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APPLICATION OF GENOME EDITING IN ENTOMOLOGY

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ABSTRACT

Genome editing mediated by programmable CRISPR/Cas system is a new, emerging technology that holds greater potential in developing genetic pest management strategies via precision guided sterile insect technique; genetic improvement of pollinators, natural enemies etc. The seemingly innocuous prokaryotic immunity has now been translated into a mammoth site specific editing technology for its use in medical, agricultural, diagnostic fields etc. Until recently most of the genome editing work was dependent on RNA directed site specific cleavage by Cas9 enzyme followed by error prone, non-homologous end joining (NHEJ) resulting in random mutations. Later development of improved editing systems like base editor and prime editor have enabled obtaining site-specific, pre determined mutations without a double stranded break. Similarly many engineered Cas9 variants and other Cas proteins belonging to different class and types such as Cas3, Cas12a, Cas13a have improved the existing editing tool box. Currently efforts are being made to design and implement suitable gene drive methods for various pests such as spotted wing Drosophila, *Drosophila suzukii* Matsumura, Diamondback moth, *Plutella xylostella* (Linnaeus) , many species mosquito, Med fly, *Ceratitis capitata* Wiedem etc.

Key words: CRISPR/Cas systems, genome editing, double strand break, guide RNA, gene knockout, insect pest management, PAM sequence, non-homologous pairing

Insect pests are the major limiting-factor in crop production costing farmers billions of dollars in loss annually worldwide. Synthetic chemicals continue to be the main stay in insect pest management albeit posing serious environmental and health issues. Therefore, in order to reduce this over dependence, scientists continued to explore newer avenues for mitigating the pestilence in an effective, ecofriendly way. In this regard, the recent developments in genomics, molecular biology, bioinformatics have enabled scientists to develop and apply novel tools such as genome editing, gene drive etc in insect pest management. Genome editing mediated by zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regularly interspersed short palindromic repeats and associated proteins (CRISPR/Cas9) essentially generate target-specific mutations. CRISPR/Cas9 induces a double stranded break (DSB) at the desired genomic loci and is repaired mostly by the error-prone repair mechanism known as non-homolgous end joining (NHEJ) thus resulting in creation of different kinds of mutations at the target site that alter the target gene function. This approach is quite opposite to the random mutations generated by physical, chemical and radiation means which also has associated fitness cost. CRISPR/Cas9 mediated genome editing is widely used due to ease of operation and availability. Currently varieties of Cas proteins (both engineered and natural variants) are employed in performing various tasks such as precise editing, base editing, prime editing, gene switch etc. Presently it has become possible to edit any genome without a double stranded break leading to precise editing, known as DNA-free editing. It took nearly 20 years to achieve today's level of precision in genome editing from the simple, inquisitive observation on presence of repeat elements and their role in adaptive immunity in prokaryotes. It was the profound, intelligent work of Jennifer's team at University of California, Berkley and Zhang's team at Broad Institute, MIT

in the year 2012 and 2013, respectively to have had optimized and demonstrated the potential of primarily the prokarotic adaptive immunity into a programmable eukaryotic genome editing tool. Currently CRISPR/Cas9 mediated genome editing is poised to make a paradigm shift touted as a panacea in many fields that include insect pest management. Insect pests, insect vectors, parasitoids, predators, pollinators, honey bee, silkworm could potentially be edited for various traits such as resistance to pathogens, enhanced ecosystem services etc. Currently many pest species such as *Helicoverpa armigera* (Hubner), *Spodoptera litura* Fab., fruit flies, stored grain pests have been edited for field level management. Countries like USA, Canada, Japan have eased the deregulation process for the genome edited organisms including plants for field use.

