

## Critical Review

# Insect allies—Assessment of a viral approach to plant genome editing

Kevin Pfeifer,<sup>1,†</sup> Johannes L. Frieß,<sup>2,†</sup> and Bernd Giese<sup>2</sup>

<sup>1</sup>Institute of Synthetic Bioarchitectures, University of Natural Resources and Life Sciences, Vienna, Austria

<sup>2</sup>Institute of Safety and Risk Sciences (ISR), University of Natural Resources and Life Sciences, Vienna, Austria

<sup>†</sup>These authors contributed equally

## Abstract

The Insect Allies program of the Defense Advanced Research Projects Agency has already sparked scientific debate concerning technology assessment-related issues, among which the most prevalent is that of dual use. Apart from the issues concerning peaceful applications, the technology also provides the blueprint for a potential bioweapon. However, the combination of a virus-induced genetic modification of crop plants in the field using genetically modified insect vectors poses a greater risk than the hitherto existing use of genetically modified organisms. The technology's great depth of intervention allows a number of sources for hazard and a tendency towards high exposure, but it is also encumbered with notable deficits in knowledge. These issues call for a thorough technology assessment. This article aims to provide an initial characterization from a technology assessment perspective, focusing on potential sources of risk for this novel invasive environmental biotechnology at an early stage of research and development. *Integr Environ Assess Manag* 2022;18: 1488–1499. © 2022 The Authors. *Integrated Environmental Assessment and Management* published by Wiley Periodicals LLC on behalf of Society of Environmental Toxicology & Chemistry (SETAC).

**KEYWORDS:** Agriculture, CRISPR, Gene drive, Horizontal Environmental Genetic Alteration Agents (HEGAA), Technology assessment

## INTRODUCTION

In late 2016, the Defense Advanced Research Projects Agency (DARPA), an agency of the US Department of Defense responsible for the support of interventive technologies for national security, published a funding call for applications to a new project named Insect Allies. Recently, DARPA had identified crop health and yield as a matter of national security. With this funding opportunity, DARPA was looking for applicants to develop a system that could “enable expression of crop traits within a single growing season at scale by delivering a modified virus to target plants by a mobile insect vector” (DARPA, 2016). According to Reeves et al. (2018), the Insect Allies Project, also known as Horizontal Environmental Genetic Alteration Agents (HEGAA), aims to develop genetically modified (GM) insects as vectors for GM plant viruses to infect crop plants. The GM virus is planned to contain sequences that confer traits to the crops

necessary to withstand “naturally occurring threats to the crop system, including pathogens, drought, flooding, and frost, but especially by threats introduced by state or non-state actors” (from the DARPA homepage; DARPA, 2016). The expression of the conferred trait is planned to be transient and non-inheritable. The GM insects are planned to be sterile and carry a dead switch to ensure confineability. From the Broad Agency Announcement (Biological Technologies Office, 2016), it becomes apparent that it is envisioned to use multiple viruses to confer at least three transgenic sequences to achieve a gain of function in the targeted crop.

Although neither the idea of using plant viruses as genetic tools (for a review, see Mushegian & Shepherd, 1995; Venkataraman & Hefferon, 2021), nor the use of insects to infect plants with viruses (Abrahamian et al., 2020; Ziegler et al., 2000), nor even the genetic modification of mature plants (e.g., vacuum-assisted agroinfiltration; Bechtold & Bouchez, 1995; Gleba et al., 2005) were new, the proposal to use these techniques, which are limited to the laboratory, to genetically manipulate plants in the wild was a novelty and sparked debate in the scientific community (Kupferschmidt, 2018; Partan & Goldstone, 2018; Reeves et al., 2018). Until now, if GMOs are to be released as part of an application, they are either planted in a field, kept in a tank (US Food & Drug Administration, 2020) or, if not strictly confined to a

**Correspondence** Bernd Giese, Institute of Safety and Risk Sciences (ISR), University of Natural Resources and Life Sciences, Vienna 1190, Austria.

Email: bernd.giese@boku.ac.at

Published 12 January 2022 on [wileyonlinelibrary.com/journal/ieam](https://onlinelibrary.com/journal/ieam).

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

specific area, at least limited in their potential to become persistent in a wild population (e.g., female specific release of insects carrying a dominant lethal allele [fsRIDL] with its only temporary persistence of transgenes in the target population; cf Alphey, 2014). With gene drives, certain types of transmissible vaccines, and now with HEGAA, there are plans to push these boundaries (Giese, 2021). For risk governance and regulation, this means that, for the first time, we are considering ongoing genetic manipulation that takes place in the field rather than in the laboratory (cf Simon et al., 2018). There is no experience among the competent authorities who have to decide on the approval of such techniques. Gene drives still rely on inheritance; however, with HEGAA, we are going a step further by aiming for horizontal, and thus potentially much faster, spread in wild populations. The debate in the scientific community is not about the idea of growing genetically engineered crops, a practice that has been established since the 1980s, but rather that this application has particular potential for dual use, could easily result in unintended spread, and furthermore, could circumvent established regulatory mechanisms put in place to test the safety of genetically engineered crops for consumption, ecosystems, and the economy (Reeves et al., 2018). In this article, we will outline the current practices in the development of GM crops and characterize the technological power and failure potential of applications proposed in the Broad Agency Announcement “Insect Allies” from the perspective of technological assessment, focusing on safety aspects. Without sufficient means to control and even limit an application in case of malfunction, it would be irresponsible to consider its use. This paper examines the extent to which HEGAAs meet this requirement and what critical aspects currently remain.

## US GM AGRICULTURE

Currently, approximately 44% of the total landmass of the continental United States is used for crop production (The World Bank, 2021), where soybeans (34%), maize (34%), and wheat (15%) make up the largest percentage (FAOSTAT, 2020). As of 2018, the USA was the world's largest producer of maize and soy, with ~392 million and 124 million tons/year, respectively, accounting for 35% of the world's supply of each. The US was also the fourth largest producer of wheat, supplying 7% of the world's wheat demands (~51 million tons/year; FAOSTAT, 2020). Although most products are used domestically, a relevant share is exported internationally (wheat: 26 million tons [53% of production]; soybeans: 64 million tons [57% of production]; maize: 51 million tons [14% of production]; FAOSTAT, 2020). In the light of the importance of these crops to the US economy and the global market, DARPA and the US government's interest in exploring potential safeguards for the supply chain is self-evident.

In 2016, a crop loss of \$2 billion was recorded for the US. Owing to the large scale of monocultures in the US, adverse weather conditions can gravely affect yields (Olen & Auld, 2019). The events mainly responsible for these losses

were excess moisture (40%) and drought (15%); natural disasters and biotic factors accounted for only 2% of the damages each. Therefore, the main areas of application of the Insect Allies program account for 57% or \$1.14 billion of all annual US crop losses. Unlike losses caused by abiotic factors, biotic factors seem to play only a minor role in crop loss (Olen & Auld, 2019). A possible explanation could be that, in the US, 92% of maize and 94% of soy are GM crops with insect- or herbicide-resistance genes (Economic Research Service, United States Department of Agriculture, 2020). These numbers also reveal how widely GM foods are accepted and used in the US agricultural industry. Although historically, GM crops contained one additional gene conferring a specific trait, crops with multiple genetic alterations became a major product of the US agricultural system with the advent of gene stacking technologies. The reason stacked crops have become more prominent can be found in US legislation. The development of a completely new genetic crop is subject to a long and costly process of research and development (Low et al., 2018) and must conform to a vast number of governmental regulations (Library of Congress, 2020). Once a GM crop has been developed and licensed for commercialization, it can be crossed with another GM crop through traditional breeding methods, creating a stacked crop, expressing both novel properties. This process can be repeated to produce crops containing several new genes. US legislation allows the commercialization of these stacked crops, expressing a combination of licensed GM properties, without a renewed licensing process (National Academies of Sciences Engineering and Medicine, 2016, p. 471). In addition, the cultivation of stacked crops is further incentivized because farmers must dedicate only 5% of the cultivation area to refuge crops as opposed to 20% for single-trait GM corn crops (ISAAA, 2020; Storer et al., 2012). These refuge crops are meant to reduce the selective pressure on the pest organisms to develop resistances against the toxins expressed by the GM crops.

