Background paper: CRISPR/Cas – description of the possibilities

CRISPR/Cas technology is a rapidly evolving field of research. At the same time, there has been a sharp rise in the number of publications on the technology from its advent in 2012 until the present day. Gene scissors are mainly used in basic research to better understand complex interactions in organisms. At the same time, the technology is continually being further developed for use in as many organisms as possible and for different purposes.

This backgrounder explains how gene scissors can be used in plants to make apparently small changes in DNA (i.e. SDN-1 applications) which can, nevertheless, lead to complex alterations in their metabolism. In addition, it describes new methods of introducing the gene scissors into plants cells as well as CRISPR/Cas system developments, which can be used to make more or less unrestricted changes in the genome of plants. Finally, it provides an overview of what and how CRISPR/Cas is most commonly used in plants at present.

SDN-1 applications enable complex changes in the genome

Modification of several gene copies (i.e. identical DNA sequences)

The CRISPR/Cas system makes it possible to change all DNA regions with a similar sequence. The recognition component of the gene scissors, also called guide RNA or gRNA, is developed in such a way that the gene scissors can recognize and cut several gene regions with the same DNA sequence. Plants, in particular, often have a redundant genome, which means genes are frequently present as multiple sets (as gene copies or genes of a gene family) or as variants (so-called alleles). If a gene is available in multiple versions, the gene scissors can recognize and modify all or several of the gene versions.

Many plants are polyploid, which means they contain multiple sets of chromosomes, e.g. wheat has a six-fold set of chromosomes, also known as hexaploid. This means there are six copies of genes in wheat. If a gRNA, which the gene scissors use to identify the target sequence in the genome, is introduced together with the gene scissors into the plant cell, the target sequence of the DNA can be recognized and cut several times. Ultimately, up to six changes can be made at the same time [1]. With the help of a single gRNA, several identical alleles in polyploid plants can be changed simultaneously [2; 3].

Gene scissors can also simultaneously change multiple copies of a gene. This means that entire gene families can be changed or even knocked out [4; 5]. Gene families arise when genes duplicate multiple times and may contain several hundred genes. The genes of a gene family can either stay together in so-called gene clusters or be distributed over the entire genome (on the original chromosome, but also on different chromosomes). The genes in these gene families can have completely identical DNA sequences or differ in individual bases, which can be attributed to mutation events. Gene families are common in plants and play an important role in the development of new plant species.

Multiplexing (changing several different DNA sequences)

In multiplexing, several gRNAs are introduced into the plant cells together with the gene scissors in order to simultaneously change different regions of DNA [6; 7]. Multiplexing not only changes individual copies of the different target genes, but also all identical copies of the respective DNA sequence.

Scientists consider multiplexing to be particularly important because many traits in plants are determined by more than one gene, e.g. responses to certain stress conditions. In one study, the gene scissors were directed to three different target sites in wheat and changed up to 18 DNA sequences at the same time [8]. In another instance, eight different genes were processed in a rice variety in such a way that it not only resulted in a higher yield, but also led to changes in growth and fragrance [9]. Scientists using the CRISPR/Cas variant, CRISPR/Cpf1 (also called MbCas12a) from the bacterium *M. bovoculi*, were able to simultaneously change up to 16 different genes in rice [10]. These genes regulate various agronomically relevant traits as well as bacterial resistance.

Multiplexing can be used to delete parts of the genome. Gene scissors can recognize and cut at two different target sites. The "excised" part between the two target sites can then be deleted. This is used, for example, to delete several target genes located next to each other in a DNA region [11-13].

The complexity of possible gene scissors applications, such as multiplexing, also increases the likelihood of unwanted changes in metabolic pathways, e.g. when a gene is knocked out that is involved in several processes in the cell and whose functions are not fully understood. This can have an unintentional influence on biochemical processes in the plants which may only become apparent much later (e.g. under certain stress conditions). In addition, the complexity of the interventions may increase the risk of unintended changes in the genome, i.e. technical errors that can occur during the genome editing procedure. Both the unwanted effects on other metabolic pathways and unintended changes in the DNA are explained in more detail in the backgrounder on the risks associated with CRISPR/Cas.

