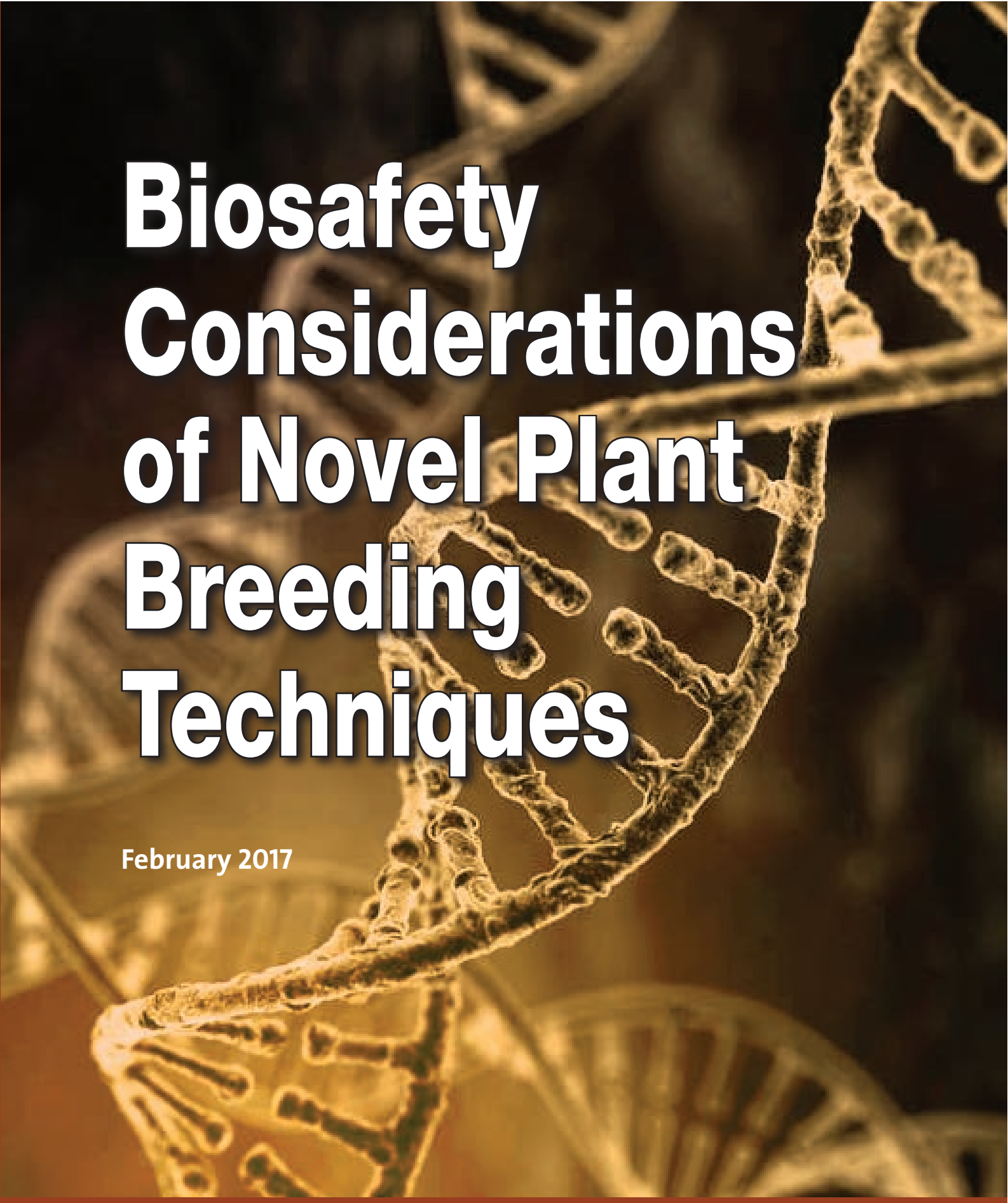

Biosafety Considerations of Novel Plant Breeding Techniques

February 2017

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Contents

On 7 April 2015 the African Centre for Biosafety officially changed its name to the African Centre for Biodiversity (ACB). This name change was agreed to by consultation within the ACB, to reflect the expanded scope of our work over the past few years. All ACB publications prior to this date will remain under our old name of African Centre for Biosafety and should continue to be referenced as such.

