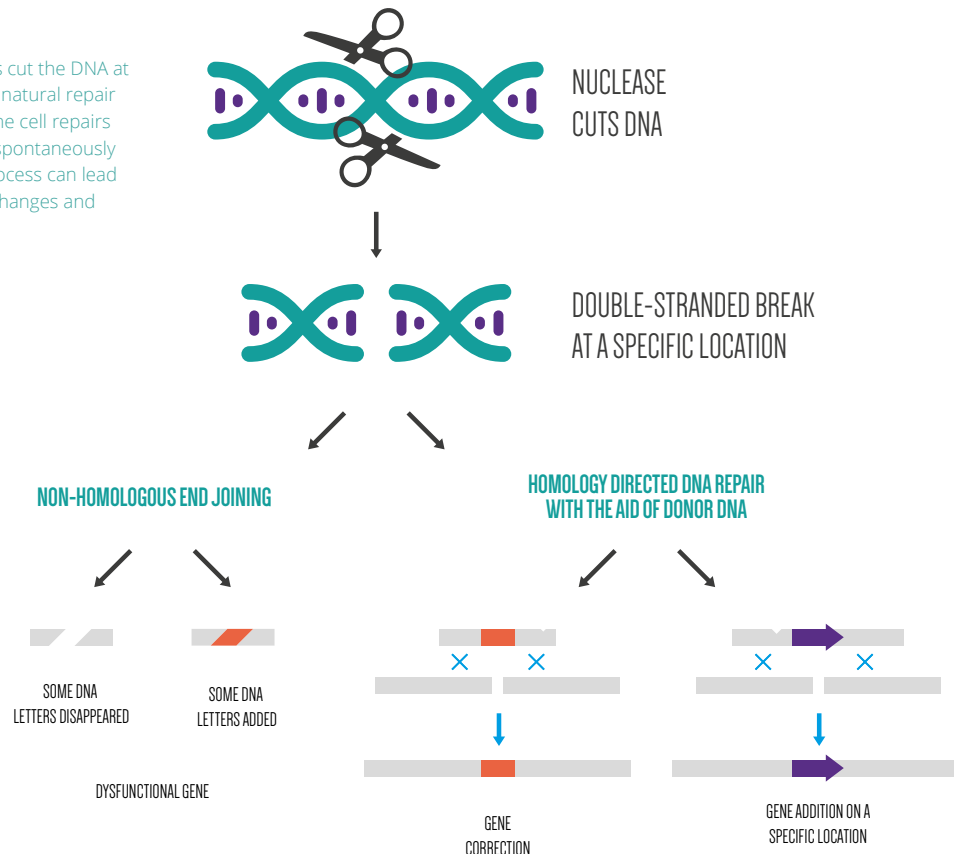
## CRISPR/Cas

CRISPR/Cas is, similar to oligonucleotide-directed mutagenesis, a method used to generate a mutation in a previously determined place in the plant DNA. CRISPR/Cas is a form of site-directed nuclease (SDN) technology. Before going into the details of CRISPR/Cas, the general principles of SDN technology are explained. Nucleases are enzymes that cut DNA. They can be compared with scissors (albeit on a molecular scale) that snip the

DNA. However, specific nucleases do not just snip away randomly at the DNA. They only do so when they recognize specific sites in the DNA. Such a recognition site is determined by a sequence of four or more DNA building blocks, based on the nuclease.

There is a great variety of nucleases, each of which recognizes different DNA sequences. When DNA is cut in half, a plant's natural repair mechanisms will try to repair the break. This can lead to two possible scenarios. In the first scenario, the ends are reconnected to each other through a process that is known in scientific jargon as 'non-homolo-

Figure 12. Nucleases cut the DNA at a specific place. The natural repair mechanism within the cell repairs the cut. Errors that spontaneously occur during this process can lead to interesting DNA changes and thus new traits.



gous end joining'.<sup>41</sup> During this process errors are often made, however, resulting in one or more DNA building blocks disappearing or being added, causing one or more mutations (Figure 12).