Changes in areas of the genome that are less accessible to plant breeders

There are areas in the genomes of plants that are difficult for traditional breeders to access. These are, for example, regions of DNA in which naturally homologous recombinations only rarely occur during meiosis (i.e. the formation of germ cells). During meiosis, the number of chromosomes is halved and the egg cell and pollen are formed. Homologous recombination is a process during meiosis in which pieces of homologous chromosomes can be exchanged (by crossing over). Exchanging certain regions of DNA increases genetic diversity. There are areas at specific sites on the genome of plants where homologous recombination takes place more frequently (so-called recombinational hot spots) than others (so-called recombinational cold spots). Breeders often have to work with linked genes that cannot be separated from each other because they are located in recombinational cold spots.

Genes that are located very close together on a chromosome are also called 'linked' genes because they are very likely inherited together. If two genes are located on different chromosomes or on the same chromosome, but at a greater distance, then they are not linked and can be inherited separately from one another. In some plant species, many regions of DNA are linked, e.g. in tomatoes, where over 25% of the genome is linked [14].

Breeders may want to separate linked genes if, for example, one of the two genes mediates an undesirable trait in the plants. The gene scissors can be used to selectively modify individual genes or gene variants that cannot be accessed through homologous recombination and that are linked to other genes. For example, one of the two genes can be knocked out by CRISPR/Cas inducing small changes in the DNA sequence, and thereby cancelling the common inheritance of certain traits. In a study on tomatoes, for example, gene scissors were used to separate two genes that determine both the shape of the fruit and the connection between the fruit and the stem [15]. The CRISPR/Cas application in this case eliminated the common inheritance of these two traits, making it possible to grow a tomato with a specific shape which is easier to harvest.

CRISPR/Cas has, in addition, already been applied in the model plant *Arabidopsis thaliana* to rearrange a large area of a chromosome. It enables the relocation of DNA regions in recombinational cold spots to other DNA regions in which homologous recombinations occur more frequently [16].

The gene scissors can also be used to change DNA regions that are in the immediate vicinity of the centromeres of chromosomes. The centromere divides a chromosome into two parts and is the area where the two halves of a chromosome pull apart during cell division. In these areas, the DNA is packed more densely than in others and there is hardly any exchange of DNA during the meiosis. The proportion of such areas is quite high: in maize and barley, around 20% of all genes are located near the centromere [17; 18]. Various gene scissors applications can also change these areas [19; 20; 16].

Traits generated by changes in these parts of the genome were mostly unachievable with previous breeding processes, which is why such genome-edited plants must undergo suitable risk assessment (more on this in the background paper on the risks of CRISPR/Cas).

Changes in preferentially protected areas of the genome

According to classical evolutionary biology, new mutations can occur naturally in all genes with the same frequency, regardless of the gene function and the consequences for the fitness of the respective organism. In the last few years, a number of scientific papers have shown that new mutations more frequently occur naturally in certain areas of the genome than in others. This was shown in work on *Arabidopsis thaliana* [21; 22], a model plant used in basic research, but also in human cells [23; 24] and yeast cells [25].

Mutations are, therefore, not evenly distributed in the genome. Fewer mutations occur in many functionally important parts of the genome, for example, in gene areas. The mutation rate depends on a certain repair mechanism, the so-called DNA mismatch repair (MMR),

which is responsible for repairing newly occurring mutations in such areas. Scientists specifically knocked out the MMR in *A. thaliana* so that damage to the DNA could no longer be repaired [22]. They were able to show that mutations accumulate particularly in gene areas in these plants.

There are also differences in the distribution of mutations within the gene areas themselves: more mutations occur in the outer areas than in the inner areas, which often mediate gene functions. This pattern can even be detected in genetic variants, so-called polymorphisms, in wild populations [21].

It was also shown that certain epigenetic markers can be detected in gene areas that are particularly protected by the MMR. Mutations occur less frequently there. Epigenetic markers are inheritable factors that are not alterations in the DNA sequence, but are attached to DNA and regulate gene activity. These epigenetic markers serve as recognition signals for the MMR and ensure that the repair mechanism is particularly active in these areas of the genome. It should be emphasized that, in theory, new mutations can occur anywhere in the genome. However, mutations occur much less frequently in certain areas of the genome than in others due to the MMR repair mechanisms.

