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Transferring the laboratory to the wild: An emerging era of environmental genetic engineering

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Introduction

The last 30 years of commercialisation of genetically modified organisms (GMOs) have thus far been restricted to a limited number of species, predominantly maize and soy. Developers are reacting to plateauing global adoption rates of these commercialised first-generation genetically engineered (GE) crops, which are plagued by declining trait efficacy and sustained market rejection, by reinvigorating efforts to usher in new crops and organisms.

New genetic engineering techniques such as genome editing and new delivery techniques have facilitated an emerging trend to genetically engineer organisms in the wild, moving the engineering process to agroecosystems and beyond, essentially converting the environment into the laboratory. Previous techniques originally developed as research tools in contained-use settings, or for gene therapy in clinical settings, may be released into the environment to genetically engineer agricultural and wild organisms unchecked. These developments expand the range of species that can be engineered, increasing scalability and speeding up the development process, and can potentially circumvent regulations, premised on false claims of increased safety and precision of genome editing techniques (see Box 1).

Such 'environmental genetic engineering' (Heinemann, 2019) raises heightened concerns with regard to controllability, the risk of spread and exposure, and unintended adverse effects that cannot be eliminated inside a laboratory prior to release. Genetic engineering in the wild also raises unprecedented regulatory challenges, removing our ability to risk-assess these engineered organisms and products before they are introduced into the environment.

This briefing presents examples of research and applications in the field of environmental genetic engineering, including the development of gene drive organisms (GDOs), horizontal environmental genetic alteration agents (HEGAAs) that deliver viruses carrying genome editing machinery

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Box 1: Releasing genetic engineering laboratory process into the wild relies on false premises of precision and safety

The overarching scientific justification for the use of genome editing technologies, such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) systems, is the false premise that these systems are controllable, precise and free of unintended effects, and thus will perform *just* the expected modification in the field. However, even if the techniques could be guided only to the intended sequence of DNA in a genome, there can be millions of different species in a treated environment that have the same sequence in their genomes. Each of these 'non-target' species could be genetically modified at the same time.

Moreover, genome editing techniques can induce unintended effects (see Agapito-Tenfen et al., 2018), including off-target modifications of unintended regions of the genome, e.g., mutations, complex rearrangements, translocations, insertions and deletions (e.g., Kosicki et al., 2018).

Crucially, unintended on-target effects are also associated with any genome editing nucleases that induce double-stranded DNA breaks (e.g., CRISPR, double nickases, transcription activator-like effector nucleases (TALENs) and zinc finger nucleases). These unintended effects include mutations, chromosomal recombination events (e.g., Bruner et al., 2019, documented in insects), high-frequency production of aberrant protein products (e.g., Tuladhar et al., 2019), and unintentional incorporation of foreign genetic material, creating unintended transgenic organisms (e.g., Ono et al., 2015). A recent study documented the surprising incorporation of foreign DNA derived from common laboratory reagents that edited mouse cells were exposed to, including bacterial DNA (derived from the standard use of bacteria as a production source of the genetic engineering machinery), as well as goat and bovine DNA (derived from the animal serum present in the culture medium used to grow the mouse cells) (Ono et al., 2019). Also incorporated were goat and bovine retrotransposons (jumping genes), exposing the potential for genome editing to facilitate infectious transfer of unwanted pathogens, including viruses.

These studies expose a fundamental flaw of genome editing: no matter how much off-target effects are reduced, or even eliminated through increased 'precision' and 'specificity', unintended effects at the target site cannot be controlled, and unwanted DNA is regularly incorporated as part of the cell's attempts to repair the double-stranded DNA breaks induced by genome editing.

Additional unintended effects independent of 'specificity' and 'precision' are also documented, such as the observation that CRISPR-treated human cells are associated with mutations in the tumour-suppressing protein p53 (Ihry et al., 2018) or loss of p53 function (Haapaniemi et al., 2018). These results suggest that genome editing is associated with disruption of DNA repair mechanisms.

Repeated edits by genome editing are also possible, in contrast to older techniques such as radiation where repeated exposure to a mutagen would kill organisms, thus increasing the potential depth of interventions that are possible with genome editing.

Such findings are precisely the opposite of industry claims that genome editing is mere 'tweaking' on par with natural genetic variation and, further, does not constitute genetic modification as no foreign sequences are inserted. How such effects will play out when genome editing is deployed directly in the field, without the ability to assess for unintended on- and off-target effects, is of serious concern.

directly to crop fields, the delivery of genome editing machinery to crops via pollen-mediated transfer, the application of RNA interference products directly to crops and farmed animals, and developments in 'penetration' techniques to deliver genetic engineering tools to organisms.