In the other scenario, the break can be repaired with a piece of DNA which, at the break ends, has a sequence homologous with the break. This is called 'homology-directed DNA repair'.<sup>41</sup> Through this method, a new DNA fragment can be introduced in the plant's DNA or a fragment can be added to its cells so that the original DNA sequence is repaired but with one or more intentional errors. With both repair mechanisms, the original DNA code is changed in a specific place, which is the principle upon which CRISPR/Cas and other SDN technologies (see case study on 'The four flavors of nuclease technology') are based.

Most nucleases cut DNA based on a short recognition sequence. When these nucleases are simply let loose on the plant DNA, the DNA will be cut at too many places. However, the smaller the sequence, the greater the chance that this sequence is available in the DNA by coincidence. To have a nuclease only exercise its cutting function at a specific place, it must have either a sufficiently long recognition sequence or be coupled to another molecule that ensures its accuracy. In CRISPR/Cas, the accuracy of the scissors (nuclease) is obtained by coupling it to an RNA molecule. In other variants of the SDN technology (see text box 'The four flavors of nuclease technology') the specificity of the molecular scissors is formed in another way.

In CRISPR/Cas, CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats and Cas for CRISPR-associated.<sup>42</sup> The technology was developed based on the CRISPR/Cas defense

system of bacteria against viruses.<sup>43</sup> When bacteria are infected with a virus, the RNA molecules of the bacterial CRISPR/Cas system bind to the RNA of the virus. This bond calls in bacterial nucleases to cut the viral RNA and thus destroy the virus. In the CRISPR/Cas system it is therefore the bacterial RNA molecules that determine the accuracy of the Cas nucleases.



Figure 13. In the CRISPR/Cas method the specificity of the nuclease (orange) is determined by an RNA-molecule (purple).

Once the CRISPR/Cas defense system was understood, scientists were able to simplify the process and combine the guiding role of the RNA molecules into a single RNA molecule.<sup>43</sup> This development means that it is now possible to elicit DNA mutations in specific genes with a simple and flexible fusion of RNA with a nuclease, not only in bacteria but also in plants and animals.<sup>44-46</sup> With the other SDN technologies (see text box 'The four flavors of nuclease technology') developing a specific nuclease is still laborious. As a result, the CRISPR/Cas technology has taken off much more than the other SDN variants. CRISPR/Cas is already the most popular SDN technology, both for scientific research and for commercial applications. The CRISPR/Cas system has been successfully applied in the model plants *Arabidopsis* and tobacco but also in crops such as wheat, corn, rice and tomatoes.<sup>47</sup>

Before mutations can be generated using CRISPR/Cas (as well as for the other SDN technologies), the necessary components (for CRISPR/Cas that is a nuclease coupled to a leading RNA molecule) must be incorporated into the plant cell. This can occur in a number of ways. One possibility is through GM technology. The genetic information for the production of the specific nuclease is then stably built into the plant DNA. The GM plant created in this way will make the CRISPR/Cas molecules, subsequently they can execute their function. After the creation of the mutation(s), those molecules and corresponding DNA are no longer of use. The GM plant will undergo a cross-breeding program in which offspring are selected that have inherited the mutation but no longer contain the CRISPR/Cas genes..

Alternatively, the nuclease and the corresponding RNA molecule can be transferred to the plant in the form of RNA with the help of plant viruses, or directly as RNA-protein complexes.<sup>48,49</sup> In both cases, no hereditary material is built into the plant DNA. In this case, no GM plant is made or used anywhere in the process. After completing their mutating task, nuclease and RNA are spontaneously broken down by the plant cell.

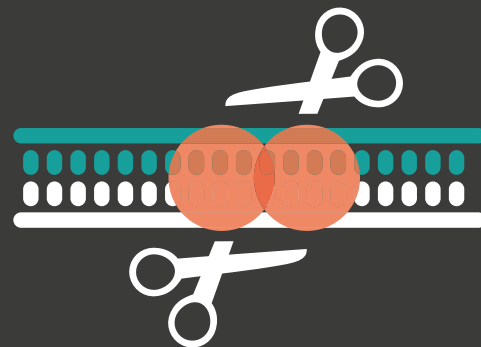
In both cases (use of a GM intermediary or not) the end result is a plant with one or more mutations in a specific gene that is no longer distinguishable from a plant developed through conventional mutation breeding. Scientifically speaking, there are no arguments to evaluate these plants at a risk-analysis level in any other way than plants obtained through conventional mutation breeding.