## CREATION OF GM PLANTS IN THE LABORATORY

The predominant techniques to produce transgenic plants are the bombardment of plant cells with DNA-coated microprojectiles and the use of microbial vectors such as *Agrobacterium tumefaciens*. The transformation efficiency of these methods is limited, and the introduced DNA is only integrated into the genome in a fraction of the transformed cells. The resulting transgenic plant cells can be grown to produce offspring expressing the desired genes (Low et al., 2018). However, these approaches to identifying, testing, and transforming potentially enhancing genes (Prado et al., 2014) often take hundreds of attempts and are costly.

The use of particle bombardment and microbial vectors can also be applied to transient expression systems, which can be created and tested more quickly than their transgenic counterparts. Transient expression systems are used in the research and production of biopharmaceuticals (Komarova et al., 2010). The introduced genetic information

is not stably integrated into the genome and thus, with high probability, not inherited to the next generation.

A third method for the creation of a transgenic plant is the use of native plant viruses (Komarova et al., 2010; Pogue & Holzberg, 2012). Viruses can be used for virus-induced gene silencing (VIGS), virus-mediated gene overexpression (VOX; Bouton et al., 2018; Ramegowda et al., 2014), and for virus-enabled gene editing (VEdGE; Mei et al., 2019). Viruses are easily modified and highly efficient at transforming the cells of specific tissues. However, the virus genome size also limits the capacity for foreign genetic material. Another problem with virus vectors is the difficulty in achieving an efficient infection rate without a transmitting host. In nature, plants are infected via vector organisms such as fungi, nematodes, aphids, leafhoppers, plant hoppers, mites, and whiteflies (Ng & Perry, 2004). Because rearing these vectors in laboratories to infect plants is costly and takes considerable effort, scientists have taken advantage of other transformation systems to introduce viral genomes into plants. For the leaf agroinfiltration method, *A. tumefaciens* is applied topically to mature leaves either directly, by the wound-and-agrospray method, or by utilizing vacuum infiltration to transfer plasmid or virus-based expression cassettes. In plasmid-based systems, transfection is limited to the infiltration site, whereas in virus-based expression systems, *A. tumefaciens* is transformed with specific viral DNA or cDNA, which is able to spread throughout the plant into all infected tissues (Komarova et al., 2010). In an improved system known as magnification, the desired plant as a whole is immersed in a culture of transformed *A. tumefaciens* and placed in a vacuum chamber to achieve maximum infiltration efficiencies to increase heterologous protein production (Bechtold & Bouchez, 1995; Gleba et al., 2005). The expression of desired proteins can be maximized by combining vacuum-assisted agroinfiltration with the use of viral genomes. For large-scale productions, the transformation of heterologous protein expressing crops is done before the plants are planted.

### MOVING TRANSFORMATION TO THE FIELD—THE INSECT ALLIES APPROACH

So far, none of the traditional transgenic or transient expression systems really allow for a quick and large-scale intervention as required for the Insect Allies project. The conceptualizer of the funding program instead proposed a project based on plant viruses and their natural vectors (DARPA, 2016). The HEGAA system shall consist of a modified vector insect, such as leafhoppers or aphids, carrying a modified plant virus and infecting the target crop with that virus to become itself modified. Although the intentions of the project are socially beneficial, there are several high-risk aspects to this technology, which so far have not been fully addressed. The major concerns brought up by the scientific community are its dual use potential, the introduction of transgenic modifications, the introduction of untested or unapproved modifications, and the unintended spread to other plant species or regions

(Kupferschmidt, 2018; Partan & Goldstone, 2018; Reeves et al., 2018).

Although the dual use potential of an HEGAA system (Reeves et al., 2018) is a valid concern, this holds true for several technologies. However, some display more potential than others, but the use of insect vectors as in HEGAA presents an invitation to clandestine applications. Reeves et al. (2018) base their criticism on the guideline of the 1976 UN convention on Environmental Modification techniques (ENMOD) and the 1972 Biological Weapons Convention (BWC), focusing on the prohibition of developing means of delivery for agents or toxins that are meant to be used for hostile purposes or in armed conflict. The use of insect vectors is criticized, because an aerosol or large-scale agroinfiltration application for transformation would be more efficient for peaceful purposes. Therefore, it could be argued that the insect-based dispersal system is merely an unnecessary and less reliable over-complication, more suited to a clandestine application to harm than as a quick and confined preventive intervention (Reeves et al., 2018).

### TECHNOLOGY CHARACTERIZATION OF HEGAA

Risk, as the probability of an adverse effect caused by exposure to a stressor, can be described by characterizing the hazard and exposure potential of the stressor and the potentially affected system (IPCS, 2004, p. 13f). As an approach in prospective technology assessment, technology characterization (TC) aims at the predictive analysis of these risk-determining factors and sources of malfunction (von Gleich, 2013). In this way, it can serve as a basis for course correction and a risk-minimized design (Frieß et al., 2019). Technology characterization represents a prospective method that provides a basis for decision making that meets the requirements of the precautionary principle by identifying possible reasons for concern or relief (Commission of the European Communities, 2000; United Nations, 2000; United Nations Conference on Environment and Development, 1992). The precautionary principle legitimizes precautionary action without clearly proven risk, if there are reasonable grounds for concern and it is unwarrantable to wait for sufficient evidence in the face of potentially adverse effects (cf. Fischer et al., 2006). By investigating available scientific information in an early assessment, TC aims to identify possible hazards and exposure potential (Frieß et al., 2020; von Gleich, 2013). Moreover, it aims to pre-estimate the nature and extent of uncertainty domains in advance. The underlying hypothesis of TC is that non-knowledge is generated in large part by the characteristics of the technology and can therefore be reduced by appropriately adapting the technology design to avoid complexity. The depth of technological intervention into matter and living beings as a primary criterion of TC provides information about the power and range of a technology (see Box 1). The “depth” increases from interventions at the level of the phenotype to interventions at the level of underlying control structures (ultimately the genotype). Power and range determine hazard and exposure, respectively. As the

**BOX 1**

## Glossary

**HEGAA**—Horizontal Environmental Genetic Alteration Agents such as described in the Insect Allies program utilize engineered vector organisms to infect the target crop with an engineered virus that alters its gene expression.

**Depth of intervention**—Refers to the depth of the technical intervention in relation to the level of the structures controlling the phenomena (phenotype). It is the source of technological power (hazard) and technological range (exposure).

**Intensity of intervention**—Refers to the technology's quantitative characteristics of mass and frequency.

**Application**—The utilization of a technology with specific characteristics and under specific conditions to achieve a purpose. The term does not refer to the application for marketing of an applicant to a competent authority, nor does it refer to the technology as a whole.

**Non-knowledge**—Knowledge gaps can be divided into different types of missing knowledge. For example, there are known unknowns, where the kind of knowledge gap is known, and unknown unknowns, where even the nature of the ignorance is unknown.

**Non- and off-target effects**—Biotechnology applications are targeted to affect specific target organisms. If other (groups of) organisms are affected, these are non-target effects. If the unintended effects occur in the target organism, these are considered off-target effects.

**CRISPR/Cas**—Clustered regularly interspaced short palindromic repeats (CRISPR) associated protein, a method of molecular biology for genome editing derived from a bacterial antiviral immunity mechanism (Jinek et al., 2012).

depth of intervention increases, so does the lack of knowledge of unintended effects and interactions. The second aspect of TC is the assessment of the technology's quantitative aspects mass and frequency in respect to the intensity of the technological intervention. The reliability of the technology is assessed as the third aspect in an inverse approach by characterizing its vulnerabilities, such as non- and off-target effects. Finally, the corrigibility of the expected effects, possibilities to limit or mitigate harm and reverse damage done, are investigated.

**Depth of intervention**

As a technology for self-spreading perpetual transient genome editing in the wild, HEGAAs can be considered to have a great depth of intervention because they not only affect the phenotype, but can potentially introduce

modifications into the target species' germline (Ellison et al., 2020). For a transgenic gain of function, three or more genes have to be introduced utilizing a virus-carrying insect vector, as per the project announcement (Biological Technologies Office, 2016). The broad variety of conferrable traits as well as the chosen viruses and insect vectors allow for high power and range in terms of hazard and exposure potential. Depending on the extent of the introduced effect, be it beneficial or detrimental, a considerable hazard potential can be achieved (Lange et al., 2013). The exposure potential depends strongly on the vector species and host range of the virus. For instance, an aphid would probably spend its lifetime on a single plant specimen (Irwin et al., 2007), whereas a leafhopper would have a wider migration radius (Power, 1992) and presumably infect a greater number of individual plants in a defined area. The virus-mediated horizontal gene transfer is a variable of technological range, considerably increasing the technology's potential for exposure. Concomitantly, exposure is scaled depending on the infectiousness of the virus, the fitness, competitiveness, longevity, and migratory behaviour of the vector species, its ability to spawn infected offspring, the transferability of the virus onto native insects and the specificity of the virus to the target crop.