We remain committed to dismantling inequalities in the food and agriculture systems in Africa and to our belief in peoples' rights to healthy and culturally appropriate food, produced through ecologically sound and sustainable methods, and to define their own food and agriculture systems

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Acronyms

DNA	deoxyribonucleic acid
GM	genetically modified
GMO	genetically modified organism
RdDM	RNA-dependent DNA methylation
RNA	ribonucleic acid

Introduction

Recent debate surrounding genetically modified organisms have focussed strongly on the development of new techniques for plant breeding. Technical advances for generating novel plant traits have now moved beyond the scope of current regulations for genetically-modified organisms (GMO), raising concern that GMO producers may be able to push such products onto the market without regulatory testing, or labelling (where labelling laws are in place). This would remove the requirement to assess any potential effects on food, feed or environmental safety, and reducing consumer choice for those wishing to avoid such products.

There are various terms and techniques being employed, but as highlighted by the EU commission there are several that have obtained consensus in the discussions: cisgenesis and intragenesis; RNA-mediated DNA methylation, agroinfiltration, grafting, reverse breeding, and genome editing techniques (CRISPR and gene drives, TALENS and oligonucleotide-directed mutagenesis). Gene editing techniques are summarised in a separate report entitled 'Biosafety Risks of Genome Editing Techniques in Plant Breeding'. Also summarised in this report are crops utilising RNA interference. Though not considered a novel plant breeding technique, these crops utilise epigenetic mechanisms and thus introduce novel risks not associated with standard GM crop traits such as current Roundup Ready or Bt insecticidal crops.

Cisgenesis and intragenesis

The new terms of cigenesis and intragenesis refer to the introduction of genetic material from a sexually related donor organism that the crop could theoretically breed with naturally in the wild. The debate surrounding cisgenesis is merely a matter of semantics that is being exploited for improved marketability of GMOs in comparison to products consisting of genetic material derived from a mix of unrelated species, a notion that has been off-putting to many consumers. The concept is however misleading, as exemplified by the description on the website cisgenesis.com of a cisgenic plant as one that contains "no foreign genes", yet they are claiming to be adding a novel trait via the introduction of genetic material not already present in the plant (Cisgenesis.com).

With cisgenesis, all the genetic material inserted is sourced from the one sequence of the donor organism (the gene and all its regulatory elements), while with intragenesis, the inserted DNA is recombinant – i.e. made up of a composite of genetic material from a mixture of genetically related sources, combined together in the laboratory. For details on classic transgenesis and its risks, please refer to 'Biosafety Risks of Genome Editing Techniques in Plant Breeding' (ACB, 2017).

The claim that inserting genetic material from a genetically related species reduces potential toxicity, ignores the fact that the technical processes involved are identical to transgenesis and thus comes with all the attendant risks. This also means that they still fall under current definitions of GMOs in the Cartagena Protocol for Biosafety and the EU directive (2001/18/EC). The EU directive is based wholly on the processes used to alter the plant's genome and covers any organism (except humans) that has been altered in a way that does not occur by natural mating or genetic recombination, implying that it is the process, rather that the products, that pose the risk. Conversely, Canada has taken a "novel traits" approach and regulates new varieties based on the risks posed by its characteristics regardless of the breeding methods used. These are plants that contain a trait which is both new to the Canadian environment and has the potential to affect the specific use and safety of the plant with respect to the environment and human health. These traits can be introduced using biotechnology, mutagenesis, or conventional breeding techniques.