With CRISPR/Cas, changes can also be induced in specially protected areas of the genome. This results from the mode of action of the gene scissors: if a DNA double-strand break caused by CRISPR/Cas is repaired so that the original state is restored [26], CRISPR/Cas can recognize the target sequence again and cut again at this site. Ultimately, the gene scissors increase the likelihood that changes can also occur in these parts of the genome. The CRISPR/Cas system thus makes it possible to change gene areas where mutations are unlikely to occur naturally.

One way of identifying areas which accumulate less mutations, is to create so-called pangenomes, which encompass the entirety of all genes and gene variants in a species. This involves the genome sequencing of many different representatives or lines of a species (i.e. its DNA sequence is determined). The pangenome of a plant species can serve as a reference genome for all naturally occurring genetic variants, and thus describe the entire genetic diversity within a species. The pangenomes of maize and barley have, for example, already been published, including some lines of the respective species [27; 28]. As a next step, wild representatives of these crops also need to be sequenced in order to further describe the natural gene pool. If certain gene variants that have been generated by genome editing also occur naturally in the gene pool, or if certain gene variants have already been generated by conventional breeding, they, nevertheless, need to undergo risk assessment as unintended changes can arise from technical errors during gene scissors applications and other technical processes necessary in the multi-step process.

***De novo* domestication**

The pangenome can, for example, be used for tomato plants [29; 30] to investigate how similar currently grown crop species are to their wild representatives. As it stands, numerous positive

traits in wild representatives, such as stress tolerances, have been lost in many crops during the course of domestication. CRISPR/Cas gene scissors can be used in wild species to induce accelerated domestication (also called *de novo* domestication). The aim is to change the genome in such a way that the plants carry certain genetic variants from cultivated plant lines [31-34]. Gene scissors have already been used several times for this purpose. As a result, such genome-edited plants have properties from wild species that have been "lost" in the course of plant breeding, and combine these with "domesticated" traits that are important for cultivation.

For example, CRISPR/Cas9 was used to modify six different genes in a wild tomato species that have proven to be important for yield and nutritional value of domesticated tomatoes [31]. In a further study, domestication traits, such as the grain seat in the ear and the length of the awns, were changed in a wild rice species with a four-fold set of chromosomes (i.e. a tetraploid rice) [32; 33]. Rice that is widely grown today has only two sets of chromosomes, i.e. it is diploid. An increased set of chromosomes is advantageous if a (negative) mutation occurs because there are still many more gene copies in the genome. In addition, plants with more than two sets of chromosomes can adapt better to changing environmental conditions. The authors of the aforementioned study used a tetraploid relative of a currently grown rice species. This "wild" rice has traits such as a high biomass (i.e. it grows higher), various biotic stress tolerances and heat tolerance. Many genes involved in the domestication process of rice are also present in the genome of the wild relative and were altered using gene scissors. Amongst others, the length of the awns was reduced, the loss of grain and the overall height of the plants were reduced.

In studies using gene scissors for *de novo* domestication [31; 32; 34; 35], complex alterations in the genome of the crop plants were induced by combining several SDN-1 changes. Different genes, for example, were changed at the same time by multiplexing, or multiple gene copies were changed simultaneously. Such plants must be subjected to an adequate risk assessment, on the one hand, to investigate unintended alterations that may occur through the application of the technology and, on the other hand, to detect unwanted effects on other metabolic pathways. More information about this can be found in the background paper on the risks of CRISPR/Cas.

Further developments in CRISPR/Cas technology

The following section contains a summary of how CRISPR/Cas has been further developed in order to use it for as many applications in plants as possible. The classic CRISPR/Cas9 system has been adapted to new applications and new variants of the gene scissors are being developed.

Recent advances in the classic CRISPR/Cas9 system

The structure of the classic CRISPR/Cas9 system can be modified so that the gene scissors can still bind to the target sequence but can no longer cut DNA. This form of gene scissors is known

as dead Cas9 or dCas9. Various enzymes can be attached to these gene scissors. Thus, dCas9 acts as a platform that “carries” an attached enzyme to a specific genomic region. These enzymes can induce different responses at the target sequence, which are described in more detail below.