**Intensity of intervention**

Mass and frequency as quantitative aspects of the technology application also depend on the properties of vector species, but additionally, the requirements of a release have to conform to the urgency of deployment, the number and size of the fields. And indeed, the aspired application case calls for a quick spread of the desired traits among the target plant species. The Broad Agency Announcement demands that “insects should cover and feed on 50% of the target plants within a defined space less than 48 h with a level of feeding on non-target plants that does not sustain viral transmission” (Biological Technologies Office, 2016, p. 7). Considering the application of HEGAA against weather conditions such as drought, the application area might be considered as hundreds of square kilometres. The number of vector insects depends on how many plants one insect may infect in its potentially shortened lifetime but could easily range in the billions (see Box 2). This constitutes a large mass of applied GMOs, which to an extent may pose problems in both production and environmental impact. These problems could be mitigated by spreading the high load-out over time by administering a lower mass at greater frequency of application. Both approaches represent a high intensity of intervention for HEGAA technology.

At the current stage of development, the details of HEGAA approaches that are ready for first trials remain unclear; therefore, we are restricted to a preliminary characterization of the hazard and exposure potential. Nevertheless, we will discuss several possible HEGAA strategies in the context of their reliability to gain insight into the level of uncertainty, non-knowledge, and controllability associated with this technology at the current stage of development.

## BOX 2

Sample calculation on the required number of infected GM insects

The state of Iowa has the largest corn cultivation area in the US with 13.9 million acres (USDA, 2019) or 56 251 304 271 m<sup>2</sup>. We assume that a single insect could reliably infect five square meters of corn plants in a field per day (probably an overestimation as it would likely take multiple insects to reliably infect a single plant, and also assumes a very mobile insect that switches plants often). Also, we assume that the whole area would have to be infected within 7 days (a longer sustained drought would result in too extreme crop loss). This means each insect can effectively over 7 days infect 35 m<sup>2</sup>

$$n_{\text{Insects}} = \frac{A}{l \times t} = \frac{5.6 \times 10^{10} \text{ m}^2}{5 \frac{\text{m}^2}{\text{days}} \times 7 \text{ days}} = \frac{5.6 \times 10^{10} \text{ m}^2}{35 \text{ m}^2} = 1.6 \times 10^9. \quad (1)$$

Thus, 1.6 billion insects would be required in a best-case scenario for Iowa alone. Also, the Broad Agency Announcement demands: Insects should cover and feed on 50% of the target plants within a defined space less than 48 h with a level of feeding on non-target plants that does not sustain viral transmission. For quick interventions, it would likely be necessary to sustain a library of insects and viruses for different crops with different transgenic traits. For the rapid mass-rearing, a collaboration of multiple facilities may be required. To put this calculation into perspective, for field trials of the sterile insect technique on *Drosophila suzukii*, a weekly release size of two million flies is estimated (Liou, 2021).

## Reliability

The reliability of a given technology can be assessed by examining its vulnerabilities and potential pathways to failure. For the envisioned HEGAA applications, three tiers corresponding to the three involved entities (virus, vector, host) and their specific combinations represent the sources of failure and determine the ability of the approach to deal with imponderables. With regard to the origin of relevant cause-and-effect chains that might be associated with each of these tiers, we subdivide them into three levels. These are on the genetic level, on the level of the entity in relation to the other entities, and finally the relationship of each entity with the whole ecosystem. The resulting differentiation yields a 3 × 3 matrix, which lists the main sources of non-knowledge (Figure 1). However, this scheme does not consider emerging properties arising from the combination of

all three entities or all three entities and the environment. Figure 1 shows a matrix that systematically examines the vulnerabilities and sources of non-knowledge inherent in HEGAA technology.

*Virus.* The main molecular vulnerability arising from the virus is its limited genomic capacity. To produce a virus that, despite its limited capacity of genetic material, can transfer resistance traits onto a host plant compatible with the host's genetics is a powerful feature but poses a grand research challenge. Virus-induced gene silencing systems have previously been constructed in laboratories (Ali et al., 2015). The virus in that study introduced an sgRNA into a plant constitutively expressing Cas9. Using this approach, a recent publication by Ellison et al. (2020), funded by the Insect Allies project, used a viral vector to introduce an sgRNA targeting the native phytoene desaturase (PSD) into a Cas9 overexpressing *Nicotiana benthamiana*, causing photo-bleaching in growing plants (Naing et al., 2019). Although use of PSD silencing as a reporter phenotype is a fairly common practice in plant genetics, the possibility to induce this lethal phenotype by the introduction of a single sgRNA is new (Schäfer et al., 2013). These experiments clearly demonstrate how easily a combination of VIGS and HEGAA could be misused to devastate a crop. The idea of cultivating fields of Cas9-expressing crops awaiting the dispersion of sgRNA carrying HEGAA would pose an easy target for malignant outside forces and thus only exposes the food supply to unnecessary risks. However, in this article, we don't want to address security issues caused by the dual use of HEGAA but instead focus on safety issues of peaceful application. Beneficial traits, such as drought resistance on the other hand, rely on the expression of multiple transgenes, which certainly exceed the viral capacity (Shinozaki & Yamaguchi-Shinozaki, 2007). A potential pathway to circumvent the limited loading capacity could be to split the transgenic sequences onto multiple viruses as proposed in the Broad Agency Announcement (Biological Technologies Office, 2016). The high mutation rate in viruses can not only affect the GM transmissibility but may potentially cause a host of other problems for the proposed system (Acosta-Leal et al., 2011). As the genes inserted into the virus genome are not vital to the virus, mutations in these genes would not be corrected through natural selective pressure. A mutation in the heterologous-expressed gene may simply render the desired protein non-functional, but at worst could alter its function and properties (for an overview of frequency and character of mutations in an ssRNA plant virus, see Tromas & Elena, 2010). On the other hand, when the virus is carrying Cas9 and gRNA, a mutation may render the gene editing aspect of the technology non-functional or cause off-target DNA cleavage (see Acosta-Leal et al., 2011 on plant virus evolution; Naeem et al., 2020 on CRISPR/Cas off-target effects). Therefore, a single mutation in the virus genome has the potential to decrease the fitness of the target crop plant instead of improving it. One way to reduce the likelihood of that would be multiplexing




		Biological level		
		Genome	Organism	Ecosystem
Part of the HEGAA-system	Virus 	<ul style="list-style-type: none"> <li>- Limited genomic capacity</li> <li>- High mutation rate</li> </ul>	<ul style="list-style-type: none"> <li>- Broad host range of single strand viruses</li> <li>- Superinfections could cause unexpected viral mutations</li> <li>- Host and vector specificity</li> </ul>	<ul style="list-style-type: none"> <li>- Infection of non-target vectors</li> <li>- Infection of non-target hosts</li> </ul>
	Vector 	<ul style="list-style-type: none"> <li>- Mutation of GM-stylet surface proteins*</li> <li>- Failure of dead switch</li> <li>- Bt-resistance**</li> </ul>	<ul style="list-style-type: none"> <li>- Reinfection by the virus***</li> <li>- Parthenogenesis</li> <li>- Loss of sterility</li> </ul>	<ul style="list-style-type: none"> <li>- Target plant specificity</li> <li>- Long range dispersal</li> <li>- Gene flow</li> </ul>
	Crop 	<ul style="list-style-type: none"> <li>- Mutations at Cas9 cleavage sites****</li> <li>- Complex genomic rearrangements</li> <li>- Transgene compatibility</li> <li>- Expression in the right quantity and tissues at the right time</li> </ul>	<ul style="list-style-type: none"> <li>- Inheritability of transgenic element</li> <li>- Development of a gene drive by transgenes in germline cells</li> </ul>	<ul style="list-style-type: none"> <li>- Spread of transgenes by horizontal or vertical transfer</li> <li>- Feeding damage by GM-vector</li> </ul>

FIGURE 1 Matrix of non-knowledge sources concerning the genomic, organismal, and ecosystem levels (horizontal) of the virus, vector, and host crop (vertical). \*In the case of such a modification for increased confineability. \*\*Considering the wide use of Bt crops in the US. \*\*\*In the case that non-circulative viruses are used. \*\*\*\*If the application relies on the action of the CRISPR/Cas system

(Ma et al., 2015; Xing et al., 2014), where multiple gRNA sequences would ensure the faithful insertion. This would likely increase efficiency and longevity of the HEGAA approach in the environment owing to reduced resistance formation.