As with transgenesis, foreign genetic material is transformed into plant cells *in vitro*, a process that is in itself, mutagenic. Second, the genetic material is randomly inserted into the host genome having the potential to disrupt gene

expression and thus compositional profiles of the plant. Despite no marker genes (e.g. antibiotic resistance markers) or vector-backbone sequence being theoretically present in a final cisgenic plant, small DNA elements, the so-called T-DNA borders that are co-inserted when *Agrobacterium tumefaciens* is used for transformation, remain in the final product. There are known recombination hotspots (sites that tend to break and join) in the T-DNA borders. This may increase propensity for instability of the cisgene/intragene, and instability of the genome that has been suggested to increase potential for horizontal gene transfer of the transgene.

The concerns regarding unintended changes to the plant genome and composition, therefore remain the same as with transgenesis. Such disruptions have the potential to alter behaviours and performance, higher susceptibility to disease, alter invasiveness/fitness, composition of signalling molecules, nutrients, toxins and allergens. An example of this is the altered levels of proteins and metabolites in the glyphosate-tolerant NK603 maize, which has altered levels of various molecules including potentially toxic polyamines that were 28-fold higher than its non-GM counterpart, resulting from the genetic modification procedure (Mesnage *et al.*, 2016). Introducing the gene via conventional breeding would circumvent these issues and therefore distinguishes cisgenesis from being considered a fast-tracked version of a natural breeding method.

Further, it cannot be assumed that because a gene and its product are non-toxic in their natural context, that when taken out of context and expressed outside of its own genome, it will remain non-toxic. This is exemplified by a study that took a gene from one edible species of pea and transferred it to another genetically related species. Mice who consumed the genetically modified (GM) crop suffered immune responses due to the protein product of the transgene being processed differently in the GM crop (Prescott et al., 2005).
When it comes to detection of the plant, a prerequisite for GMO approval in some regions such as the EU and an important aspect of global trade between regions with different regulatory approval requirements, cisgenic plants can still be detected with standard methods used for the detection of classic GM crops.

Examples of cisgenic crops in development include a blight-resistant potato and apple scab-resistant apples, both developed by collaborative researchers led by Wageningen University.

Techniques that modify the epigenome

Recent crop breeding techniques include modification of the epigenetic status of a plant. For a background on epigenetics see Box 1.

There are various techniques now being introduced to modify the epigenome. These include:

1. RNA-dependent DNA methylation (RdDM)

As described in Box 1, RNA-dependent DNA methylation (RdDM) involves non-coding RNAs that direct the cell to specific DNA sequences for silencing of a gene of interest. The RNA molecules match the DNA sequences, acting as a guide molecule to direct the cellular machinery to the gene of interest. In the case of plant breeding, the guide RNAs can theoretically synthesised in the lab to be of any desired sequence. The aim is for the generation of epigenetic tags that can persist for several generations.

The generation of such a plant involves the identical processes of introducing genetic material, as is involved in transgenesis, and thus comes with all the attendant risks such as the mutagenic process of transformation procedures. The principle is that plants can be backcrossed to remove any transgenic DNA, while the epigenetic changes persist.

Potential negative effects include silencing of the target gene that have knock-on, undesired effects on other pathways, thus affecting

Background to epigenetics

If one can describe the genome as the hardware of a cell, then the epigenome is like the software, working as tags on the DNA that alter the activity of genes, switching them on or off, without altering the DNA sequence itself. It functions as an added layer of additional information on top of the DNA sequence. Such chemical tags can be placed on DNA itself. For example, DNA can be tagged with tiny molecules called methyl groups that stick to some of its bases, called **DNA methylation**. Proteins then specifically seek out and bind to these methylated areas, and shut it down so that the genes in that region are inactivated in that cell. Methyl groups and other tags can also be added to proteins called histones that are closely associated with DNA, and mediate the DNA/histone structure (called chromatin), and therefore how accessible genes are to the cellular machinery required to turn them on or off. These are called **histone modifications**.