Horizontal environmental genetic alteration agents

One of the most aggressive environmental engineering applications being developed is the use of viruses to deliver genome editing machinery directly to organisms, termed 'horizontal environmental genetic alteration agents' (HEGAAs).

Arguably the most controversial of HEGAA projects is the Insect Allies programme, funded by the United States military research arm,the Defense Advanced Research Projects Agency (DARPA). Insect Allies plans to use insects as vectors to deliver GE viruses directly to crop fields to modify those crops, potentially by delivering genome editing machinery to the crop. DARPA's Insect Allies website describes the project as developing "countermeasures against potential natural and engineered threats to the food supply with the goals of preserving the U.S. crop system", with cited examples of drought, flooding, pathogens and frost. The DARPA work plan published in 2016, however, describes aims that go beyond modifying the US crop system, to include crops of "global agricultural importance (including rice, cassava, cowpea, tree fruits, etc.)" (DARPA, 2016). DARPA states that such a technology provides an alternative to pesticide application, slash and burn, selective breeding and quarantine that would be employed for rapidly emerging threats.

The work plan describes three areas that will be addressed concurrently (DARPA, 2016). First, the genetic engineering of plant viruses; second, the viral delivery by insect vectors; and third, the rapid transformation of a mature plant. Molecular control mechanisms such as conditional lethality systems, e.g., antibiotic-dependent survival or light and/or temperature sensitivity, are also being investigated. Without such kill switches, there is a risk of the virus spreading and mutating, as is the case with viral pathogens, yet it remains unclear how such measures will be proven to work at expected scales of release. There appear to be no foolproof kill-switch mechanisms, and they may well be susceptible to being reversed or inactivated. Such added complexities may further complicate any unintended effects.

The HEGAA approach circumvents the need for industrial infrastructure ordinarily used to generate GE plants, such as lengthy, costly procedures of tissue culturing and regeneration of modified plant tissue, purportedly allowing for rapid emergency responses *en masse* to stressors. However, scientific experts have published concerns on the limited scope provided by HEGAAs to enhance US agriculture (Reeves et al., 2018). Instead, they counter that Insect Allies provides a realistic opportunity for dual-use applications developing targetable bioweapons. Genetic engineering tools such as genome editing have been demonstrated to be far more efficient at destroying genes than inserting or editing genes (Mao et al., 2008). As such, it is much easier to kill or sterilise a plant (or other agriculturally relevant organism, such as earthworms, fungi or livestock) by destroying single genes, than it is to alter a complex trait such as drought or flood tolerance by inserting or 'editing' the many genes involved.

The use of insect vectors is justified by DARPA to get around limitations in current technologies for mass delivery of CRISPR machinery to crop fields, such as sprays, which it claims are constrained by the need for water and irrigation infrastructure for sustained delivery. However, using insects removes all predictability for controlling dispersal in agricultural settings, where a variety of farming practices tend to coexist. Omitted from the DARPA work plan (DARPA, 2016) is the most likely scientifically plausible explanation for choosing insects over sprays, which is that viruses are usually unable to penetrate the tough plant cell walls unless there is a wound via which they can enter (though this problem may be overcome; see Heinemann and Walker, 2019). Insects such as those proposed for the project, including aphids, whiteflies and leafhoppers, cause such wounds when feeding, which is how viruses typically enter plants. However, the global infrastructure and the rapid response to evolving threats that would be required (e.g., rapid infection of insects with the correct viruses, global insectaries for mass rearing etc.) make this technology impractical for its proposed applications.

Recent DARPA statements claim that the project aims to only induce 'transient expression' that does not alter chromosomes or the germline (The Scientist, 2018). However, this contradicts earlier statements, e.g., that "The project relies

on a fairly new technology called CRISPR-Cas9 that can modify a DNA sequence in plants, animals and humans" (The Lantern, 2017). Transient modification of crops with viruses has already been developed, leaving question marks regarding DARPA's interests in pouring at least US$27 million as of 2016 into already existing technologies. Indeed, transient expression systems with viruses have already been trialled (USDA, 2017).