Outside the natural rate of mutation, it has been demonstrated that, when a host is superinfected with multiple viruses, these viruses can exchange genetic information to create a new variant, combining properties of both or multiple viruses (Tollenaere et al., 2016). As a result of hybridization between different viral strains, the hazard and exposure potential of these pathogens may change, for example, in terms of host range, vector preference, cellular tropism, or transmission rate (Elena et al., 2014; Tollenaere et al., 2016). Such hybridization may become relevant to the HEGAA approach, for example, if the intended ssRNA or ssDNA viruses are intentionally designed to be restricted to a single target plant species. According to the mixing vessel theory, this may provide viruses with an altered host range that allows infection of new hosts. An historical example of the potential dangers of the mixing vessel theory are the influenza A viruses (Ma et al., 2008). Assuming that the host range of the originally restricted GM virus is expanded by such a hybridization event, the virus has the potential to infect non-target plant species. If these species contain genomic sequences with sufficient similarity to the corresponding sequences in the target species, the genomic alteration originally intended only for the target plant species may also be realized in the non-target species. If the trait is inherited, this advantage may lead to an increase in the

respective plant population, which in turn could disturb the ecosystem. The opposite effect is true for the spread of a trait, which results in a loss of fitness. In any case, this hybridization potential, combined with the fact that ssRNA and ssDNA viruses have a broad host range (McLeish et al., 2019; Moury et al., 2017), highlights an important weakness of the HEGAA system in terms of its controllability.

Transient expression depends strongly on the chosen virus vector but, because most plant viruses are +ssRNA viruses that normally do not integrate into the host genome, this vulnerability is less problematic (Hull, 2002). Furthermore, tissue tropism may be mostly neglected because most plant viruses can be considered more or less pantropic (Harper et al., 2014). A more complicated issue arises on the environmental scale, where the spread of the GM virus needs to be confined to the specific vector and host species.

On an environmental scale, the host and vector specificities of the virus are the main vulnerabilities. Research on the molecular mechanisms behind the virus' ability to both infect its host plants and to be transmitted by their insect vectors revealed the mechanisms behind non-circulative transmissions in aphids and has allowed for the development of non-aphid-transmissible viruses (Atreya et al., 1995; López-Moya et al., 1999). Harrison and Robinson (1988) found that a highly conserved N-terminal 3-amino acid motif (DAG motif) on the coat protein of various plant viruses was responsible for the transmissibility in aphids. Through a mutation of this DAG motif, the virus could no longer be transmitted by aphids, because it was no longer recognized by receptors in the host's stylet (Atreya et al., 1995). In

theory, this mutation could be used to reduce the risk of transmission through wild insects, but there are two problems with this idea. First, the aforementioned high mutation rate and selective pressure results in a high chance of a random mutation reversing the mutation of the DAG motif, creating a transmissible virus that can spread freely to wild type and other vector species. Second, the DAG motif occurs in viruses with a short transmissibility time frame, which brings its own limitations to the technology, as argued further below. However, the specificity to the target host remains unaddressed in both the project announcement and published research. The host range breadth (HRB) was determined to depend on four different viral properties: genome nature, number of genome segments, mode of vertical transmission, and vector type (Moury et al., 2017), where single stranded viruses exhibited a higher HRB than double stranded ones.

*Vector insect.* Concerning the insect vectors, the project suggests that they be sterile and short-lived to improve confineability. This is reminiscent of the Release of Insects carrying a Dominant Lethal allele (RIDL), which introduces a conditional viability, dependent on tetracycline. This dead switch, however, is encumbered with its own vulnerabilities (Benedict & Robinson, 2003; Evans et al., 2019; Frieß et al., 2020; Massonnet-Bruneel et al., 2013; Phuc et al., 2007).

One variant of the RIDL technology is the Friendly *Aedes*, where genetically engineered male *Aedes* mosquitoes are to be released in the Florida Keys to reduce the dengue burden. In these mosquitoes, the molecular dead switch kills female offspring in the larval stage. This technique as well constitutes an environmental intervention. However, gene transfer is strictly vertical and thus limited to the target species and, owing to the lethality of the GM trait, the intervention is self-limiting. For a more thorough technological characterization of the RIDL technology, see Frieß et al. (2020).

A related issue arising with sterility especially in aphids is parthenogenesis, which, for the prevalent cyclic parthenogenesis (Davis, 2012), may be considered an additional safeguard against unintended gene flow. But its multifactorial regulation, dependent on dark period duration and cell cycle regulation, makes parthenogenesis a property that is difficult to control. To limit transmissibility, a surface protein modification of the aphid is envisioned to allow the specific binding of the GM virus only via the so-called helper component (for a review, see Valli et al., 2018). But although reliable genetic engineering can be challenging in model organisms, it may be further exacerbated when it is to take place in non-model arthropods with under-researched genetic makeup. Interestingly, this is addressed by a new publication funded by the Insect Allies project: Chen et al. (2019) recently sequenced the genome of maize leaf aphid. This genome could be a key to adapting the host transmission mechanism specifically to a GM virus.

Some of the better understood virus transmission systems are those of aphids (Ng & Perry, 2004) and other members of the old homoptera taxon (Ferreles & Moreno, 2009). Aphids pick up plant viruses when feeding on infected plants and can transmit these viruses to new hosts through feeding events. Viruses that are transmitted by retention in the stylet or foregut are transmissible for seconds or days depending on the virus, whereas viruses that circulate through the insect's body can cause it to remain infectious indefinitely. For an HEGAA system, the virus should be transmissible long enough to allow for production, transport, and dispersal of infected aphids but short enough to minimize undesired spread throughout the ecosystem. For instance, 50% of winged aphid's migrants terminate their flight past 90 m, whereas 0.1% can migrate up to tens of kilometres, 10% average around 1–1.5 km (Parry, 2013; Pleydell et al., 2018). The viruses that best fit these criteria are non-circular, semipersistent, and circular non-propagative viruses, which currently include 12 known viral genera, only some of which have been developed into genetic expression systems (Ng & Perry, 2004). By selecting a virus from one of these two transmission types, one would limit the time in which the released aphids can infect plants, but the issues of reinfection and unintended dispersal into wild aphids remain great sources of non-knowledge. Non-persistent viruses can be transmitted by just a brief tasting, and aphids carrying these viruses normally do not settle on or colonize plants, thus promoting a faster spread of the virus. Although these characteristics are advantageous when spreading the virus to target plants, they also increase the potential of transmitting the virus to non-target crops via potential non-target vectors (Department of Employment Economic Development and Innovation, 2009). Reinfection may be problematic for non-persistent stylet-borne and semipersistent foregut-borne viruses, also spreading the virus to non-target vectors and eventually non-target hosts (Carr et al., 2020; Deshoux et al., 2020; Hogenhout et al., 2008). A circulative propagative virus on the other hand has the advantage of ease of handling and reliability when infecting vector organisms in the laboratory. Furthermore, feeding times of up to several hours may be necessary for non-target vectors to acquire such persistent viruses from infected hosts, which increases confineability (Department of Employment Economic Development and Innovation, 2009). Still, this approach would call for additional safeguards to ensure only the GM vectors can spread the GM virus. This demonstrates that the insect vector should not only be chosen to be specific to the target host plant, but it should present a balance of mobility. A single specimen should be able to infect multiple plants quickly but also be confined to the target area.

Most non-knowledge is derived from the potential of uncontrolled spread, persistence, and gene flow of the GM vectors. It is unclear how well released GM vectors will fare in competition with wild conspecifics and other biotic factors such as competitors and predators. In this respect, it may be prudent to consider the widespread use of Bt

crops and thus equip the vector with corresponding resistances. This would give the vectors a competitive advantage but also make gene flow containment much more paramount.

Finally, the vector species must be chosen so that it wounds the host plant to reliably infect the host but not do so much damage as to severely lower the host's viability and yield. Luckily, corn aphids, for instance, do not seem to cause detrimental damage to maize (Department of Agriculture and Fisheries, 2018).