In some cases, certain types of non-protein-coding RNA molecules can mediate certain epigenetic processes. RNA is a nucleic acid like DNA, of which there are various species. Classically, they were known as the intermediate molecule in the expression of DNA to proteins, termed messenger RNA. Now many classes of RNAs have been identified that do not encode proteins (non-coding RNAs), but instead are involved in many regulatory functions in the cell.

Certain classes of non-coding RNAs (ncRNA) can mediate the methylation of DNA – **RNA-dependent DNA methylation** (RdDM). They can also act via **RNA interference** – the binding to complimentary messenger RNAs that are destined to be translated into proteins (see Figure 1).

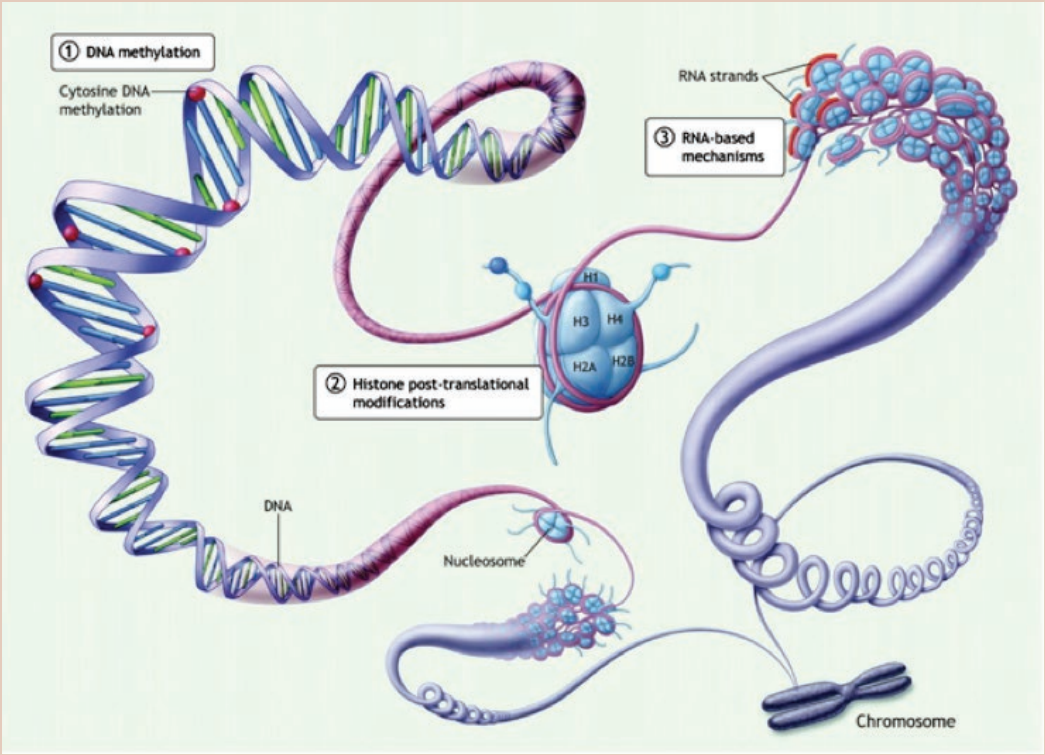

Figure 1: Introduction to epigenetic mechanisms

Three main mechanisms for epigenetic regulation of gene expression: 1) DNA methylation, where chemical tags called methyl groups are added to gene regulatory regions called promoters that switch gene expression on or off; 2) Histone modifications of histone proteins. DNA is wrapped around histone proteins. Adding chemical tags to histone proteins, alters the structure of the DNA/histones, together called chromatin. This alters the accessibility of the DNA to the cellular machinery that regulates gene expression, regulating gene expression; 3) RNA-based mechanisms where non-coding RNAs direct gene silencing complexes to gene promoters. Non-coding RNAs can also bind and repress messenger RNAs, blocking their translation into proteins (not illustrated here, shown in figure 1) (adapted from Lee et al., *2014).*

the behaviour or composition of the plant. Pleiotropic effects on other pathways caused by the expression of the target gene is possible.