Base Editing

The sequence of the bases determines the sequence of the DNA (i.e. the genetic code). The four bases of DNA are adenine (A), guanine (G), cytosine (C) and thymine (T). In base editing, enzymes which can change specific bases of the DNA at the target site are attached to dCas9. Base editing can be used for targeted conversion of one base to another base without cutting the target sequence or using a DNA template [36; 37]. For example, cytosine can be converted to thymine and adenine to guanine. Base editing has already been used several times in plants [38-40]; amongst other things, several different target genes were changed simultaneously (i.e. base editing multiplexing) [41; 42]. The technology is used above all to induce traits such as herbicide tolerances [42; 41; 43]. Unintentional changes have, however, also been observed in base editing. These are described in more detail in the background paper on the risks of CRISPR/Cas.

Prime Editing

In prime editing, modified CRISPR/Cas9 gene scissors cut just a single-strand of the DNA at the target sequence [44]. This type of gene scissors is also called nickase. The recognition component of the gene scissors is changed in prime editing in such a way that it has a further function in addition to recognition of the target site: it also serves as a repair template to introduce a targeted change in the DNA. The recognition component in prime editing is called pegRNA (i.e. the abbreviation for *prime editing gRNA*). An enzyme, the so-called reverse transcriptase, is linked to the gene scissors during prime editing. This enzyme acts as a kind of translator that converts the pegRNA into DNA. This piece of DNA then serves as a DNA template for repairing the DNA single-strand break and is copied into the genome at this site. As a result, the two DNA strands at the target sequence no longer match each other. The gene scissors also cut the unchanged DNA single strand, and the DNA template in the other DNA single strand is then used to repair this cut. Many different changes can be introduced into the DNA with prime editing, e.g. deletions, insertions; or individual bases can be changed to any other type of base.

Prime editing has already been used several times in plants [45-47], but the efficiency with which changes can be introduced at the target sequence is still quite low [48; 49]. This depends on various factors, such as the reverse transcriptase used (i.e. the translator of the pegRNA) or the design of the corresponding pegRNA [50]. Scientists are already working on further developing prime editing in plants and improving its efficiency [51]. So far there are only a few studies that have systematically examined the risks of the procedure [52; 53]. More information can be found in the background paper on the risks of CRISPR/Cas.

Alterations of epigenetic markers through CRISPR/dCas applications (Epigenome Editing)

Epigenetics encompasses mechanisms and, in some cases, hereditary changes in the genome that are not based on changes in the DNA sequence. This can affect the phenotype of an organism, i.e. its observable characteristics or traits. Amongst other things, epigenetics regulates the activity of genes, for example, during the development of organisms or in response to the environment. For this purpose, epigenetic markers are transferred to the DNA and/or proteins that are closely associated with the DNA (i.e. histones). These epigenetic markers can be likened to little switches that are attached to genes and can be turned on or off. They are attached by certain enzymes to the DNA or histones in different areas of the genome. So-called methyl groups can be transferred to DNA, for example, and are typical epigenetic DNA markers, which most frequently turn off gene activity. Various epigenetic markers can be attached to the histones, including methyl or phosphate groups.

Epigenome editing uses the non-cutting gene scissors dCas9 that are either directly or indirectly linked to enzymes that change epigenetic markers around the DNA target site [54-56]. Scientists use epigenome editing to switch genes on or off. Currently, it is mainly used and being further developed for basic research in plants. Both the methylation of DNA [57] and the epigenetic markers of histones [58; 59] are changed by epigenome editing. An investigation of unintended effects is of particular importance here, as the enzymes attached to the dCas9 can also alter epigenetic markers at other parts of the genome (you can find more information in the background on the risks of CRISPR/Cas).

<u>CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa)</u>

The CRISPR/dCas9 gene scissors can also be guided to certain regulatory areas of DNA in the genome, which can hinder gene activity spatially. Thus the corresponding protein is no longer produced [60]. In this application, the DNA sequence is not cut by dCas9 and the “blunt” gene scissors do not change the sequence of the DNA sequence. Such applications are known as CRISPR interference or CRISPRi. Alternatively, certain regulatory factors can be coupled to dCas9, which can activate gene activity due to the spatial proximity to target genes (CRISPR activation/CRISPRa) [61; 58] or inactivate (CRISPRi) [60; 62].