HISTORY OF CRISPR/Cas

Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas systems are currently in the spotlight of active research in biology. The first repeat sequences (now CRISPRs) were identified 30 years ago in *Escherichia coli* while working on the gene involved in isozyme conversion of alkaline phosphatase (Ishino et al*.,* 1987). During that time, it was impossible to predict the biological significance due to lack of genomic resources. The actual function of this unique sequence remained a mystery until the mid-2000s. In 1993, CRISPR for the first time was observed in archaea, *Haloferax mediterranei*, and subsequently observed in a number of bacterial and archaea. In the early 2000s, the identification of sequence similarity between the spacer regions of CRISPRs and bacteriophages, archaeal viruses, and plasmids finally revealed the function of CRISPR as a prokaryotic immune system. Various genes previously envisaged to encode DNA repair proteins were identified as being necessarily associated with CRISPR and were designated Cas (CRISPR-associated) genes. Comparative genomic analyses further suggested that CRISPR and Cas proteins work together and constitute an acquired immunity system to protect prokaryotic cells against invading viruses and plasmids, very similar to the eukaryotic RNA interference. CRISPR sequences were found in nearly all archaeal genomes and in about half of bacterial genomes, rendering them the most widely distributed family of repeated sequences in prokaryotes. Till date CRISPR sequences have not been identified and reported from any eukaryotic genome.

DISCOVERY OF CRISPR FUNCTION

However, with increasing number of sequences available, it was found that many mesophilic organisms also contained CRISPR sequences. Importantly, it was pointed out that the phages and plasmids do not infect bacterial host strains containing the homologous spacer sequences. The two groups also suggested that the CRISPRs could initiate capturing of pieces of foreign invading DNA to constitute a memory of previous infections. This was confirmed further by noticing a correlation between the number of spacers of phage origin and the degree of resistance to phage infection, and they suggested that CRISPR could be used to produce antisense RNA. Importantly, it was pinpointed that the CRISPR-Cas system, with its memory component, rather resembles the adaptive immune system of vertebrates, with the crucial difference being that the animal immune system is not inheritable. The function of the CRISPR-Cas system as a prokaryotic immune system was finally proven in 2007 in *Streptococcus thermophilus*. Incorporating the phage sequence into the spacer region of the CRISPR of *S. thermophilus* resulted in this strain resistant to the phage. In the contrary this bacterial resistance became void when the corresponding protospacer sequence was deleted.

COMPONENTS OF CRISPR/CAS9

Based on the structure and functions of Cas-proteins, CRISPR/Cas system could be divided into Class I (type I, III and IV) and Class II (type II, V and VI). The class I system consist of multi-subunit (4 to 7 Cas proteins) Cas-protein complexes, while the class II systems utilize a single Cas-protein. Class I system is widespread in bacteria and archaea, comprising 90%

of all identified CRISPR-Cas loci while the remaining 10% belong to class II, which use single multi domain effector proteins found exclusively in bacteria. Since the structure of type II CRISPR/Cas-9 is relatively simple, it has been well studied and extensively used in genetic engineering. It is a two component system comprising of a guide RNA (gRNA=crRNA) and CRISPR associated proteins (Cas9). The Cas9 protein, the first Cas protein used in genome editing, has been isolated from *S. pyogenes* (spCas9). It is a large (1368 amino acids) multi-domain DNA endonuclease responsible for cleaving the target DNA to form a double stranded break by recognizing the protospacer adjacent motif (PAM). The RuvC and HNH domains are used to cut each single-stranded DNA, while PAM interacting domain confers PAM specificity and is responsible for initiating binding to target DNA. The Guide RNA is made up of two components viz. crRNA and trans-activating crRNA (tracrRNA). The crRNA is 18–20 base pair in length that specifies the target DNA by pairing with the target sequence, whereas tracrRNA is a long stretch of loops that serve as a binding scaffold for Cas9 nuclease. It has now become programmable by combining crRNA and tracrRNA to form a single guide RNA (sgRNA) in order to target almost whichever gene sequence of any organism.

CLASS II SYSTEM SUITABLE FOR GENOME EDITING

The simple architecture of the effector complexes has made class 2 CRISPR-Cas systems an attractive choice for developing a new generation of genome editing technologies. Several distinct class 2 effectors have been reported, including Cas9 in type II, Cas12a (formerly Cpf1), Cas12b (C2c1) in type V, and Cas13a (C2c2) and Cas13b (C2c3) in type VI. The most common and best studied multidomain effector protein is Cas9, a crRNA-dependent endonuclease, consisting of two unrelated nuclease domains, RuvC and HNH, which are responsible for cleavage of the displaced (non-target) and target DNA strands, respectively, in the crRNA-target DNA complex. Type II CRISPR-Cas loci also encode a trans-activating crRNA (tracrRNA) which might have evolved from the corresponding CRISPR. The tracrRNA molecule is also essential for pre-crRNA processing and target recognition in the type II systems