Furthermore, omitted from the DARPA work plan is the explicit prohibition of inducing heritable changes to either target or non-target plants. While it was previously assumed that viruses could not infect adult undifferentiated plant tissue that can develop into germline cells, recently published studies have shown that this is not always the case. Some viruses have indeed been reported to access the germline, a mechanism that is actively being explored as a means for rapid genetic modification of plant species. A recent study demonstrated access and modification of meristem tissue (Ali et al., 2015), showing that the tobacco rattle virus "can serve as a vehicle to deliver genome engineering reagents to all plant parts, including meristems, [and] provides a general method for easily recovering seeds with the desired modifications, obviating the need for transformation and/or tissue culture". Moreover, it is not necessary for viruses to infect the seed germplasm to pass down genetic modifications; instead, they can have transitory access to tissues that lead to seed development. Such findings contradict 'transient expression' claims by DARPA scientists, including that "Most plant viruses are not seed-transmissible, which means if a plant is infected by the virus, no matter what the virus does, even if [it] genetically modifies plants stably using CRISPR-Cas9, the virus does not get to the seeds" (The Scientist, 2018).

This project also raises clear concerns over the controllability, persistence and temporal-spatial spread of insect vectors, their associated viruses and the resultant genetic modifications in the open environment. Restricting virus exposure to target plants is extremely difficult, considering that some of the viruses being investigated, such as the tobacco rattle virus, can infect 400 species from 50 families. As described in Box 1, genome editing machinery is associated with a myriad of unintended effects, none of which can be pre-assessed if HEGAAs are released into the open environment. Further, such viruses will be very difficult, if not impossible, to detect and trace.

Gene drive organisms

Gene drive technologies are a form of genetic engineering designed to skew the natural patterns of inheritance such that most, if not all, offspring of a target organism inherit a particular GE trait, 'driving' it through a population. This technology has received a lot of attention because of its unprecedented biosafety, regulatory, societal and ethical implications (e.g., Courtier-Orgogozo et al., 2017; Simon et al., 2018; ACB, 2018; ETC Group, 2018; ETC Group and Terre à Vie, 2018; CSS, ENSSER and VDW, 2019; Meghani, 2019). It was also a major focus of the recent negotiations at the UN Convention on Biological Diversity, where strict conditions were placed on any environmental release, in line with the precautionary approach and the requirement for full, prior and informed consent from affected communities.

Gene drive organisms (GDOs) are being developed to disrupt genes that reduce 'fitness', possibly even essential for survival, to bias the sex ratio of offspring such that the overall population is reduced or eliminated, or to reduce disease transmission in disease vectors. Proof-of-concept GDOs have already been developed in numerous organisms, including mosquitoes and mammals, with the first GDO demonstrated in 2015 in yeast (DiCarlo et al., 2015), flies (Gantz and Bier, 2015), and later in mosquitoes (Gantz et al., 2015; Hammond et al., 2016; Kyrou et al., 2018) and mice (with only partial efficacy) (Grunwald et al., 2019). The most advanced gene drive project, and the one considered to be the most likely first application, is that of the Target Malaria consortium, led by Imperial College, London, which also operates in Burkina Faso, Mali and Uganda (Target Malaria, 2018).

The 'driving' of a genetic trait throughout a population – the rapid spread of a modified gene and its associated trait – is achieved by inserting transgenes into an organism that code for the genetic engineering machinery. Whereas before, GMOs were genetically engineered in the laboratory and then released into the environment, GDOs are engineered in the laboratory to carry the genetic engineering machinery (e.g., CRISPR/Cas9) so that it is then passed down to future generations, carrying out genetic engineering at each generation for perpetuity. As such, GDOs essentially convert the field into the laboratory, performing ecosystem-wide genetic engineering. This raises unprecedented biosafety challenges for predicting and controlling unintended effects.

While current risk assessment procedures are largely focused on the issue of controlling GMO spread, GDOs are designed for exactly that purpose. Further, for GE crops, unintended effects on new crop varieties can be assessed before release, and the crops are planted for single seasons only. As such, first-generation GMOs produced in the laboratory under controlled conditions are not an adequate example for predicting potential hazards that may emerge with GDOs in future generations.

Potential hazards and uncertainties include, for example, how GDOs will behave in genetically diverse, wild populations with potential interspecies breeding, with the added complexities of unintended molecular effects, e.g., heritable off-target effects (Hayes et al., 2018) (see Box 1), which vary with differing genetic backgrounds (Canver et al., 2018). Other concerns include unintended on-target effects such as incorporation of foreign genetic material (see Box 1); ride along of additional sequences (Courtier-Orgogozo et al., 2017); toxicity of the genome editing machinery; and resistance development. Such complex processes that occur continuously over time and space cannot be pre-assessed in the laboratory, and may impact issues such as outcrossing potential; genome stability; transgene stability/efficacy; or wider health impacts such as toxicity of biting GE female mosquitoes, and epidemiological interruptions of disease parameters such as acquired immunity; and ecological impacts such as ecosystem function and species interactions, and niche replacement (including with disease-carrying species). All these potential impacts will not have been considered to the same extent as with current GMOs to date. The ecological consequences of eradicating entire populations are also very difficult to predict and potentially harder to reverse, with potential severe ecosystem effects (Hochkirch et al., 2018).