New CRISPR/Cas variants

The most commonly used gene scissors are CRISPR/Cas9, in which the cutting component Cas9 is derived from the bacterium *Streptococcus pyogenes*. However, other CRISPR/Cas systems from different bacteria are also used to expand the possibilities of gene scissors applications, e.g. CRISPR/Cpf1 [63] or CRISPR/Cas13 to modify RNA [64].

The gene scissors variants differ in that they recognize a very specific short DNA sequence in the genome of the target organism, which is also known as the "PAM sequence" (PAM stands for protospacer adjacent motif). The PAM sequence precedes the actual target sequence and serves as a stop signal for the gene scissors. The enzyme complex stops at the PAM sequence and uses the gRNA to check whether the preceding DNA sequence matches it. When the target sequence has been recognized, the gene scissors cut the DNA there.

The CRISPR/Cas variants are derived from different types of bacteria which recognize specific PAM sequences around their target areas in the genome. The classic gene scissors CRISPR/Cas9 recognize the DNA sequence containing the sequence “NGG”, where the “N” stands for any base of the DNA bases and the “G” for guanine. The CRISPR/Cas variant CRISPR/Cpf1 recognizes the sequence “TTTV” as its PAM recognition sequence in the genome of target organisms, whereby the “T” stands for thymine and the “V” for one of the three bases guanine, cytosine or adenine.

CRISPR/Cpf1 recognizes a different PAM sequence enabling scientists to change other areas of the genome that cannot be altered with the classic gene scissors CRISPR/Cas9. In addition, CRISPR/Cpf1 is more efficient than CRISPR/Cas9 and causes fewer unintended off-target effects in the genome (for more information see background information on the risks of CRISPR/Cas).

There is ongoing research aiming to develop new gene scissors variants that work without a specific PAM sequence [65]. However, such variants are still error-prone and need to be further developed for more precise applications.

In addition to expanding the possibilities of changing as many target areas as possible with the gene scissors variants, further development of the variants is intended to reduce the occurrence of unintended changes in DNA, such as off- and on-target effects. For example, CRISPR/Cas9 gene scissors, which occur naturally in different types of bacteria (e.g. *Streptococcus pyogenes* and *Staphylococus aureus*). Synthetic CRISPR/Cas9 variants produced in the laboratory (e.g. SpCas9 HF1, HypaCas9 and HiFi Cas9) have also been further developed and used [66].

RNA Editing

RNA can also be altered by using specific CRISPR/Cas variants. RNA plays a major role in cells: RNA functions in the cells as an intermediate product during the translation of gene sequences into the corresponding proteins. The DNA sequence is used as a template to transcribe different RNAs that are then translated into proteins. Proteins are made up of amino acids, which are the building blocks of proteins. A single amino acid is determined by certain combinations of three bases in DNA. This means that the DNA sequence determines the exact order of the amino acids in a protein.

Scientists have found CRISPR/Cas variants that cut RNA instead of DNA [64; 67; 68]. CRISPR/Cas13 works similarly to CRISPR/Cas9, except that CRISPR/Cas13 cuts RNA instead of DNA. The recognition component of CRISPR/Cas13 is called crRNA (crRNA stands for CRISPR RNA). In the transformed cell, CRISPR/Cas13 is directed to the target RNA with the help of the crRNA and then cuts it. As a result, fewer RNA molecules are present in the cell and fewer proteins are formed.

CRISPR/Cas13 has already been used in various applications in plants [69; 70]. In tobacco plants, for example, the genetic information for the formation of the RNA gene scissors, CRISPR/Cas13a, was permanently integrated into the genome of the plants to protect the

plants from infection with the Turnip Mosaic Virus (TuMV) [69]. TuMV has a genome that consists of RNA. Thus, in the event of an infection with TuMV, CRISPR/Cas13a recognizes the invading viral RNA genome and cuts it. The transgenic plants are more resistant to the TuMV.