Employing RNA species to target a desired gene is understood to be associated with off-target activity, potentially binding to similar sequences elsewhere in the genome. This could have negative effects such as the production and accumulation of toxins and allergens, disease susceptibility, or lowered nutrient content.

There does yet not appear to be any crops near commercialisation with this novel technique. The consequences for such a technology are not yet tested or understood. Crops generated with such as technology should not bypass regulatory testing.

An example of a crop developed with RdDM, is a maize plant with male sterility, developed by Pioneer HiBred, though whether it will be commercialised is not yet known (Cigan *et al*., 2005).

2. CRISPR/Cas9-based acetyltransferases

A novel technique only developed in the last couple of years, employs the CRISPR-Cas9 system, typically used for gene editing techniques (see Biosafety Risks of Genome Editing Techniques in Plant Breeding', (ACB 2017)). Though not discussed in recent debates surrounding novel plant breeding techniques, with such rapid evolution of CRISPR-based technologies, it is now gaining attention as a potential plant breeding tool.

CRISPR-based systems involve the employment of guide RNAs that direct DNA cutting enzymes (nucleases) to specific DNA sequences for gene editing. In nature, bacteria use it to target viral pathogens. The CRISPR/Cas9-based acetyltransferase system does not however cut and edit DNA. Instead, the Cas9 cutting activity is inactivated, and instead is engineered to be fused to a protein that adds epigenetic tags that activate the expression of genes – termed acetyltransferases. Such acetyltransferases add an acetyl group to histone proteins that then affect gene expression through altering how easily the genes are accessible to the gene expression machinery of the cell. This is a form of histone modification as described in Box 1.

The technical aspects and thus risks parallel those associated with RdDM.

With regards to detection, a typical enforcement laboratory will not be able to differentiate between naturally induced epigenetic patterns and those induced by the deliberate use of RdDM or CRISPR-Cas9 acetytransferases, so products from this technique cannot be routinely detected or identified.

3. RNA interference

Other GM crops that take advantage of epigenetic modifications in plants are those utilising RNA interference (RNAi). This technique is not one of the six novel breeding techniques under discussion for GM legislation, as such crops are generated via classic transgenic approaches with a permanent introduction of genetic material. Nonetheless, these crops deserve specific attention due to the alternate risks that come with employing epigenetic mechanisms in the plant. Further, several crops utilising RNA interference are now commercialised, including the non-browning Arctic® apple commercialised in Canada by Okanagan Specialty Fruits, as well as several potatoes by J.R. Simplot that confer blight-resistance, reduced spot bruising and reduced acrylamide now approved for planting in the US.

In order to generated crops that utilise RNAi, classic transgenic techniques are used to insert genetic material encoding for the expression of a non-coding RNA designed to target a particular messenger RNA that is meant to be silenced such that no protein is synthesised (see Figure 1). Silencing a gene of interest may have unintended effects such as the interruption of other pathways in the cell, and thus alter the behaviour and/or composition of the plant.

A specific risk associated with RNAi over classical transgenic crops is the potential for off-target effects of the non-coding RNA (ncRNA) introduced into the GMO. The ncRNA may well bind to other messenger RNAs that encode for other proteins not intended for silencing, thus disturbing the gene expression and potentially the composition of the plant. Negative effects such as the production and accumulation of toxins and allergens, disease susceptibility, lowered nutrient content could occur.

It is also now shown in multiple studies, that ncRNAs survive mammalian digestion, even in humans, and go on to modify gene expression in the organism. There is therefore potential for the ncRNAs from consumed GM crops, to be passed into the bloodstream and interfere with our genes or that of other organisms. It is becoming increasingly clear that many ncRNAs are used as communication molecules that can be active between organisms of different species and even kingdoms. Disrupting such intricate interactions could have unpredictable and wide-ranging consequences.