*Host crop plant.* Regarding the host plant, there are some obvious choices. The chosen crop should represent a major crop that is widely cultivated such as the above-mentioned soybeans, maize, and wheat. Then the transferred trait should be tailored to the native physiology of that host to facilitate tissue- and stimulus-specific expression of the conferred genes. This will likely pose a challenge to genetic engineering. Imperfect compatibility may result in reduced fitness and thus reduced efficiency of the application and so potentially even more drastically reduced crop yields. Furthermore, as has been repeatedly stated in interviews and press statements by Insect Allies, the traits transmitted by the virus are said to be non-inheritable (Aschenbach, 2018; Kupferschmidt, 2018; Olena, 2018). However, the study published by Ellison et al. (2020) demonstrates how heritability can be achieved. A tobacco rattle virus was used to introduce a modified sgRNA with increased cell-to-cell mobility to induce VIGS. Although the unmodified sgRNA-silenced plants only display a heritability of the silencing effect in 1/438 seedlings, silencing with the modified sgRNA was inherited to up to 100% of progeny. These findings highlight the risk of HEGAA-induced transgenic mutations in plants that could even develop into a gene drive (Westra et al., 2016, p. 17f) in both the targeted agricultural and non-target plants. Such changes may result in uncontrolled spread of edited sequences and transgenes by horizontal or vertical gene flow, even into other species (Courtier-Orgogozo et al., 2017). Genetically, there are more sources of vulnerability than just the unintended inheritability. Mutations in the cleavage site could reduce the effectiveness of the HEGAA system, whereas unforeseen mutations in the rest of the crop genome could increase off-target effects. Furthermore, recombination errors after Cas9 cleavage could cause complex genomic rearrangements or unintended integration of the viral genome. All of which could reduce the fitness of the target plant or, at the very least, reduce the manifestation of the desired phenotype (Chiba et al., 2017; Feschotte & Gilbert, 2012; Kosicki et al., 2018; Xu et al., 2019).

### **Corrigibility**

Despite the potentially far-reaching and hardly predictable effects that a failure of the technology may trigger, there is no true means to correct a failed application. There is however the possibility of mitigation in the form of pesticides to reduce the number of vector organisms.

But to this end, it must first be possible to detect unintended consequences, which depends mainly on monitoring efforts. Those may entail the constant visual supervision of the crop's health; genetic profiling of the harvest, wild type, and other insects in the target and neighbouring areas to check for properties of the GM virus; probing of harvest seeds to ensure the virus was not transmitted into germ line cells; and monitoring of the vector population's mortality. However, the limited mitigation options underscore the importance of reliable confinement measures.

### **Options for improved confinement and containment**

So far, this article has only addressed the fundamental strategy outlined by DARPA for the HEGAA technology. To expand on this view, we will now introduce and examine other possible avenues of application, namely the dispersal via duster planes or spraying, a split approach, and a design with multiple viruses, illustrated in Figure 2.

The fundamental strategy detailed above will likely lead to a spotty infection of crops because of the difficulty of directing the vectors to specific plants and because of the yet unrestricted host range of neither vectors nor virus to spread beyond the target host.

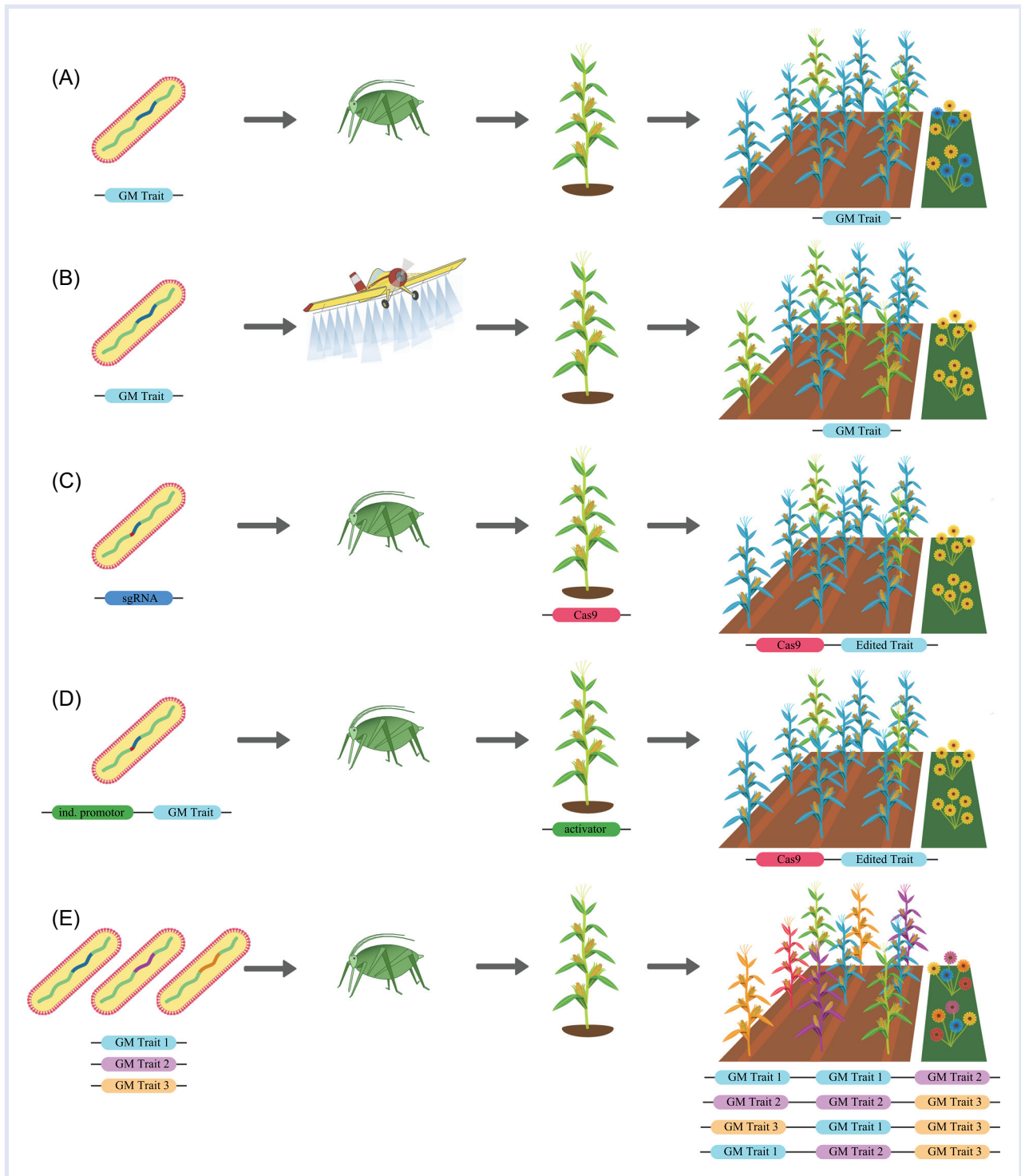
The spraying or duster plane approach would disperse a virus in a solution and would allow for a comparably accurate delivery to a target area. In this approach, infection would occur through existing wounds on the plants and by the native fauna feeding on the crop plant. Therefore, especially on Bt crops, the infection density would be reduced compared with the fundamental approach. Non-circulative viruses would likely be best suited to this approach, possibly adding an additional layer of confineability.

In the split approach, the virus would either carry an sgRNA, whereas the crop host would be pre-modified to constitutively express Cas9, or vice versa. This would be a versatile system because desired phenotypes could be controlled through the sgRNA transmitted by the virus. This system could either be used to both silence and activate a gene, by either targeting the gene directly or by targeting the gene's transcription repressors, respectively. It would also present a high confineability because non-target hosts lack an essential component of the system. The versatility and ease of use of such a system is also its major drawback because malignant actors could exploit the system by introducing sgRNAs with detrimental consequences for the crop plants. Another safer split approach, at the cost of versatility, could consist of an activator gene in the virus and a repressed cargo gene in the host.

This vulnerability could be avoided by expressing both the Cas9 gene and sgRNA from the virus genome as demonstrated by Ma et al. (2020), utilizing the circulative propagative, negative strand RNA *sonchus yellow net rhabdovirus* (SYNV). Of course, this would then constitute neither a split approach nor a transient modification.

