Breakthrough of the Year, 2015

Science

TIME

The Gene Machine

What the CRISPR experiments mean for humanity

By Alice Park
Agenda

1. Genetic engineering defined
2. Gene editing defined
3. Examples and applications
4. Our work with CRISPR
5. Society, regulation, markets
Coauthor Estefania Elorriaga

PhD student, Molecular and Cellular Biology, OSU

Research on gene editing in poplar and eucalypts
What is genetic engineering (GE)

• Direct modification of DNA
  – Vs. indirect modification in breeding and genomic selection

• Asexually modified in somatic cells
  – Then regenerated into whole organisms, usually starting in Petri dishes
Steps to create a GE plant

- *Agrobacterium*-mediated transformation
- Biolistics or gene gun
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A big deal?

- Ability to modify native genes efficiently
- The theoretical becomes practical

“CRISPR/Cas9 is a game-changing technology that is poised to revolutionize basic research and plant breeding.”
Gene editing described

• Technique that allows specific changes to the genome
• Employs methods of genetic engineering but generally does not leave the editing agent in the genome
  – Editing agent enters cell but does not become part of genome
  – Editing agent sexually segregated away (progeny chosen with the edit, but not the editing agent)
  – Or agent somatically excised after editing
CRISPR gene editing system can be used for multiple purposes

• Mutations to destroy gene function
• Directed changes to sequence to change function
  – Proteins, RNAs, regulatory regions
• Gene or chromosome scale rearrangements (inversions, translocations)
• Ability to readily multiplex and mutate numerous genes at once
• Gene insertions directed at specific places
• Very low off-target rate in plants
• Conversion of alleles in successive generations (gene drive) – a useful means for control of serious diseases, pests, invasive exotic species?
Multiplex CRISPR: 62 genes targeted

Genome-wide inactivation of porcine endogenous retroviruses (PERVs)

Luhua Yang1,2,3,4,1,1, Marc Gueli1,2,3,1,1, Dong Niu1,2,3,1,1, Haydu George4,5,1,1, Emal Lesha1,1, Dennis Grishin1, John Aach1, Ellen Shrock1, Weiheong Xu1,1, Jürgen Poci1,1, Rebeca Cortazio1,1, Robert A. Wilkinson1, Jay A. Fishman1, George Church1,2,3,4,1

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Science 27 Nov 2015;
Vol. 350, Issue 6264, pp. 1101-1104
DOI: 10.1126/science.aad1191

Virally cleansing the pig genome

Transplants from pigs could be a solution to a shortage of human organs for transplantation. Unfortunately, porcine endogenous retroviruses (PERVs) are rife in pigs and can be transmitted to humans, risking disease. L. Yang et al. integrated CRISPR-Cas into the pig cell genome, where continuous induction of the Cas9 editing enzyme resulted in the mutation of every single PERV reverse transcriptase gene. This prevented replication of all copies of PERV, viral infection, and transmission to human cells.

Science, this issue p. 1101
Application to mint

- CRISPR most useful in crop systems where breeding is difficult, clonal varieties of high value, there are traits of value under simple genetic control (few genes)

- Mint breeding
  - Few elite clones
  - Complex ploidy (multiple chromosome sets)
  - Fertility very low
  - Undirected mutagenesis methods important

- Possible mint targets
  - Oil yield and quality
  - Disease resistance (Verticillium, others)
  - Any trait where the genic basis is known – growing with science
    - Genome sequence, QTL, and gene expression data growing
    - Molecular biology of terpenoid pathways – novel high value products?
Polyploid gene editing is effective

Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts

**Abstract**

**Key message**

Altered starch quality with full knockout of *GBSS* gene function in potato was achieved using CRISPR-Cas9 technology, through transient transfection and regeneration from isolated protoplasts.
CRISPR/Cas systems are the dominant form of gene editing

- CRISPR stands for Clustered, Regularly Interspaced, Short Palindromic Repeats
- The CRISPR/Cas system is an adaptive defense system in prokaryotes to fight against alien nucleic acids