## THE FOUR FLAVORS OF NUCLEASE TECHNOLOGY

*Alongside the CRISPR/Cas technology described above, there are another three variants of site-directed nuclease technology. They differ in the way in which the DNA-cutting nuclease is guided to a specific DNA sequence.*

### **Meganuclease technology**

*Meganucleases originate from micro-organisms such as bacteria and yeasts and recognize relatively large DNA sequences (18 to 30 building blocks) in comparison with standard nucleases.<sup>50</sup> Only very specific fragments that seldom occur in plant DNA are cut by meganucleases. Accuracy is therefore ensured entirely by the meganucleases, and it is not necessary to fuse them with a RNA molecule (as CRISPR/Cas) or a DNA-binding protein such as a zinc finger (see below).*



*Meganucleases (orange structures) recognize highly specific fragments that rarely occur in the DNA. The specificity of this method is thus determined by the recognition sequence of the meganucleases.*

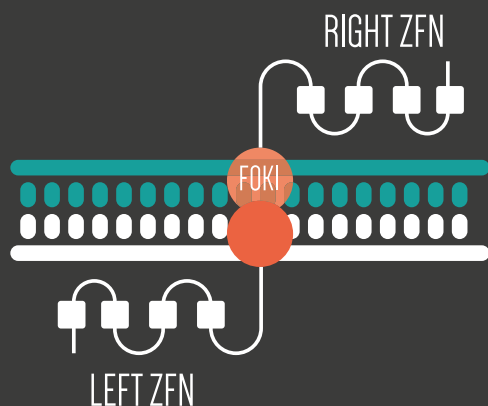
*A disadvantage of this technique is that the number of meganucleases available in nature is limited. That means it is quite possible*



that when a specific DNA sequence needs to be cut, there is no meganuclease available to fulfil this task. This is why, for several years, scientists are trying to change the recognition sequences of existing meganucleases to be able to employ them much more effectively.<sup>50</sup> 'Dimers' – two nucleases joined together – are often chosen in this case. This adjustment is not an easy matter because it can also negatively influence the activity of the nuclease, as a result of which the applicability of the meganuclease technology becomes limited.

### Zinc finger nuclease technology

Zinc finger nucleases (ZFN) are proteins composed of a zinc finger part and a nuclease part. By coupling the nuclease to a zinc finger—a protein that binds with great accuracy on a specific DNA fragment—the nuclease will only be able to conduct its cutting function at that location. The specificity of the ZFN technology is therefore fully determined by the zinc finger domain. Zinc finger nucleases work in pairs, one on each side of the double-stranded DNA, to increase accuracy. It is theoretically possible to adjust the recognition sequence of the zinc finger to such an extent that the ZFN can cut any sequence in the plant



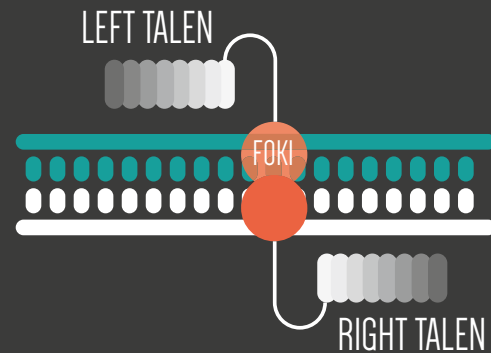
DNA. However, practice has shown that it is not that easy to make efficient and successful adjustments.<sup>51</sup> Unlike CRISPR/Cas, zinc finger nuclease technology

does not use RNA molecules but two DNA-binding proteins..

### TALEN technology

TALEN stands for Transcription Activator-Like Effector Nucleases.<sup>52</sup> Just as with zinc finger nucleases, the word 'nucleases' can be also found in TALEN. Also here the idea behind fusing a nuclease and a TAL effector is to not allow the nuclease to cut everywhere but only at one specific place in the plant DNA. As ZFNs, TAL effectors work in pairs.