MECHANISMS OF CRISPR/CAS9

The mechanism of CRISPR/Cas-9 genome editing can be generally divided into three steps: recognition, cleavage, and repair. The designed sgRNA directs Cas9 and recognizes the target sequence in the gene of interest through its 5′crRNA complementary base pair component. The Cas9 protein remains inactive in the absence of sgRNA. The Cas9 nuclease makes double-stranded breaks (DSBs) at a site three base pair upstream to PAM. PAM sequence is a short (2–5 base-pair length) conserved DNA sequence downstream to the cut site and its size varies depending on the bacterial species. The most commonly used nuclease in the genome-editing tool, Cas-9 protein recognizes the PAM sequence at 5′-NGG-3′ (N can be any nucleotide base). Once Cas-9 has found a target site with the appropriate PAM, it triggers local DNA melting followed by the formation of RNA-DNA hybrid. Then, the Cas-9 protein is activated for DNA cleavage. HNH domain cleaves the complementary strand, while the RuvC domain cleaves the non-complementary strand of target DNA to produce blunt-ended DSBs and is repaired by the host cellular machinery. Non-homologous end joining (NHEJ), and homology directed repair (HDR) pathways are the two mechanisms to repair the double stranded break (DSB) and the NHEJ facilitates the repair of DSBs by joining DNA fragments through an enzymatic process in the absence of exogenous homologous DNA but it is an error-prone mechanism that may result in small random insertion or deletion (indels) at the cleavage site leading to the generation of frame shift mutation or premature stop-codon. HDR is highly precise and requires the use of a homologous DNA template. It is most active in the late S and G2 phases of the cell cycle. In CRISPR-gene editing, HDR requires a large amount of donor (exogenous) DNA templates containing a sequence of interest. HDR executes the precise gene insertion or replacement by adding a donor DNA template with sequence homology at the predicted DSB site.

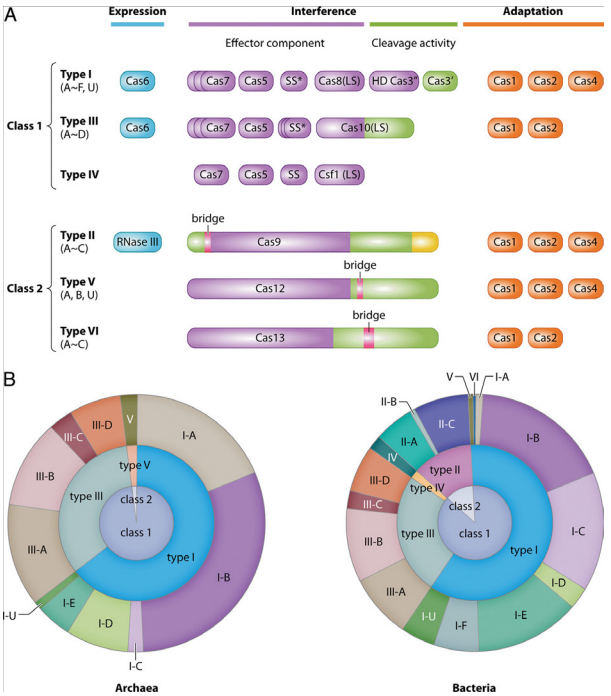

Fig. 1. Classification of CRISPR/CAS system (Makarova et al., 2020)