Image credit: http://pnabio.com/products/RGEN.htm
“The story starts in the Mediterranean port of Santa Pola on Spain’s Costa Blanca, ...in a laboratory working on Haloferax mediterranei, an archaeal microbe with extreme salt tolerance...the salt concentration of the growth medium appeared to affect the way in which restriction enzymes cut the microbe’s genome...the [derived] fragments...did not resemble any family of repeats known in microbes..”
Lessons from CRISPR discovery

(Lander 2006, Cell)

• Breakthroughs often emerge from unpredictable origins
  – Motivations included curiosity, and military and industrial applications

• Growing “hypothesis-free” omic discovery

• Best science work early in career – often pre-30

• Seminal work not from eminent research centers

• Leading journals rejected all the early papers

• Science is a slow, global enterprise, with multiple authors and institutions contributing
Science journalist Carl Zimmer explains CRISPR DNA editing in 90 seconds.
A video with a more technical look at CRISPR
Sandman CRISPR!

"Bring Me a Gene"
Summary of CRISPR Cas-mechanism

Two major types of edits

1. Deleting a gene
2. Inserting a gene
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RNA interference (RNAi)

A natural mechanism of gene suppression.

Many products on the market. Gene editing products do similar things without the transgene present.
Non-browning “Arctic Apple”
RNAi suppression of native polyphenol oxidase gene expression

Courtesy of Jennifer Armen, Okanagan Specialty Fruits, Canada
Gene-edited CRISPR mushroom escapes US regulation

A fungus engineered with the CRISPR–Cas9 technique can be cultivated and sold without further oversight.

Emily Waltz

14 April 2016
Non-bruising PPO- and invertase-mutant potatoes produced by gene editing

NON-BRUISING GE POTATO CLEARED FOR SALE BY USDA

The GE potato that withstands bruising and browning has been cleared for sale by the U.S. Department of Agriculture. According to USDA, the GE potato is not considered as "regulated article" under federal law because it does not contain genes from plant pests.

The GE potato was developed by Calyxt, Inc. by introducing a TALEN reagent into potato gene glycol mediated transformation followed by temporary lentiviral vector to achieve PPO gene knockout and regeneration of protoplast plants. Thus, there is no foreign genetic material inserted into the potato plant.

Improwing cold storage and processing traits in potato through targeted gene knockout

Benjamin M. Clasen1, Thomas J. Stoddard1, Song Luo1, Zachary L. Demorest1, Jin Li1, Frederic Cedrone2, Redeat Tibe1, Shawn Davison1, Erin E. Ray1, Aurelie Daulhac1, Andrew Coffman1, Ann Yabandith1, Adam Retterath1, William Haun1, Nicholas J. Baltes1, Luc Mathis1, Daniel F. Voytas1 and Feng Zhang1†*1

1Calyxta, Inc., New Brighton, MN, USA.
2Calyxta, SA, Paris, France.
Pioneer’s CRISPR-edited waxy corn of high commercial value, unregulated.
CRISPR- modified grapefruit resistant to citrus canker

• Other examples:
  – Blight resistant rice (Zhou et al., 2014, Nucl. Acids Res.)
  – Fungus-resistant wheat (Wang et al., 2014, Nat Biotech)
  – Virus-resistant cucumber (Chandrasekaran et al., 2016, Molecular Plant Pathology)
Mutated yield-related genes in wheat leads to larger and more numerous grains

Reassessment of the Four Yield-related Genes Gn1a, DEP1, GS3, and IPA1 in Rice Using a CRISPR/Cas9 System

“...The T2 generation of the gn1a, dep1, and gs3 mutants featured enhanced grain number, dense erect panicles, and larger grain size, respectively....” Li et al. (2016)
Sugarcane, a polyploid, with lower lignin for use as biofuel

- Brown coloration seen in plants with high lignin reduction (22 to 32% reduction)
- Altpeter, 2016, Plant Molecular Biology
Other applications