Similar to the ZFN technology, TALENs are composed of a nuclease (FokI) and a DNA binding part that guides the nuclease to a specific DNA sequence.



Almost all breeding techniques are based on systems that exist in nature, including the TALEN technology. TAL-effectors are DNA-binding proteins that originate from *Xanthomonas bacteria*.<sup>52</sup> These bacteria cause several plant diseases. During the infection process of the plant by *Xanthomonas*, the bacteria inject TAL effectors in the plant cells. The TAL effectors are then transported to the nucleus of the plant cells to bind to the plant DNA, and more specifically to promoters of certain genes. This binding triggers the activity of plant genes that benefit the infection by the bacteria. *Xanthomonas* thus uses TAL effectors to increase the plant's sensitivity. Interestingly, the DNA recognition sequence of TAL effectors can be adjusted to develop TAL effectors that can recognize almost all

DNA sequences. By binding specific TAL effectors to a nuclease, DNA breaks can be caused—and therefore mutations can be generated—in almost all genes. Just as with the aforementioned site-specific nuclease techniques, the needed adjustments of the DNA recognition sequence of the TAL effectors limits its applicability.

A good example of the power of nuclease technology is the development of mildew resistant wheat.<sup>53</sup> The wheat genome is huge and complex. Common wheat (*Triticum aestivum*) for example has six copies of each gene. To make wheat resistant to mildew, six copies of a certain gene have to be silenced. Using radiation or chemicals (conventional mutation breeding, see previously) for this purpose is simply not feasible because it doesn't allow a targeted approach. Chinese researchers tried the TALEN technology and succeeded. The six wheat DNA sequences were simultaneously cut at a specific location and lost their functionality as a result.<sup>53</sup> SDN technologies can thus contribute great value to the development of new varieties with new traits.

### **What type of DNA modifications can be obtained with CRISPR/Cas?**

Depending on the adjustment that occurs in the DNA, SDN technology has three variants<sup>54</sup>:

**SDN-1:** this method will only cause a break in DNA in a previously determined place. The plant's natural repair mechanism will repair the break. This is often imperfect, leading to the disappearance or addition of one or more base pairs or the use of the wrong base pairs. The function of a gene can disappear through these mutations.

**SDN-2:** the working mechanism of this is similar to SDN-1, but here a type of instruction manual is given to the plant (in the form of a small piece of DNA) to instruct it how to repair the break in the DNA. It is therefore determined in advance which building blocks of the original plant DNA must be changed or added. This variant to SDN technology is still at the research stage, however.

**SDN-3:** the working mechanism is again similar to that of SDN-1 but now a large piece of DNA is added to the plant DNA. The result of this SDN variant differs greatly therefore from SDN-1 and SDN-2. However, SDN-3 is still at the early stages of research. Moreover, this technique cannot be categorized under mutation breeding 2.0 and will be discussed later (see page 37).

## Cis-/intragenesis

### Cisgenesis

All genetically modified plants that are cultivated commercially today are transgenic. 'Trans' refers to 'coming from another group'. With the help of genetic modification, in transgenic crops, a DNA fragment is added from a species with which the crop in question cannot cross-breed; for example a maize gene is built into a rice plant. Building a bacterial Bt gene into cotton to obtain resistance to insects (see VIB Fact series 'Bt-cotton in India'<sup>20</sup>) or building in a viral coat protein into papaya to develop virus resistance (see VIB Fact series 'Virus resistant papaya in Hawaii'<sup>20</sup>) also leads to transgenic plants.

In cisgenesis, as in transgenesis, extra DNA is stably built into the plant DNA. The major difference between transgenesis and cisgenesis is the origin of the DNA. With cisgenesis, the extra DNA originates from a plant with which the acceptor plant (the plant that will receive the extra DNA) can cross-breed. 'Cis' refers to 'within the same crossable group'. For example, adding a tomato gene to a tomato. Cisgenesis aims to obtain the same result as through conventional crosses, but in a much faster and more efficient manner. A representative application of cisgenesis is the development of a potato resistant to potato blight. Wild species of potatoes from Mexico and the Andes have genes that make them resistant to this disease. The genes can be transferred by cross-breeding to the potato cultivars we eat, but this process is complicated and takes time. Moreover, with cross-breeding, undesirable traits are also inherited. With cisgenesis, the resistance

genes can be added to the DNA of our Bintje and Nicola potatoes in one step without losing the characteristic variety traits. Such cisgenic potatoes are currently being developed in Belgium.<sup>20</sup>