As per the demands of the project's Broad Agency Announcement, where the research teams must





**FIGURE 2** Different HEGAA approaches. (A) The fundamental approach may result in spread to non-target hosts. (B) Dispersal by duster planes or spraying is likely less effective but more confined. (C) The split approach is well confinable but results in permanent modifications. (D) Introducing a gene that is under the control of a promoter whose activator is expressed by a GM plant. (E) The multi-virus approach may result in multiple phenotypes with similar confinement issues as the fundamental approach

demonstrate successful delivery of at least three genes by one or more insect-mediated viruses (Biological Technologies Office, 2016), there may be a multi-virus approach. Figure 2E depicts an example with three viruses, each propagating a different trait. The result may likely be a

panoply of phenotypes where crops are infected with all possible combinations of none, some, or all the viruses. Similarly, these combinations may occur outside the target area and target host for the same reasons mentioned for the fundamental approach.

Finally, at least with respect to climate change adaptation, it is worth noting that studies have also demonstrated that growing drought-resistant maize under non-stress conditions has no yield penalty when compared with wild type maize grown under the same conditions (Adee et al., 2016). It would therefore be simpler to grow drought-resistant maize every season as a preventive measure against drought damage than to introduce these genes into fully grown plants via the HEGAA approach.

## CONCLUSION

In this article, we outlined the current developmental state of the technology and examined it in the framework of a prospective technology characterization. HEGAA represents a technology with potentially great depth of intervention, because it can enable remote modification of the germ line of target plant species in the field. It tends to require a high intensity of intervention with either many insects released or many releases repeated to meet the requirements announced by DARPA. The hazard and exposure potential of an HEGAA approach can vary greatly depending on the viruses, vector insects, target plant species, and genetic modifications selected and their effects. However, at the current stage of development, the most critical aspect is the compromised reliability of the HEGAA approach, owing mainly to its complex design with three different species. The identified sources of non-knowledge and vulnerabilities from interfering processes can not only limit the effectiveness of an application. Rather, they are a cause for concern because of the numerous effects that can increase the potential for hazard and exposure. Combined with the current inadequacy of corrective measures, it is clear that there is an urgent need for early analysis of whether HEGAA approaches can be inherently contained and controlled by their specific technology design. To this end, we have briefly discussed four alternative approaches. Although some are advantageous, it remains an open question whether HEGAA or modified HEGAA approaches can become a manageable technology for rapid and large-scale interventions for peaceful applications with acceptable risk potential.

## ACKNOWLEDGMENT

On behalf of all authors, the corresponding author hereby states that no funding was received for the preparation of this article, and for the preparation of this article there was no assistance from individuals or organizations not identified among the list of authors.

## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

## DATA AVAILABILITY STATEMENT

Data, associated metadata, and calculation tools are available from corresponding author Bernd Giese (bernd.giese@boku.ac.at).

## REFERENCES

- Abrahamian, P., Hammond, R. W., & Hammond, J. (2020). Plant virus-derived vectors: Applications in agricultural and medical biotechnology. *Annual Review of Virology*, 7, 513–535. <https://doi.org/10.1146/annurev-virology-010720-054958>
- Acosta-Leal, R., Duffy, S., Xiong, Z., Hammond, R., & Elena, S. F. (2011). Advances in plant virus evolution: Translating evolutionary insights into better disease management. *Phytopathology*, 101, 1136–1148.
- Adee, E., Roozeboom, K., Balboa, G. R., Schlegel, A., & Ciampitti, I. A. (2016). Drought-tolerant corn hybrids yield more in drought-stressed environments with no penalty in non-stressed environments. *Frontiers of Plant Science*, 7, 1534. <https://doi.org/10.3389/fpls.2016.01534>
- Ali, Z., Abul-faraj, A., Li, L., Ghosh, N., Piatek, M., Mahjoub, A., Aouida, M., Piatek, A., Baltes, N. J., Voytas, D. F., Dinesh-Kumar, S., & Mahfouz, M. M. (2015). Efficient virus-mediated genome editing in plants using the CRISPR/Cas9 system. *Molecular Plant*, 8, 1288–1291. <https://doi.org/10.1016/j.molp.2015.02.011>
- Alphey, L. (2014). Genetic control of mosquitoes. *Annual Review of Entomology*, 59, 205–224. <https://doi.org/10.1146/annurev-ento-011613-162002>
- Aschenbach, J. (2018). *The Pentagon is studying an insect army to defend crops. Critics fear a bioweapon*. Stuff. Retrieved January 14, 2021, from: <https://www.stuff.co.nz/world/americas/107618542/the-pentagon-is-studying-an-insect-army-to-defend-crops-critics-fear-a-bioweapon>
- Atreya, P. L., Lopez-Moya, J. J., Chu, M., Atreya, C. D., & Pirone, T. P. (1995). Mutational analysis of the coat protein N-terminal amino acids involved in potyvirus transmission by aphids. *Journal of General Virology*, 76, 265–270. <https://doi.org/10.1099/0022-1317-76-2-265>
- Bechtold, N., & Bouchez, D. (1995). In plant Agrobacterium-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. In I. Potrykus & G. Spangenberg (Eds.), *Gene transfer to plants* (pp. 19–23). Springer. [https://doi.org/10.1007/978-3-642-79247-2\\_3](https://doi.org/10.1007/978-3-642-79247-2_3)
- Benedict, M. Q., & Robinson, A. S. (2003). The first releases of transgenic mosquitoes: An argument for the sterile insect technique. *Trends in Parasitology*, 19, 349–355.
- Biological Technologies Office. (2016). *Broad agency announcement Insect Allies*.
- Bouton, C., King, R. C., Chen, H., Azhakanandam, K., Bieri, S., Hammond-Kosack, K. E., & Kanyuka, K. (2018). Foxtail mosaic virus: A viral vector for protein expression in cereals. *Plant Physiology*, 177, 1352–1367. <https://doi.org/10.1104/pp.17.01679>
- Carr, J. P., Tungadi, T., Donnelly, R., Bravo-Cazar, A., Rhee, S.-J., Watt, L. G., Mutuku, J. M., Wamonje, F. O., Murphy, A. M., Arinaitwe, W., Pate, A. E., Cunliffe, N. J., & Gilligan, C. A. (2020). Modelling and manipulation of aphid-mediated spread of non-persistently transmitted viruses. *Virus Research*, 277, 197845. <https://doi.org/10.1016/j.virusres.2019.197845>
- Chen, W., Shakir, S., Bigham, M., Richter, A., Fei, Z., & Jander, G. (2019). Genome sequence of the corn leaf aphid (*Rhopalosiphum maidis* Fitch). *GigaScience*, 8, 4. <https://doi.org/10.1093/gigascience/giz033>
- Chiba, S., Wakabayashi, T., Tachi, Y., Takaki, N., Terashima, A., Okumura, S., & Yoshida, T. (2017). Method to reduce long-lived fission products by nuclear transmutations with fast spectrum reactors. *Scientific Reports*, 7, 13961. <https://doi.org/10.1038/s41598-017-14319-7>
- Commission of the European Communities. (2000). *Communication from the commission on the precautionary principle*.
- Courtier-Orgogozo, V., Morizot, B., & Boëte, C. (2017). Agricultural pest control with CRISPR-based gene drive: Time for public debate: Should we use gene drive for pest control? *EMBO Reports*, 18, e201744205. <https://doi.org/10.15252/embr.201744205>
- DARPA. (2016). *Insect allies*. Retrieved November 17, 2019, from: <https://www.darpa.mil/program/insect-allies>
- Davis, G. K. (2012). Cyclical parthenogenesis and viviparity in aphids as evolutionary novelties: Cyclical parthenogenesis and viviparity in aphids. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution*, 318, 448–459. <https://doi.org/10.1002/jez.b.22441>