• Soybean seeds with improved fatty acid content/profile (Haun et al. 2014, Plant Biotechnol Journal)

• Tobacco with improved glycosylation profiles for safer/faster production of pharmaceutical proteins (Li et al., 2016, Plant Biotechnol Journal 14)
Recombinetics creates hornless cattle

Open Season Is Seen in Gene Editing of Animals

By AMY HARMON  NOV. 26, 2015

A calf, left, approximately the same age as the first two genetically modified calves to have their DNA edited so that they do not grow horns, right. Jenn Ackerman for The New York Times
Directed gene modification
Homology-directed repair leads to herbicide-resistant crops

- Chlorsulfuron-resistant maize (Svitashev et al., 2015, Plant Physiology)
- Chlorsulfuron-resistant potato (Butler et al., 2016, Front. Plant Science)
- Chlorsulfuron-resistant soybean (Li et al., 2015, Plant Physiology)
- Chlorsulfuron-resistant rice (Sun et al., 2016, Molecular Plant)
- Glyphosate-resistant rice (Li et al., 2016, Nature Plants)
Promoter replacement using CRISPR increased grain yield in maize

Promoter replacement of the endogenous ARGOS8 gene leads to increased grain yield during flowering stress and no yield loss during well-watered condition.

ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions

Jinrui Shi, Huirong Gao, Hongyu Wang, H. Renee Lafitte, Rayeann L. Archibald, Meizhu Yang, Salim M. Hakimi, Hua Mo, Jeffrey E. Habben
A strong promoter, modified by gene editing, increased anthocyanin biosynthesis in tomato.

Čermák, T. et al., 2015, Genome Biol 16.
Gene drives for suppression of crop pests?

**Normal inheritance**
- Altered gene
- Wild type
- Male
- Female

Altered gene without gene drive: One copy inherited from one parent. 50 percent chance of passing it on.

Altered gene does not spread

**Gene drive inheritance**
- Gene drive
- Wild type
- Cut
- Repair

Altered gene as gene drive: One copy converts gene inherited from other parent. More than 50 percent chance of passing it on.

Altered gene is almost always inherited
REWIRING THE CODE OF LIFE

Through DNA editing, researchers hope to alter the genetic destiny of species and eliminate diseases.

By Michael Specter
Agenda

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CRISPR-Cas9 targeting of floral genes for genetic containment

• **Goal:** To develop robust male and female containment technologies for vegetatively propagated forest trees

• **Why:** Regulatory, market, and public acceptance with exotic and native trees can be costly or impossible – even for field research

• **Advantage of gene editing:** Expected to be more predictable and stable than alternative genetic containment methods that depend on modified gene expression

• **High efficiency:** Biallelic knock-outs needed in one or more genes
Target genes for bisexual sterility

• **LEAFY** – floral meristem prior to organ differentiation

• **AGAMOUS** – Male and female organ development and floral determinacy
Strong *lfy* mutants appear to have no flowers

Parcy et al. 2002; Moyroud et al. 2010
Flowers in strong \textit{ag} mutants are missing both stamens and carpels

\textit{Arabidopsis} \hspace{1cm} \textit{Ranunculid}

\textbf{WT}

\textbf{ag mutants}

Parcy et al. 2002; Galimba et al. 2012
LEAFY and AGAMOUS homologs in poplar studied in prior work
Field trials of RNAi-poplars
Sterility, normal growth of *LEAFY*-RNAi poplars

Klocko et al. 2016, *Nature Biotechnology*
Overview of CRISPR methods in poplar
Overview of CRISPR methods in poplar.
Experimental constructs – single and double targets per gene

Nuclease constructs

Control construct
Targeting two sites in the single-copy LFY gene in poplar

Target site for LFYsg1 (in exon 1)

Target site for LFYsg2

"PtLFY"

...CATGCACCAGTGAAA**GATCACAGAGAGAGAGACAA**GGGGGCAGATAGAT**ATGG**GATCCGGAGGCTTT
CACGGCGAGTTTTGTTCAAATGGGACACGAGAGCAATGCGCCTAACCCTCTGCTTTGAAATGGT
**GCCCCCGCCTACGAGCCAC**CGGCTGCGGCGTTTGCTGTAAGGCCAAGGGAGCTATGTGGGCTAGAGG
AGTTGTTTTCAAGCTTATGCTATTAGGTACTACACGGGAGCGAATGCTGAACCTGGGTTTCACAGTGA...