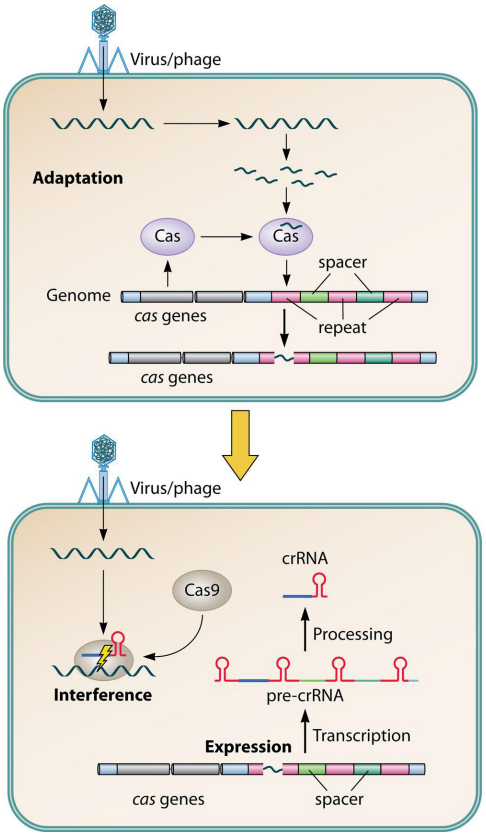

Fig. 2. Schematic diagram of prokaryotic CRISPR/Cas9 system (Ishino et al., 2018)

DEVELIVERY METHODS FOR Cas9 AND gRNA

Microinjection is the major method applied in insects. Reagents (DNA, RNA or protein) are usually injected into early-stage embryos when there are few cells so that they can access the nuclei of both somatic and germline cells. Then transfection is mostly used with insect cell lines. Yet another method is to transfect cells with a microfluidic membrane deformation method. In addition, virus mediated Cas9-gRNA delivery is also an option. These methods of delivery are proven practicable primarily in mammals and zebrafish. This is essential in guiding the attempt to adapt them in insects. As well as direct delivery, the generation of transgenic lines expressing Cas9 and gRNA is also possible due to the insects' relatively short life cycle.

Table 1. List of online tools available to find the CRISPR targets in insects

Name of the tool	Link
CCTop	http://crispr.cos.uni-heidelberg.de
Optimized CRISPR design-MIT	http://crispr.mit.edu
CHOPCHOP	http://chopchop.cbu.uib.no
CRISPOR	http://crispor.tefor.net
CRISPR Optimal Target Finder	http://tools.flycrispr.molbio.wisc.edu/targetFinder
CRISPR scan	http://www.crisprscan.org
E-CRISP	http://www.e-crisp.org/E-CRISP
CRSPRs	http://www.flynai.org/crispr
Cas9 Target Finder	http://shigen.nig.ac.jp/fly/nigify/Cs9/Cas9TargetFinder.jsp
Cas-OFFinder	http://www.regenome.net
CROP-IT	http://www.adlilab.org/CROP-IT/homepage.html

IDENTIFICATION OF THE EDITS

Endonucleases such as the CEL1 nuclease family (CEL1 assay or surveyor assay) or T7 endonuclease I (T7E1 assay) can recognize and cleave malfunctions. Fragments with desired mutations are amplified via PCR and subjected to endonucleases later. Endonuclease cleavage occurs at the mismatch sites and results in shorter fragments, visible through electrophoresis. This method is easy, fast, cost-effective and scalable. Another high-throughput method to detect mutation is the high-resolution melt analysis (HRMA) (Bassett et al*.,* 2013). Mismatches change the melting temperature of the fragment of interest; therefore, mutations can be detected if the melting curve differs from the wild type. HRMA detects mutations with higher sensitivity and less time is required compared to the endonuclease assays. For species with high polymorphism such as *A. aegypti*, T7E1 and HMRA are prone to false positive. In this case, sequencing is a reasonable choice for mutation detection. In addition, sequencing provides information on the exact mutation, which is often used for precise mutation identification after T7E1 or HMRA. In addition, observing a mutagenesis-induced phenotype is a straight-forward way of evaluating efficiency, such as the yellow pigmentation from yellow-knock-out fruit flies (Baena-Lopez et al*.,* 2013). However, this is only realistic for certain targets.

CRISPR/CAS9 BASED EDITING IN INSECTS

Diptera

A. aegypti is the main vector for major arboviruses such as yellow fever, dengue and chikungunya viruses, which cause significant human health impacts. The CRISPR/Cas9 system has been used in *in vivo* gene disruptions of ECFP in ECFP transgenic *A. aegypti* lines (Dong et al*.,* 2015). Injection of *in vitro* transcribed Cas9 mRNA and sgRNA into embryos introduced mutations with 5.5% knockout efficiency. Kistler et al. (2015) increased the CRISPR/Cas9 mutation rate up to 24 per cent by identifying active sgRNAs and proper Cas9 mRNA concentrations in five *A. aegypti* genes. Up to 90 per cent of editing effectiveness was found in *A. aegypti*, in which a large cohort of sgRNAs were evaluated in early embryos, with only highly ranked candidates going forward to experiments based on germline. It should be noted that injection of plasmids encoding Cas9 and sgRNA did not increase observed mutations, which might result from the weak promoters used to drive Cas9 and sgRNAs or intrinsically low efficiency of the CRISP/Cas9 system in *A. aegypti.*