In order to comply with the definition of cisgenesis, the genes that are added must also come with their original regulatory units; i.e. the switches that decide when a gene is activated or silenced and to what extent. All the hereditary information for a particular trait is therefore fully incorporated and the gene has the identical sequence (including introns) to the crossable plant it comes from. Cisgenic plants may not include bacterial selection genes (such as antibiotic resistance genes) or selection genes from non-crossable species. Selection genes can be used during the process of genetic modification but they must then be eliminated from the plant DNA.

Because natural cross-breeding barriers are not crossed, cisgenesis has wider acceptance among the public.<sup>55</sup> However, from the point of view of environmental and food safety, there is no difference at all between cisgenesis and transgenesis. It is always the trait for which the introduced gene encodes that determines whether a crop is safe and not whether that gene comes from the same species or a crossable species. The distinction between cisgenesis and transgenesis may be important, though, from a regulatory point of view. Depending on the legislation—and this varies across the world—certain applications of cisgenesis may or may not be regulated.

## Intragenesis

Also with intragenesis, a certain DNA fragment is stably built into the plant DNA and, again, DNA is used from a crossable species or the same species. So just like cisgenesis, it is a specific type of genetic modification. The difference from cisgenesis is, however, that the original composition of the DNA fragment is not retained. With intragenesis, new combinations of the existing DNA are made.<sup>56</sup> The most common case is that gene X is combined with the gene switch (or promoter) of gene Y. This kind of modification is nothing new since many organisms (including plants) have undergone spontaneous DNA-rearrangements during evolution, resulting in the formation of new DNA combinations. In nature however this occurs at random and the individuals that undergo

undesirable rearrangements disappear. With intragenesis, the element of chance is removed.

Intragenesis is an interesting breeding method to allow a certain trait that already occurs naturally in the plant (for example the production of a certain vitamin in the leaves) to be expressed in the harvestable part (for example the seeds) as well. Or the reverse: an undesirable trait in the harvestable part can be eliminated or reduced by silencing the gene in the specific plant organ by using another gene regulator. By giving certain genes other switches, the expression of the existing hereditary information can be optimized to human requirements without the need to build in DNA foreign to the species.<sup>56,57</sup>