- Department of Agriculture and Fisheries (Queensland Government). (2018). *Insect pest management in maize*. Retrieved November 28, 2021, from: <https://www.daf.qld.gov.au/business-priorities/agriculture/plants/crops-pastures/broadacre-field-crops/insect-pest-management-specific-crops/insect-pest-management-maize#other>
- Department of Employment, Economic Development and Innovation. (2009). *Aphid-transmitted viruses in vegetable crops—Integrated virus disease management* (Information Note). Queensland, Australia.
- Deshoux, M., Masson, V., Arafah, K., Voisin, S., Guschinskaya, N., van Munster, M., Cayrol, B., Webster, C. G., Rahbé, Y., Blanc, S., Bulet, P., & Uzest, M. (2020). Cuticular structure proteomics in the pea aphid *Acyrtosiphon pisum* reveals new plant virus receptor candidates at the tip of maxillary stylets. *Journal of Proteome Research*, 19, 1319–1337. <https://doi.org/10.1021/acs.jproteome.9b00851>
- Economic Research Service, United States Department of Agriculture (USDA ERS). (2020). *Adoption of genetically engineered crops in the US*.
- Elena, S. F., Bernet, G. P., & Carrasco, J. L. (2014). The games plant viruses play. *Current Opinion in Virology*, 8, 62–67. <https://doi.org/10.1016/j.coviro.2014.07.003>
- Ellison, E. E., Nagalakshmi, U., Gamo, M. E., Huang, P., Dinesh-Kumar, S., & Voytas, D. F. (2020). Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. *Nature Plants*, 6, 620–624. <https://doi.org/10.1038/s41477-020-0670-y>
- Evans, B. R., Kotsakiozi, P., Costa-da-Silva, A. L., Ioshino, R. S., Garziera, L., Pedrosa, M. C., Malavasi, A., Virginio, J. F., Capurro, M. L., & Powell, J. R. (2019). Transgenic *Aedes aegypti* mosquitoes transfer genes into a natural population. *Scientific Reports*, 9, 1–6.
- FAOSTAT. (2020). *Food and Agriculture Organization of the United Nations (FAO)*. FAOSTAT Database. Retrieved August 20, 2020, from: <http://www.fao.org/faostat/en>
- Fereres, A., & Moreno, A. (2009). Behavioural aspects influencing plant virus transmission by homopteran insects. *Virus Research*, 141, 158–168. <https://doi.org/10.1016/j.virusres.2008.10.020>
- Feschotte, C., & Gilbert, C. (2012). Endogenous viruses: Insights into viral evolution and impact on host biology. *Nature Reviews Genetics*, 13, 283–296. <https://doi.org/10.1038/nrg3199>
- Fisher, E., Jones, J. S., & von Schomberg, R. (Eds.). (2006). *Implementing the precautionary principle—Perspectives and prospects*. Edward Elgar Publishing.
- Frieß, J. L., Giese, B., & von Gleich, A. (2020). Technology characterisation. In A. von Gleich & W. Schröder (Eds.), *Gene drives at tipping points: Precautionary technology assessment and governance of new approaches to genetically modify animal and plant populations* (pp. 1–28). Springer International Publishing. [https://doi.org/10.1007/978-3-030-38934-5\\_1](https://doi.org/10.1007/978-3-030-38934-5_1)
- Frieß, J. L., von Gleich, A., & Giese, B. (2019). Gene drives as a new quality in GMO releases—A comparative technology characterization. *PeerJ*, 7, e6793. <https://doi.org/10.7717/peerj.6793>
- Giese, B. (2021). The viral era: New biotechnologies give humans an unprecedented control over nature and require appropriate safeguards. *EMBO Reports*, 22. <https://doi.org/10.15252/embr.202153229>
- Gleba, Y., Klimyuk, V., & Marillonnet, S. (2005). Magnification—A new platform for expressing recombinant vaccines in plants. *Vaccine*, 23(17–18), 2042–2048.
- Harper, S. J., Cowell, S. J., Robertson, C. J., & Dawson, W. O. (2014). Differential tropism in roots and shoots infected by *Citrus tristeza virus*. *Virology*, 460–461, 91–99. <https://doi.org/10.1016/j.virol.2014.04.035>
- Harrison, B. D., & Robinson, D. (1988). Molecular variation in vector-borne plant viruses: Epidemiological significance. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 321(1207), 447–462.
- Hogenhout, S. A., Ammar, E.-D., Whitfield, A. E., & Redinbaugh, M. G. (2008). Insect vector interactions with persistently transmitted viruses. *Annual Review of Phytopathology*, 46, 327–359. <https://doi.org/10.1146/annurev.phyto.022508.092135>
- Hull, R. (2002). *Matthew's plant virology*. (4th ed.). Gulf professional publishing.
- International Programme on Chemical Safety (IPCS). (2004). *Risk assessment terminology*. <https://apps.who.int/iris/bitstream/handle/10665/42908/9241562676.pdf?sequence=1%26amp;isAllowed=y>
- Irwin, M. E., Kampmeier, G. E., & Weisser, W. W. (2007). Aphid movement: Process and consequences. In H. F. van Emden & R. Harrington (Eds.), *Aphids as crop pests* (pp. 153–186). CAB. <https://doi.org/10.1079/9780851998190.0153>
- ISAAA. (2020). *Stacked traits in biotech crops*.
- Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, 337, 816–821. <https://doi.org/10.1126/science.1225829>
- Komarova, T. V., Baschieri, S., Donini, M., Marusic, C., Benvenuto, E., & Dorokhov, Y. L. (2010). Transient expression systems for plant-derived biopharmaceuticals. *Expert Review of Vaccines*, 9, 859–876. <https://doi.org/10.1586/erv.10.85>
- Kosicki, M., Tomberg, K., & Bradley, A. (2018). Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nature Biotechnology*, 36, 765–771. <https://doi.org/10.1038/nbt.4192>
- Kupferschmidt, K. (2018). *Crop-protecting insects could be turned into bioweapons, critics warn*. Science. <https://doi.org/10.1126/science.aav6274>
- Lange, M., Yellina, A. L., Orashakova, S., & Becker, A. (2013). Virus-Induced Gene Silencing (VIGS) in plants: An overview of target species and the virus-derived vector systems. In A. Becker (Ed.), *Virus-induced gene silencing: Methods and protocols, methods in molecular biology* (pp. 1–14). Humana Press. [https://doi.org/10.1007/978-1-62703-278-0\\_1](https://doi.org/10.1007/978-1-62703-278-0_1)
- Library of Congress. (2020). *Restrictions on genetically modified organisms: United States*. Library of Congress, Law. Retrieved January 27, 2021, from: <https://www.loc.gov/law/help/restrictions-on-gmos/usa.php>
- Liou, J. (2021). *Sterile insect technique to control emerging global invasion of drosophilid fruit fly*. International Atomic Energy Agency (IAEA). Retrieved May 6, 2021, from: <https://www.iaea.org/newscenter/news/sterile-insect-technique-to-control-emerging-global-invasion-of-a-drosophilid-fruit-fly>
- López-Moya, J. J., Wang, R. Y., & Pirone, T. P. (1999). Context of the coat protein DAG motif affects potyvirus transmissibility by aphids. *Journal of General Virology*, 80, 3281–3288. <https://doi.org/10.1099/0022-1317-80-12-3281>
- Low, L.-Y., Yang, S.-K., Kok, D.-X. A., Ong-Abdullah, J., Tan, N.-P., & Lai, K.-S. (2018). Transgenic plants: Gene constructs, vector and transformation method. In Ö. Çelik (Ed.), *New visions in plant science* (pp. 41–61). InTech Open. <https://doi.org/10.5772/intechopen.79369>
- Ma, W., Kahn, R. E., & Richt, J. A. (2008). The pig as a mixing vessel for influenza viruses: Human and veterinary implications. *Journal of Molecular and Genetic Medicine*, 3, 158–166.
- Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., Wang, B., Yang, Z., Li, H., Lin, Y., Xie, Y., Shen, R., Chen, S., Wang, Z., ChenYuanling, Guo, J., Chen, L., Zhao, X., Dong, Z., & Liu, Y.-G. (2015). A Robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. *Molecular Plant*, 8, 1274–1284. <https://doi.org/10.1016/j.molp.2015.04.007>
- Ma, X., Zhang, X., Liu, H., & Li, Z. (2020). Highly efficient DNA-free plant genome editing using virally delivered CRISPR-Cas9. *Nature Plants*, 6, 773–779. <https://doi.org/10.1038/s41477-020-0704-5>
- Massonnet-Bruneel, B., Corre-Catelin, N., Lacroix, R., Lees, R. S., Hoang, K. P., Nimmo, D., Alphey, L., & Reiter, P. (2013). Fitness of transgenic mosquito *Aedes aegypti* males carrying a dominant lethal genetic system. *PLoS One*, 8, e62711.
- McLeish, M. J., Fraile, A., & García-Arenal, F. (2019). Evolution of plant-virus interactions: Host range and virus emergence. *Current Opinion in Virology*, 34, 50–55. <https://doi.org/10.1016/j.coviro.2018.12.003>
- Mei, Y., Beernink, B. M., Ellison, E. E., Konečná, E., Neelakandan, A. K., Voytas, D. F., & Whitham, S. A. (2019). Protein expression and gene editing in monocots using foxtail mosaic virus vectors. *Plant Direct*, 3, e00181. <https://doi.org/10.1002/pld3.181>