Exon 1 Exon 2 Exon 3

PtLFY locus
Targeting two identical sites in the two paralogous AG genes in poplar

>**PtAG1**

...GGATCAGCTAGCTAGACTGCAGCT**ATG**GAATATCAAAATGAATCCCTTGAGAGCTCCCCCCTGAAGAGCAGC
TAGGAA**GGGGAAAGGTGGAGATCAAG**CGGATGCAGGAACACCACACAAATC**GCCAAGTCACTTTCTGCAAAA**
AGGCCGCAGTGGTTTGCTCAAGAAAGGCCCTACAATTATCTGTCTTTTGTGATGCTGAGGTTGCACTCATCG...

Target site for AGsg2
Target site for AGsg1

>**PtAG2**

...GATCAGCTAGCTAGGAGCAGCT**ATG**GCATACCAAAATGAATCCCTTGAGAGCTCCCCCCTGAAGAGCAGC
TGGGRA**GGGGAAAGGTGGAGATCAAG**CGGATGCAGGAACACCACACAAATC**GYCAAGTCACTTTCTGCAAAA**
AGGCCGAATGGTTTGCTCAAGAAAGGCCCTATAATTATCTGTCTTTTGTGATGCTGAGGTTGCACTCATCG...

Exon 1

**PtAG1** and **PtAG2** loci
High mutation rates observed

• Cas9-only control events
  – No mutations

• CRISPR-Cas events
  – 80% with mutations
  – 50% knock-outs!
Large and small mutations – many routes to non-functioning gene

Mutations

Target site for LFYsg2

Target site for LFYsg1

Partial peptide sequence

Early stop codons
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Social dimensions

- Largest barriers to use are social, not technical
- Ethics: Is the method OK to use at all?
  - Impossible to trace with certainty
  - Do we need to add tracer DNA?
- Should it be regulated and labeled like GMOs?
- Should all mutagenesis uses of gene editing be excused from regulation?
- What about market forces and self-labeling?
It’s “too easy,” need to restrict use by public?

'Any idiot can do it.' Genome editor CRISPR could put mutant mice in everyone's reach

By Jon Cohen | Nov. 3, 2016, 10:00 AM

In the beginning of 2013, Michael Wiles sat down with high-level managers of the Jackson Laboratory in Bar Harbor, Maine, and told them about a novel way to cut DNA that had amazing power. The lab, called JAX for short, genetically engineers mice that it sells to researchers under a trademarked brand: JAX® Mice, it likes to boast, “are the highest quality and most-published mouse models in the world.” Wiles evaluates and develops technologies for the lab, and he was convinced that this new strategy that bacteria and archaea use to protect the way. JAX engineered mice. “Of about a dozen
Did a Swedish researcher eat the first CRISPR meal ever served?

By Jon Cohen | Sep. 7, 2016, 2:30 PM

In what Swedish plant scientist Stefan Jansson declares “maybe” a historic event, he cultivated, grew, and ate a plant that had its genome edited with CRISPR-Cas9. Umeå University, where Jansson studies how trees know it’s autumn and how proteins allow plants to harvest light, released a 5 September press release about his meal, a pasta dish that included 300 grams of cabbage he grew from seeds that had been genetically modified with CRISPR-
Major wars over CRISPR patents ongoing
Global regulatory quandry

Regulatory uncertainty over genome editing

Huw D. Jones

Genome editing opens up opportunities for the precise and rapid alteration of crops to boost yields, protect against pests and diseases and enhance nutrient content. The extent to which applied plant research and crop breeding benefit will depend on how the EU decides to regulate this fledgling technology.