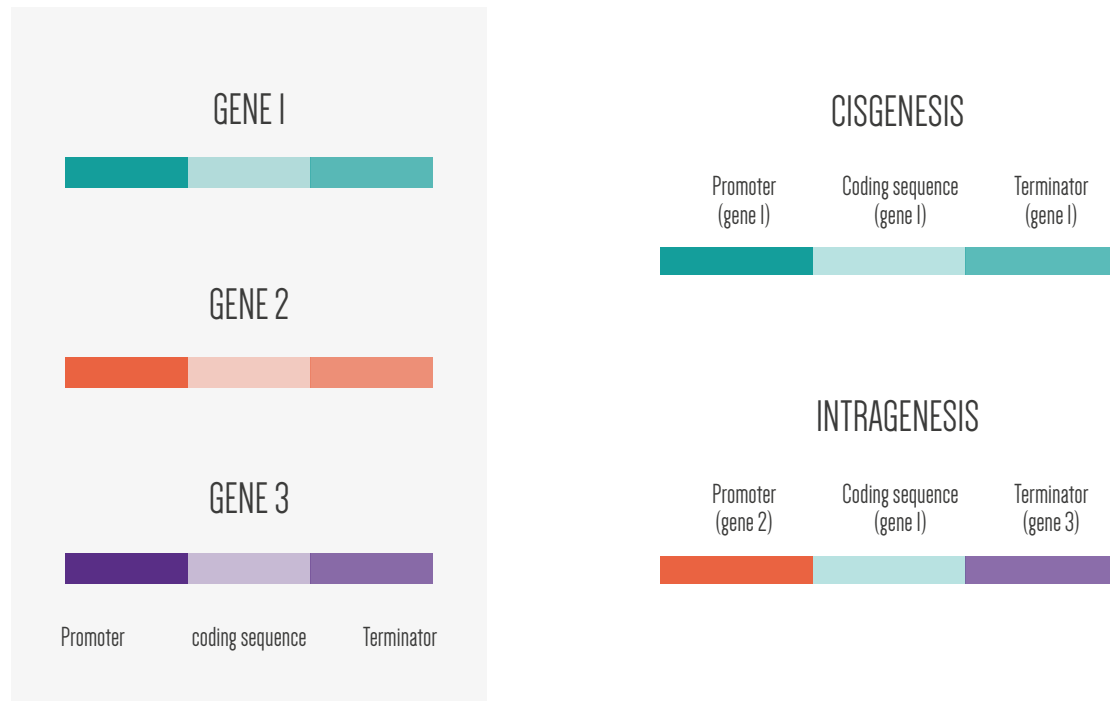


Figure 14. With cisgenesis the original arrangement within the DNA fragment is maintained. With intragenesis new combinations are made using the original DNA fragment.





## GMO 2.0

### SDN-3

Site-directed nuclease technology has already been discussed (see page 31) and as stated, there is a distinction between SDN-1, SDN-2 and SDN-3 (see text box 'What type of DNA modifications can be obtained with CRISPR/Cas?'). The SDN-3 version is discussed here because this method allows -for relatively large DNA fragments- to be built stably into plant DNA, in contrast to the very limited DNA modifications of SDN-1 and SDN-2.

The SDN-3 method does not specify the type of DNA to be built in. This can be either the DNA of a crossable plant—in which case a cisgenic or intragenic plant is obtained—but it can also be DNA from another species—in which case a transgenic plant is made. It must be ensured however that the DNA fragment contains at both ends a

sequence homologous to the site in the plant DNA where it must be built in. This is to obtain homology-directed repair of the DNA (see above, page 31).

The DNA that must be added can be delivered in a number of ways. In the first method, the DNA fragment can be transiently introduced into a plant cell (mechanically through the gene gun or using *Agrobacterium*) and subsequently stably built into the DNA at the site indicated by the SDN (site X, Figure 15).

In the second method, the extra DNA can be built in at a random site first (site Y, Figure 15). When the SDN comes into play, it will cause three breaks in the DNA instead of one: at site X (the desired site in the plant DNA) and left and right of

the DNA to be added (site Y), resulting in the extra DNA effectively being cut out. In a number of cases, the DNA repair mechanism of the plant will repair the two breaks to the extent that the extra DNA cut out appears at site X instead of again at site Y (Figure 15). Just as with SDN-1 and SDN-2, the site-specific nuclease (as well as the DNA donor construct) is selected out during the further breeding process and no longer appears in the final product.

In comparison to conventional GM technology, SDN-3 technology has the great advantage one

can determine in advance the exact location in the plant DNA where the extra DNA will be introduced. With conventional GM technology, this cannot be achieved. This advantage of the new technology becomes all the more interesting when a new trait needs to be inherited along with an existing trait. By incorporating the gene for the new trait before or after the gene of an existing trait, both genes will be inherited together by the offspring. However, because of the current low efficiency of the SDN-3 technique and the technical difficulties involved, use of this technique is limited at present to the level of proof of concept.<sup>58,59</sup>