The issue of controllability is another fundamental concern that has been raised by gene drive developers and reiterated by biosafety experts, who warn that GDOs are likely to be "highly invasive" (Esvelt and Gemmell, 2017; Noble et al., 2018; Simon et al., 2018). This is the case with 'global' gene drives, which can spread to all populations that are connected by gene flow, potentially across national borders. No risk assessments – from those that include contained use, to field trials – can ever capture unintended adverse effects that could arise from an open release. Nevertheless, there is active research being conducted to assess the potential release of gene drive mosquitoes in the Ssese Islands, Uganda for field trial purposes (e.g., Lukindu et al., 2018).

Molecular containment measures have been suggested as methods to control and limit gene drive spread either spatially, temporally or by limiting activity to genetically distinct local populations. However, such strategies are largely theoretical, and suffer from many of the limitations and uncertainties of the gene drives they are designed to undo, e.g., potential for resistance development, incomplete efficacy, inactivation, reversion back to global gene drives, and off-target effects. They also leave behind genetic engineering scars, with resultant organisms still being genetically modified. As it stands, the ability to contain and control gene drives is not yet possible, necessitating strict international regulations to be put in place even for contained-use settings (Lim and Lim, 2019).

Pollen-mediated delivery directly to crop fields

Syngenta has developed a new 'Hi-Edit' technology that delivers CRISPR genome editing machinery directly to crops via pollen. This allows for the direct genetic modification of crops pollinated by Hi-Edit crops. By combining pollen-mediated delivery with haploid induction, the developers claim to produce elite inbred crop lines that are genome-edited and free of transgenes, all within two breeding generations. This technology has also since been reproduced (Wang et al., 2019). Syngenta's work was demonstrated in maize, pollinating both maize and wheat crops. Wheat is a species that is generally recalcitrant to tissue culturing procedures ordinarily required for genetic engineering including by genome editing. Because wheat is able to hybridise with maize, pollen from maize provides a means by which wheat can be CRISPR-edited. The company claims to be developing similar methods for other crop species such as cabbage, broccoli, cauliflower, kale, tomatoes and soybeans (Syngenta, 2019).

This technology is attractive to developers for numerous reasons. First, it broadens the number of crop varieties amenable to genome editing, and also avoids lengthy and costly genetic engineering processes in those already amenable. Second, the combined use of haploid induction, a technique for rapidly generating hybrid lines, removes the need for the 6-10-generation breeding process usually required to introgress an engineered

trait into a desired crop variety. Third, due to haploid induction, the resultant edited plants will not inherit the transgenes encoding the editing machinery, only the edited trait, helping developers argue that these products do not fall within the scope of biosafety regulation. Though this technology, unlike others such as HEGAAs and gene drives, will likely be restricted to breeding programmes and not large-scale environmental applications, it still raises concerns about potential pollen escape and subsequent modification of conventional crop varieties, along with the unintended effects of these techniques (see Box 1).

RNA interference applied products

An emerging biotechnology being explored for pest control and other food production or preservation applications is the activation of a naturally occurring regulatory system that exists in many eukaryotic organisms including animals, called RNA interference (RNAi). Similar systems also are found in bacteria (Shabalina and Koonin, 2008). RNAi functions as a form of gene silencing via multiple pathways (Heinemann et al., 2013). The gene is 'silenced' because a protein is no longer produced from it.

The discovery that double-stranded RNA (dsRNA) molecules, which activate the RNAi pathway, can be moved across kingdoms, across cellular boundaries between hosts and interacting pathogens and pests, and that organisms can take up dsRNAs from the environment, led to research and development for utilising the RNAi pathway for pest control and crop trait modification (Lungren and Duan, 2013; NASEM, 2016).

While GE crops that express dsRNA molecules have already been commercialised, novel applications using synthetic dsRNAs are being developed. Some products in the pipeline include Bayer's (previously Monsanto) various BioDirect products, designed to target glyphosate resistance, canola flea beetle,the Colorado potato beetle and the honeybee varroa mite. Sprays targeting plant fungal pathogens are being developed by BASF (Koch et al., 2016). A spray is also being developed against the diamondback moth (TechAccel, 2017). Products are in development by Viaqua Therapeutics to incorporate dsRNA molecules into animal feed in attempts to suppress disease and infection in farmed animals such as shellfish.