Agroinfiltration

This involves the genetic modification of plants to include a gene of interest, to infect/transform parts of a plant in a temporary or spatially restricted manner (for example, just leaf tissue), for a maximum of one generation. The technique involves the use of transformation procedures standard to GM, but the modifications are theoretically limited to one generation only, while the progeny would not be used. It employs the same virus as used in classical GM, where Agrobacterium tumefaciens is used as a vector for infecting plant cells. Plant tissues are infiltrated via

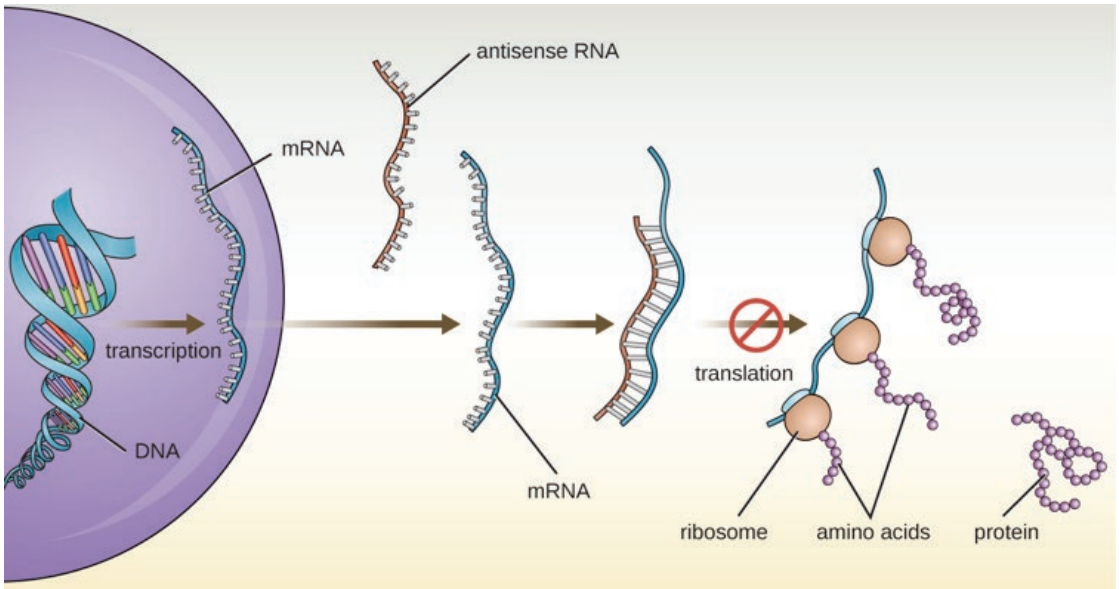

Figure 1. RNA interference mediates expression of genes by blocking the synthesis of proteins

Non-coding RNA (ncRNA) molecules play a major role in RNA interference (RNAi), a natural regulatory mechanism by which messenger RNA molecules are prevented from guiding the synthesis of proteins, often used to target viruses that have infected the cell. Taken from (Bio.libretexts.org.)

It works to target specific genes, which results from the base pairing of ncRNA molecules to mRNA molecules that have a complementary sequence, preventing protein synthesis. These ncRNAs are bound to an RNA-induced silencing complex (RISC) that binds to messenger RNA. If the ncRNA is completely complementary to the target, then the messenger RNA will be cleaved. If there is incomplete complementarity the RISK complex binds to the messenger RNA, blocking protein synthesis. RNA interference can thus be described as a form of gene silencing.

injection or, by dipping into a liquid suspension of the agrobacterium that is also carrying the gene of interest. In the case of agro-infiltration 'sensu stricto', the intention is to keep the gene expression localised, while with agro-infection, the intention is for the transgene to spread throughout the plant.