Drosophila: The first reported knockout application of CRISPR/Cas9 in insects was carried out in *D. melanogaster*, in which edits were introduced in the *yellow* gene. In *Drosophila*, there are generally four strategies for establishing the CRISPR/Cas9 system, depending on the delivery methods of Cas9 and sgRNA. In addition to the knockout technology, efficient and specific knock-in procedures mediated by CRISPR/Cas9 have greatly expanded CRISPR's application by facilitating complex genome modifications. Precise incorporation of exogenous DNA can be achieved by providing Donor DNA templates through homology-driven repair (HR). Previous studies have successfully injected donor DNA into embryos transgenic containing both Cas9 and sgRNA cascade, donor DNA with sgRNA encoding plasmids into transgenic Cas9 embryos and donor plasmids together with a plasmid encoding Cas9 and sgRNA into non-transgenic embryos.

Lepidoptera

Silkworm: B. mori reported its first successful application of the CRISPR/Cas9 system using the *BmBLOS2* gene as a target. Two 23 bp gRNAs were designed to direct the Cas9 endonuclease specifically to two sites separated by 3.48 kb of *BmBLOS2*, with a high mutagenesis rate for each site (94 and 95.6%) and large deletions achieved through the injection of Cas9 mRNA and sgRNAs transcribed *in vitro*. Similarly, four more genes (*Bm-ok, BmKMO, BmTH* and *Bmtan*) were altered at specific sites by the direct microinjection of specific sgRNA and Cas9 mRNA into embryos, with mutation frequencies of 16.7-35.0 per cent observed in the injected generation. Ma et al*.,* 2014 separately established a system of two vectors of expression for Cas9 and sgRNA, generating the heritable site-driven

edition of *Bmku70* in *B. mori*. This same system has also been used to simultaneously induce multiplex genome editing of six genes in the *BmN* cell lines. Apart from knockout, knock-in introduced by HR has been reported as well. Recent application of CRISPR/Cas9 has been found to induce high efficiency (92.5%) somatic mutations of the *Abd-B* gene in swallowtail butterfly, *Papilio xuthus* Linneaus. In addition, a highly efficient, heritable gene knockout at two clock gene loci, *cry2* and *clk* was reported in the monarch butterfly, *Danaus plexippus* (Linnaeus) , in which 50 per cent of larvae had indels at rates of 3-28 per cent. The *clk* knockout helped define its critical function in encoding a circadian clock transcription activator which monarchs used during migration.

Other insects

The CRISPR / Cas9 system has demonstrated great potential in the genetic modification of non-model species. It has been demonstrated that targeted knockouts, knock-ins, and deletions were effectively generated by CRISPR technology in *Tribolium castaneum* (Herbst). In addition, by direct injection of Cas9 mRNA and sgRNA into the *S. litura* embryos, CRISPR / Cas9 also induced efficient gene mutagenesis of the typically deficient phenotype of *abd-A* in *S. litura*, a serious agricultural pest worldwide. CRISPR/Cas9 has also been used in functional characterization of the *SlitPBP3* gene in *S. litura*. Furthermore, the *Dop1* gene was knocked out using the CRISPR/Cas9 system in the non-model *Gryllus bimaculatus* De Geer, resulting in mutants that were deficient in aversive learning with sodium chloride punishment but not in appetite learning with water or sucrose reward.

OTHER EMERGING CRISPR/CAS SYSTEMS

CRISPR/Cas12a

CRISPR/Cas12a (Cpf1) belongs to class II type V CRISPR system that is also an RNA-guided system. Although, *Francisella novicida* U112 (FnCpf1) is analogous to CRISPR/Cas9, but this system showed distinct characteristics including its 4 CRISPR/Cas-Based insect resistance in crops the efficiency of HDR to insert DNA fragment using complementary DNA ends is enhanced due to cohesive DNA ends. Cpf1 is more cost effective than Cas9 due to its requirement of a 42-nt CRISPR RNA (crRNA) instead of 100-nt scaffold crRNA required by Cas9.