Other versions of the CRISPR/Cas13 gene scissors (dCas13, dead Cas13) have also been developed, which can still bind a target RNA but are unable to cut it. dCas13 variants can be linked to different enzymes that convert some types of RNA bases into others (e.g. adenosine to inosine) [68]. Scientists can use this system to change the RNA sequence and the structure of corresponding proteins without permanently altering the genome of the target organism. The RNAs modified by CRISPR/Cas13 are then naturally degraded in the cells. Thus, such changes are not detectable in DNA. Therefore, the composition of the RNAs, the proteins and the metabolic products must be examined in risk assessment to identify altered gene products. You can find more information about this in the background paper on the risks of CRISPR/Cas.

New methods of introducing the gene scissors into plant cells

Thus far, 'old' genetic engineering methods are frequently used to introduce the DNA to form the gene scissors into plant cells (you can find further information in the background information on the description of the CRISPR/Cas technology). However, various other new methods have been developed in recent years.

For example, RNA viruses have been used to introduce gRNAs into transgenic tobacco plants [71]. The DNA to form the gene scissors is too large to be introduced with RNA viruses. Therefore, tobacco plants were generated that already carry the genetic information for the formation of the gene scissors cutting component. The gRNAs were introduced into the transgenic plants via the tobacco rattle virus and were shown to cause a change in the target gene [71]. The technique of using RNA viruses to introduce components of the gene scissors into plant cells is still in its infancy, as it requires transgenic plants that already contain the DNA to form the gene scissors. The ultimate aim is to simultaneously introduce both the DNA to form the gene scissors (or the enzyme complex) and the gRNAs with the help of the RNA viruses, and thus avoid the intermediate step via a transgenic plant. The method described here does not offer an alternative to the 'old' genetic engineering methods.

In another study, wheat was pollinated with pollen from transgenic maize plants, i.e. cross-species pollination [72]. The transgenic maize contains the genetic information to form the gene scissors and several gRNAs. After the wheat is pollinated with the maize pollen, the gene scissors are formed in the wheat embryos altering the target region(s) in the wheat's genome. The chromosomes of the maize are degraded after some time as the maize and the wheat are not compatible. The chromosomes of the wheat are doubled induced by the application of a substance called colchicine. The resulting genome-edited wheat is homozygous. Using this method results in genome-edited plants that are homozygous in regard to the alterations at the target site(s). However, it emerged that heterozygous wheat plants were also obtained. This most likely results from the timing of gene scissors formation and the amount of gene

scissors formed. The method needs further development and optimization. It should also be taken into consideration that stronger gene expression of the gene scissors can result in more off-target effects in the genome of the plants. Further information can be found in the background paper about the risks associated with CRISPR/Cas.

Working with cell cultures is still a weak point in the production of transgenic and genome-edited plants: it is only possible to develop modified plants from cell cultures with a few species. In addition, it is a lengthy process in which new and unwanted mutations can arise in the genome of the plants.
There are special areas of plants in the shoot and in the root, the so-called meristems. This is where the cells divide and thus lead to the growth and development of the entire plant. Scientists have now introduced certain genes into the seedlings of tobacco plants via Agrobacterium transformation, which regulates the development of such "division zones" (i.e. meristems) resulting in the formation of new seedlings on the seedlings [73]. This system was expanded in such a way that the CRISPR/Cas gene scissors were introduced into the tobacco seedlings together with a specific gRNA. CRISPR/Cas induced gene knockouts in two genes that are important for forming carotenoids. As a result, the genome-edited seedlings could be identified by their white colour, because they lacked the green dye chlorophyll. Some of the seedlings even developed flowers and seeds, which made it possible to pass the genome-edited changes on to the next generation. This process has been used successfully in tobacco, potatoes, tomatoes and grapes [73].

Another method that bypasses working with cell cultures and the regeneration of plants is called *In-planta* particle bombardment and has been described in various wheat lines [74; 75]. Small gold particles are coated with the genetic information of the gene scissors and are shot directly into the division zones (i.e. meristems) of the wheat embryos. In the following generations it can be determined whether the corresponding target gene was changed by the gene scissors. Particle bombardment has, however, been shown to induce restructuring of DNA sequences in the genome and of the transgenic DNA [76; 77] (more information can be found in the background paper about the risks of CRISPR/Cas).