RNAi product development poses challenges to current risk assessment protocols. For example, New Zealand's Environmental Protection Authority has recently placed such products outside the scope of its legislation, declaring that organisms exposed to dsRNAs are not GMOs because it claimed that the effects of treatment are not heritable. However, externally applied RNAi products can arguably be described as an 'environmental' genetic engineering technique, thereby creating GMOs that are covered by existing biosafety regulations, and can further result in heritable effects (Heinemann, 2019). Treatments cause heritable effects via amplification of dsRNA molecules; the production of secondary dsRNAs; and DNA deletions, chromosomal rearrangements and modification of individual nucleotides, as well as epigenetic modifications such as DNA methylation. Such epigenetic changes have been observed to be inherited over a number of generations in certain organisms. Even the developers of this technology claim that its effects are heritable (Heinemann, 2019).

Biosafety concerns are also raised by off-target activity of dsRNAs, a well-established unintended effect where dsRNAs regulate the activity of unintended genes or genes in non-target organisms. Off-target activity has been documented with GE crops expressing dsRNAs (e.g. Baum et al., 2007), and is currently not predictable in non-target organisms (Hanning et al., 2013). Further, it may not be restricted to closely related species that share similarity to the target gene (Mogren and Lundgren, 2017). Secondary dsRNA production further complicates the issue, considering that this process generates new dsRNAs that may have sequence matches to many other genes (Heinemann et al., 2013). Current understanding of RNAi pathways and its biochemistry is also largely restricted to model organisms, leaving knowledge gaps about how dsRNAs could affect non-target organisms. Controlling exposure to non-target organisms is hampered by plans for mass spraying, proposals to use viruses and bacteria to express dsRNAs, the potential for dsRNAs to be taken up by plants via water, and efforts to increase stability and persistence of dsRNAs (Mitter et al., 2017).

Development of externally applied 'penetration' technologies to deliver genome editing and gene silencing genetic engineering machinery

As reviewed by Heinemann and Walker (2019), the development of 'penetration' technology

facilitating the delivery of dsRNA, DNA or protein forms of genetic engineering machinery is also promoting environmental genetic engineering technologies. These new chemical formulations cause the direct uptake of the nuclease proteins (such as Cas9) and nucleic acids (such as CRISPR gRNA) by bacteria, fungi, plants and animals. The formulations are inexpensive and made from readily accessed commercial ingredients such as cationic lipids, or the use of sugar-based buffers for genome editing protein/RNA complexes. Additional techniques to penetrate cells and tissues, such as physical damage by, for example, sandpaper, insect wounds and light energy, have also increased efficacy of RNAi responses in plants caused by externally applied dsRNAs. Nanoparticles are also being investigated for transporting proteins and nucleic acids into organisms (Cunningham et al., 2018), and have been shown to increase persistence of dsRNAs on applied plants.

Penetration technologies can be used to transfer genome editing and silencing proteins and nucleic acids directly to harvested foods, e.g., to preserve shelf-life or to alter plant flower traits. In addition to increasing scalability and exposure to genetic engineering processes, additional biosafety concerns surrounding such penetrating techniques include alternative exposure pathways via contact or inhalation. They may also facilitate evasion of biosafety legislation when applied as topical products. The ease with which some of these techniques can be developed also increases risks of dual-use applications, or do-it-yourself formulations that may evade any assessment at all.

Conclusions

The emerging era of environmental genetic engineering is converting ecosystems to laboratories, with the potential to introduce adverse and irreversible effects. Nevertheless, it is a very active field of research, which even appears to go beyond targeting non-human organisms. For example, DARPA has recently launched its PREPARE programme for mass 'epigenetic' programming of people (DARPA, 2018; The Bulletin, 2018).

With the accumulation of scientific evidence that genome editing and other emerging genetic engineering technologies have a variety of unintended effects, careful consideration by governments and funders is vital to uphold precautionary measures to prevent irreversible risks to open environments. This is of utmost concern, considering that these techniques are broadening the scale and range of potentially exposed target and non-target organisms. Regulations need to keep pace with biosafety risks and must be fit for purpose, necessitating serious consideration of such new technologies at international and national levels. This should be facilitated by regular horizon-scanning, monitoring and assessment processes on such genetic engineering advances.

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