CRISPR/Cas13a

CRISPR/Cas13a is entirely a new type of CRISPR belonging to class II type VI which can more efficiently target endogenous RNAs in plants and viral RNAs with RNA target specificity. It is associated with RNase activity due to the presence of higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains.

CRISPR/CasX

An SpCas9 variant, xCas9 was developed by phage-assisted continuous evolution and a broader range of protospacer adjacent motifs (PAMs) is recognized by this variant. The broader compatibility to PAM (including NG, GAT, and GAA), lower off-target activity, higher editing efficiency, and specificity is demonstrated by xCas9 as compared to SpCas9. The replacement of Cas9 with xCas9 in the BE3 vector generates CBE which can edit loci containing various PAMs such as NGN, GAA, and GAT.

CRISPR/Cas14

The compact CRISPR/Cas14a system is used as a guided GE tool to cut single stranded DNA (ssDNA). Cas14a, Cas14b, and Cas14c are three subgroups of 24 different Cas14 gene variants recognized on the bases of comparative sequence analysis. As compared to Cas9 protein the size of CRISPR/Cas14a is half and it does not require a flanking sequence (PAM) near to the target site.

PROSPECTIVE APPLICATION IN INSECT PEST MANAGEMENT

Gene drive

Gene drive refers to an increase in the frequency by bias inheritance of particular genes. Based on the CRISPR/Cas9 genome editing system, a mutagenic chain reaction (MCR) method was developed to conduct gene drive in *Drosophila*, in which MCR converted heterozygous mutations to *yellow* locus

homozygosity in germlines with 96 per cent homing efficiency by copying themselves through HR on the homologous chromosome. This study showed that the MCR technology in Drosophila was highly efficient and could be applied to other insect species. MCR technology was also established in *Anopheles stephensi* Listen , where eye color gene *kh* was targeted with 99.5% efficiency. Similarly, in *A. gambiae*, three female sterility genes (AGAP005958, AGAP011377 and AGAP007280) were identified and triggered expected phenotypes with 91.4 to 99.6 per cent efficiencies. Reducing female fertility possesses the potential to significantly reduce mosquito populations. These pilot experiments verify that by suppressing mosquito populations, the CRISPR-Cas9 gene drive system is a robust and valuable gene editing tool for functional genetic research in insects and in disease control. CRISPR/Cas9 based gene drive, allows the rapid spread of a DNA cassette into a target species. A normal allele has a 50% chance of being inherited by each offspring, but a gene drive cassette has more than 90% chance of being transmitted to the next generation owing to its ability to copy itself throughout the genome. Gene drive can bypass the vagaries of evolution, by manipulating both heredity and mutations: it enhances its transmission to the next generation, and mutations happen exactly where the gene drive has been designed to cut, producing the desired DNA sequence. In theory, the release of just a few individuals within a population could lead to complete invasion of the gene drive cassette within 15 to 20 generations.

BIOSAFETY CONCERNS

All of the biological/genetic research technologies have both positive and negative impacts. Genome editing tool has raised many ethical questions and opened many risks (Gaj et al., 2013). CRISPR/ Cas9 rapid use involves many issues, including off-target mutations. It would also become riskier to modify the insects using the CRISPR/Cas9 system including mosquitoes and release them into the environment (Oye et al*.,* 2014). Therefore, releasing mutant mosquitoes without any unwanted effects on humans becomes a challenge for the insect scientists (Ledford et al*.,* 2015). In general, to avoid ecological impairment, researchers applying CRISPR / Cas9 technology in science have to consider safety. Because CRISPR technology is easy to use and cheap, it has become a very popular technology worldwide which will eventually require international standards to test genetically modified insects, release them into the environment and assign liability for damage. The regulations should set clear requirements for testing the effectiveness of edited insects in carefully controlled environments or containing settings simulating their natural environments. Finally, edited insects should only be released after public consultation and appropriate consent of potentially affected populations in typical environments, whether in wild habitat or on a farm. Addressing these concerns regarding biosafety will require far more regulatory oversight than existing anywhere in the world.

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