- Moury, B., Fabre, F., Hébrard, E., & Froissart, R. (2017). Determinants of host species range in plant viruses. *Journal of General Virology*, 98, 862–873. <https://doi.org/10.1099/jgv.0.000742>
- Mushegian, A. R., & Shepherd, R. J. (1995). Genetic elements of plant viruses as tools for genetic engineering. *Microbiological Reviews*, 59, 548–578. <https://doi.org/10.1128/mr.59.4.548-578.1995>
- Naeem, M., Majeed, S., Hoque, M. Z., & Ahmad, I. (2020). Latest developed strategies to minimize the off-target effects in CRISPR-Cas-mediated genome editing. *Cells*, 9, 1608. <https://doi.org/10.3390/cells9071608>
- Naing, A. H., Kyu, S. Y., Pe, P. P. W., Park, K. I., Lee, J. M., Lim, K. B., & Kim, C. K. (2019). Silencing of the phytoene desaturase (PDS) gene affects the expression of fruit-ripening genes in tomatoes. *Plant Methods*, 15, 1–10.
- National Academies of Sciences, Engineering and Medicine. (2016). *Genetically engineered crops: Experiences and prospects*. National Academies Press.
- Ng, J. C. K., & Perry, K. L. (2004). Transmission of plant viruses by aphid vectors. *Molecular Plant Pathology*, 5, 505–511. <https://doi.org/10.1111/j.1364-3703.2004.00240.x>
- Olen, B., & Auld, S. (2019). *Farming: Weather is leading cause of US crop loss—How do you assess the risk?* AgFax. Retrieved January 14, 2021, from: <https://agfax.com/2019/01/25/farming-weather-is-leading-cause-of-u-s-crop-loss-how-do-you-assess-the-risk/>
- Olena, A. (2018). *Questions raised about DARPA-funded crop program*. The Scientist. Retrieved January 14, 2021, from: <https://www.the-scientist.com/news-opinion/questions-raised-about-darpa-funded-crop-program-64898>
- Parry, H. R. (2013). Cereal aphid movement: General principles and simulation modelling. *Movement Ecology*, 1, 1–15.
- Partan, E., & Goldstone, H. (2018). “Insect Allies” program draws criticism. WCAI. Retrieved November 6, 2019, from: <https://www.capeandislands.org/post/insect-allies-program-draws-criticism#stream/0>
- Phuc, H. K., Andreasen, M. H., Burton, R. S., Vass, C., Epton, M. J., Pape, G., Fu, G., Condon, K. C., Scaife, S., Donnelly, C. A., Coleman, P. G., White-Cooper, H., & Alphey, L. (2007). Late-acting dominant lethal genetic systems and mosquito control. *BMC Biology*, 5, 1–11. <https://doi.org/10.1186/1741-7007-5-11>
- Pleydell, D. R. J., Soubeyrand, S., Dallot, S., Labonne, G., Chadœuf, J., Jacquot, E., & Thébaud, G. (2018). Estimation of the dispersal distances of an aphid-borne virus in a patchy landscape. *PLOS Computational Biology*, 14(4), e1006085. <http://doi.org/10.1371/journal.pcbi.1006085>
- Pogue, G. P., & Holzberg, S. (2012). Transient virus expression systems for recombinant protein expression in dicot and monocotyledonous plants. In N. K. Dhal & S. C. Sahu (Eds.), *Plant Science* (pp. 191–216). <https://doi.org/10.5772/3007>
- Power, A. G. (1992). Host plant dispersion, leaf hopper movement and disease transmission. *Ecological Entomology*, 17, 63–68. <https://doi.org/10.1111/j.1365-2311.1992.tb01040.x>
- Prado, J. R., Segers, G., Voelker, T., Carson, D., Dobert, R., Phillips, J., Cook, K., Cornejo, C., Monken, J., & Grapes, L. (2014). Genetically engineered crops: From idea to product. *Annual Review of Plant Biology*, 65, 769–790.
- Ramegowda, V., Mysore, K. S., & Senthil-Kumar, M. (2014). Virus-induced gene silencing is a versatile tool for unraveling the functional relevance of multiple abiotic-stress-responsive genes in crop plants. *Frontiers in Plant Science*, 5, 323.
- Reeves, R. G., Voienky, S., Caetano-Anollés, D., Beck, F., & Boëte, C. (2018). Agricultural research, or a new bioweapon system? *Science*, 362, 35–37. <https://doi.org/10.1126/science.aat7664>
- Schäfer, M., Brütting, C., Gase, K., Reichelt, M., Baldwin, I., & Meldau, S. (2013). ‘Real time’ genetic manipulation: A new tool for ecological field studies. *The Plant Journal*, 76(3), 506–518. <http://doi.org/10.1111/tpj.12301>
- Shinozaki, K., & Yamaguchi-Shinozaki, K. (2007). Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany*, 58, 221–227. <https://doi.org/10.1093/jxb/erl164>
- Simon, S., Otto, M., & Engelhard, M. (2018). Synthetic gene drive: Between continuity and novelty. *EMBO Reports*, 19, e45760. <https://doi.org/10.15252/embr.201845760>
- Storer, N. P., Thompson, G. D., & Head, G. P. (2012). Application of pyramided traits against Lepidoptera in insect resistance management for Bt crops. *GM Crops & Food*, 3, 154–162.
- The World Bank. (2021). *Agricultural land (% of land area) - United States*. Retrieved August 1, 2021, from: <https://data.worldbank.org/indicator/AG.LND.AGRI.ZS?locations=US>
- Tollenaere, C., Susi, H., & Laine, A.-L. (2016). Evolutionary and epidemiological implications of multiple infection in plants. *Trends in Plant Science*, 21, 80–90. <https://doi.org/10.1016/j.tplants.2015.10.014>
- Tromas, N., & Elena, S. F. (2010). The rate and spectrum of spontaneous mutations in a plant RNA virus. *Genetics*, 185, 983–989. <https://doi.org/10.1534/genetics.110.115915>
- United Nations. (2000). *Cartagena Protocol on biosafety to the convention on biological diversity*. Secretariat of the Convention on Biological Diversity.
- United Nations Conference on Environment and Development. (1992). *The Rio Declaration on Environment and Development*.
- United States Department of Agriculture (USDA). (2019). *Crop production 2018 summary*.
- US Food & Drug Administration. (2020). *AquAdvantage salmon fact Sheet*. Retrieved November 26, 2021, from: <https://www.fda.gov/animal-veterinary/animals-intentional-genomic-alterations/aquadvantage-salmon-fact-sheet>
- Valli, A. A., Gallo, A., Rodamilans, B., López-Moya, J. J., & García, J. A. (2018). The HCPro from the *Potyviridae* family: An enviable multitasking Helper Component that every virus would like to have. *Molecular Plant Pathology*, 19, 744–763. <https://doi.org/10.1111/mpp.12553>
- Venkataraman, S., & Hefferon, K. (2021). Application of plant viruses in biotechnology, medicine, and human health. *Viruses*, 13, 1697. <https://doi.org/10.3390/v13091697>
- von Gleich, A. (2013). Prospektive Technikbewertung und Technikgestaltung zur Umsetzung des Vorsorgeprinzips, *Konzepte und Verfahren der Technikfolgenabschätzung* (pp. 51–73). Springer VS.
- Westra, J., Van der Vlugt, C., Roesink, C., Hogervorst, P., & Glandorf, D. C. M. (2016). *Gene drives: Policy report*. National Institute for Public Health and Environment, Netherlands. <https://www.rivm.nl/bibliotheek/rapporten/2016-0023.pdf>
- Xing, H.-L., Dong, L., Wang, Z.-P., Zhang, H.-Y., Han, C.-Y., Liu, B., Wang, X.-C., & Chen, Q.-J. (2014). A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biology*, 14, 327. <https://doi.org/10.1186/s12870-014-0327-y>
- Xu, W., Fu, W., Zhu, P., Li, Z., Wang, C., Wang, C., Zhang, Y., & Zhu, S. (2019). Comprehensive analysis of CRISPR/Cas9-mediated mutagenesis in *Arabidopsis thaliana* by genome-wide sequencing. *International Journal of Molecular Sciences*, 20, 4125. <https://doi.org/10.3390/ijms20174125>
- Ziegler, A., Sasaya, T., Torrance, L., & Cowan, G. (2000). Aphid transmission studies using helper component proteins of Potato virus Y expressed from a vector derived from potato virus X. *Journal of General Virology*, 81, 1115–1119. <https://doi.org/10.1099/0022-1317-81-4-1115>