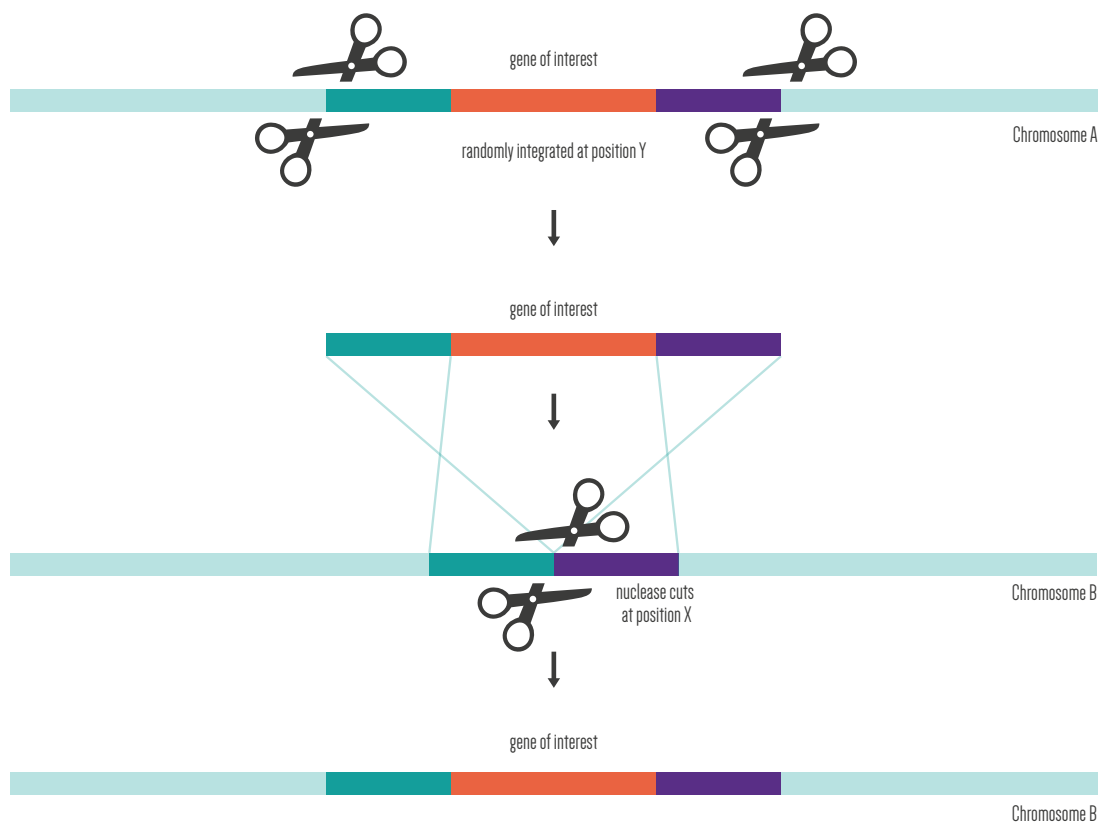


Figure 15. SDN-3 is a method to introduce an extra DNA fragment at a specific location in the DNA. The gene of interest (transiently introduced or stably built into chromosome A) has at both sides a DNA sequence (blue and purple) homologous to the place where the gene of interest should be introduced. Chromosome B is cut using a SDN method (ZFN, TALEN, CRISPR/Cas) after which the gene of interest is built in.

# 4 Conclusion

To make our food production system more efficient, sustainable and flexible, continual efforts must be made in plant breeding and cultivation techniques. Adapting crops genetically to our requirements is something we have done since the start of agriculture. New techniques for working even more efficiently will continue to be developed and form part of the breeder's toolbox. In general, it can be stated that the technology through which a plant has acquired a certain trait is secondary to the impact of the trait itself.

This VIB Fact series gives an overview of the different methods that can be used to genetically adapt plants to our requirements. This goes from the most traditional techniques such as cross-breeding and genetic modification to the newest methods, many of which do not yet have any commercial applications. A factor that several of the new breeding methods have in common is that during the development process, one or more GM plants are developed. However, the final product distinguishes itself from its GM parent plants because it contains no extra added DNA. Moreover, the changes in the DNA of the final product can no longer be distinguished from genetic changes that occur spontaneously. The latter means that the products of certain new breeding techniques can no longer be distinguished from those of traditional breeding methods when it comes to regulation.

Being alert to new products and new production technologies must be encouraged and forms a cornerstone of guaranteeing food and environmental safety. From a political and regulatory point of view, however, it is also important that innovation continues to get sufficient opportunities and that discussions about new technologies are conducted factually and on the basis of scientific data. With this dossier, we attempt to contribute to a broader knowledge and greater appreciation of plant breeding, which will hopefully make it possible to hold a serene debate and create a proportionate regulatory framework for the new breeding techniques.

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