A special CRISPR/Cas application: Genetically modified plant viruses to spread the gene scissors in the field

In this special CRISPR/Cas application, the aim is for genetically modified (GM) plant viruses to be transmitted to plants by various insects, such as aphids or grasshoppers. The infected plants are then modified by the gene scissors to make them more resistant to, e.g. pests or drought. The intention is to quickly adapt crops in the field to changing environmental conditions. The insects are used as a "transport vehicle" for the GM plant viruses. The insects infect the plants with the plant viruses, which transfer the genetic information for the formation of the CRISPR/Cas gene scissors to the plants. The gene scissors are then formed in the plants where they recognize and cut the respective target genes. The genetic modification is induced directly in the field and eludes further control mechanisms. This technology has

already been criticized by scientists, as there is not only a risk of diverse environmental effects, but also of misuse as a biological weapon [78; 79].

What is most commonly done in plants?

A look into market-oriented plant studies is informative and shows recent developments in genome editing technology [43; 80]. The number of studies using CRISPR/Cas in plants has risen sharply since 2014. The classic gene scissors CRISPR/Cas9 are the most commonly used application (in total 174 studies), only one study used the alternative CRISPR/Cas variant CRISPR/Cpf1, two used CRISPR/Cas13a and seven studies used base editing [43]. The other site-directed gene scissors, such as TALENs and zinc finger nucleases, are seldom used. About 80% of all studies transform plant cells with *Agrobacterium tumefaciens* to introduce the DNA for formation of the gene scissors into plant cells, about 10% of the studies use particle bombardment. The gene scissors are introduced as a ready formed enzyme complex in only a small number of cases (approx. 1%).

Traits that were altered in these plant studies include, for example, an increased agronomic value, altered food quality, herbicide tolerance, induction of abiotic or biotic stress tolerance and improvements for industrial purposes. Only one genome-edited soybean, developed by Calyxt, out of 231 market-oriented studies has been grown commercially so far. The soybean oil is sold in America as an edible oil. The soybean has a reduced content of saturated fatty acids and a higher proportion of oleic acid. The alteration was induced with TALEN gene scissors. The first plant modified with CRISPR/Cas, which was released for consumption in Japan in 2021, is a tomato developed by Sanatech. The fruit of these tomatoes contain more GABA, a neurotransmitter, and are supposed to have an antihypertensive effect for the consumer. Although it had not been previously possible to induce mutations in the target area of the target genes, CRISPR/Cas enabled alterations at that specific part of the tomato genome [81].

Around 100 studies used the gene scissors in market-oriented applications to knock out genes in diploid plants [82; 83], the tomato with the increased GABA content is one example [81]. In approximately 50 studies, several genes, for example, genes from a gene family [4; 84], gene copies [85; 86] and/or several alleles in polyploid plants [87; 88] were changed. In around 35 studies, multiplexing was used to alter several genes simultaneously [9; 31]. 92% of the studies used the gene scissors for SDN-1, 3% for SDN-2 and 5% for SDN-3 applications.

Summary

This background paper explains ways in which SDN-1 applications of the CRISPR/Cas gene scissors can be used to induce complex changes in the genome of plants. It also presents some further developments of the classic gene scissors CRISPR/Cas9 (e.g. Base Editing, Prime Editing etc.), gene scissors variants (CRISPR/Cas13, CRISPR/Cpf1) and some new methods to introduce the gene scissors into plant cells.

An analysis of market-oriented studies in plants shows that the classic CRISPR/Cas9 gene scissors are the most frequently used genome editing technique. It is still mainly introduced into the plant cells with the help of an 'old' genetic engineering process, i.e. Agrobacterium transformation. SDN-1 alterations are most commonly induced with gene scissors, which can be used to induce complex changes in the plants genome.

Many of the developments in gene scissor technology described in this background paper, can be assigned to basic research and are not yet ready for routine use in the laboratory. Rather, they are feasibility studies that often lack a comprehensive risk analysis.

It has been shown that apparently small SDN-1 alterations in the genome of plants can induce complex changes. Such plants must undergo comprehensive risk assessment as they can interfere with other biochemical processes in the cell, and induce unintended changes in the genome due to the combination of the gene scissors and 'old' genetic engineering techniques. Both aspects, i.e. the unintentional interference in other biochemical processes and the technical errors attributable to the gene scissors and 'old' genetic engineering, are described in more detail in the background papers on the risks associated with CRISPR/Cas.

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