Applications of such products are likely more research-based, or for the production of high levels of proteins, for example in the production of pharmaceutical drugs, and also for testing for novel traits in plants at the research stage.

Potential spread of the transgene is a concern, even for agro-infiltration 'sensu scripto' based on the properties of the types of vectors used to introduce the DNA into the cell. It is also theoretically possible, as with all the transformation procedures, that some of the DNA may integrate into the plant genome, and thus can then be inherited. This can be associated with disrupting the genome and thus potentially altering gene activity and composition of the plant, that comes with all the attendant risks of standard GM, as previously described.

Grafting

Grafting is way to combine the desired traits of two different organisms together e.g. roots that are disease resistant, with the graft with desired fruit flavour. In such cases, the fruit product would not on the whole, be GM, but the plant itself would be. However, there is exchange of molecules across the graft border that will likely include gene products such as RNAs and proteins such as signalling molecules and hormones, that may spread across the whole plant. Compounds and metabolites may well be altered and present in the fruit/product that may lead to increased presence of allergens and toxins, and lowered nutrient content.

Risks associated with such crops include the environmental exposure to GM material. The GM rootstock has all the typical risks associated with it, including potential genomic and compositional alterations that may have environmental consequences on the soil and wider ecosystem. Under current EU GMO regulations for cultivation, the GM/non-GM chimeric plant would be considered a GMO and risk assessed.

Though there are no known crops developed with this technique that are aimed for commercialisation, crops have been published in the literature that include insect resistant tomatoes, virus resistant cucumbers and peas, microbe resistant grapevines and potatoes (see Lusser *et al*., 2012).

Reverse breeding

The aim of reverse breeding is to reconstitute parental lines that are uniform and pure (homozygous) from hybrid progeny that are heterozygous. This technique therefore recreates parental lines of hybrid varieties that may no longer exist, or are no longer available. It involves classical transgenesis in the intermediate stages of the process, used to block meiosis (cell division in reproductive cells, where genetic exchange occurs to generate diversity in progeny) in the hybrid seed. Non-coding RNAs are introduced into the that utilise RNA interference to block meiosis. Using tissue culture techniques, individual gametes (reproductive cells) are generated with two sets of identical chromosomes are selected. The plants are later bred to deselect the transgene.

Utilising both transgenesis and tissue culture techniques, this technique therefore comes with the associated risks, as outlined above.

RNA interference, as with other techniques relying on the correct targeting of the non-coding RNA to its gene or RNA of interest for silencing. Off-target activity is expected, which could result in hereditary epigenetic silencing of genes for multiple generations. There is also the possibility that portions of the transgene may integrate elsewhere in the genome, even after deselecting for the transgene, can remain.

There does not appear to be any known crops currently approaching commercialisation with this technique.

Conclusions

GM crops thus far have not reduced issues of food security and sovereignty, hunger or farmer livelihoods. They have served to monopolise and privatise food systems and have introduced biosafety risks associated with genetic modification of organisms and the promotion of ever increasing amounts of chemical inputs. The novel plant breeding techniques covered in this report will only serve to exacerbate such issues. Further, they are incompletely understood, involving known and unpredictable risks associated with classic transgenesis.

Instead of moving away from the failures of GMOs, these new techniques are being pushed to escape GMO legislation. This is of great biosafey concern as such products are associated with similar risks to GMOs and in some cases, involve additional risks. Appropriate and thorough risk assessment protocols and detection methods that include the latest in molecular profiling "omics" techniques that can analyse global patterns of gene, RNA, protein and metabolite expression should be applied to all GMOs, and especially those with the potential for off-target effects such as employing epigenetic mechanisms. In cases where foreign genetic material is supposedly removed, assessments should be performed to ensure such genetic material has indeed been absent. It is recommendable that such legislation be put in place prior to the marketisation of any GMOs generated with novel techniques.

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