We are at the dawn of a new paradigm in plant breeding. Classical approaches to crop improvement based on hybridization and selection can now be complemented by targeted genome editing that exploits knowledge of specific gene sequences in a systematic way. Unlike conventional genetic modification that results from the insertion of large pieces of exogenous DNA, or maize renders the plants highly resistant to lepidopteron pests; these lepidopteron-resistant crops are grown around the world. However, this technique cannot be used to make small edits to existing genes, and can lead to the random disruption of native genes because the destination of the inserted DNA cannot be dictated.

In contrast to traditional genetic modification, genome editing makes use of one or a few bases at the cut site, resulting in a mutation. Mutations generated in this way are indistinguishable from those that occur naturally and drive evolution, as well as from those induced through the application of chemical mutagens or radiation, as employed in mutation breeding programmes since the 1940s.

Here, I focus on the potential applications and regulation of this simple 'cut and repair'
NEW US LABELING LAW FOR BIOENGINEERED FOODS

September 28, 2016

Building on the work of individual states in preparing and implementing laws to regulate the labeling of bioengineered foods, the USA has enacted a federal law providing countrywide protection and consistency for consumers.

Jim Cook, SGS Food Scientific and Regulatory Affairs Manager explains in more detail.

On July 1, 2016, the USA's first labeling law, the Vermont Genetically Engineered (GE) food labeling law Act 120 became effective but as of July 29 when President Obama signed the National Bioengineering Food Disclosure Law 1 it...
New USDA proposal on Jan 19, 2017

DEPARTMENT OF AGRICULTURE
Animal and Plant Health Inspection Service

7 CFR Part 340
[Docket No. APHIS–2015–0057]
RIN 0579–AE15

Importation, Interstate Movement, and Environmental Release of Certain Genetically Engineered Organisms

AGENCY: Animal and Plant Health Inspection Service, USDA.

ACTION: Proposed rule.

SUMMARY: APHIS is proposing to revise its regulations regarding the importation, interstate movement, and environmental release of certain genetically engineered organisms in order to update the regulations in response to advances in genetic engineering and understanding of the plant pest and noxious weed risk posed by genetically engineered (GE) organisms, thereby reducing burden for regulated entities whose organisms pose no plant pest or noxious weed risks. This would be the first comprehensive revision of the regulations since they were established in 1987.

DATES: We will consider all comments that we receive on or before May 19, 2017.

SUPPLEMENTARY INFORMATION:

Background

Overview of the Current Regulations

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) administers regulations in 7 CFR part 340, “Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which are Plant Pests or Which There is Reason to Believe are Plant Pests” (referred to below as the regulations). The current regulations govern the introduction (importation, interstate movement, or release into the environment) of certain genetically engineered (GE) organisms that are considered “regulated articles.”

Under the current regulations, a GE organism is considered to be a regulated article if the donor organism, recipient organism, vector, or vector agent is a plant pest or if the Administrator has reason to believe the GE organism is a plant pest. A plant pest is defined in §340.1 as “Any living stage (including active and dormant forms) of insects, mites, nematodes, slugs, snails, protozoa, or other invertebrate animals, bacteria, fungi, other parasitic plants or reproductive parts thereof; viruses; or any organisms similar to or allied with any of the foregoing; or any infectious agents or substances, which can directly or indirectly injure or cause disease or

article pursuant to 7 CFR part 340. Agency Actions Following Promulgation of the Current Regulations

APHIS first issued these regulations in 1987 under the authority of the Federal Plant Pest Act of 1957 (FPPA) and the Plant Quarantine Act of 1912 (PQA), two acts that were subsumed into the Plant Protection Act (PPA, 7 U.S.C. 7701 et seq.) in 2000, along with other provisions. Since 1987, APHIS has amended the regulations six times, in 1988, 1990, 1993, 1994, 1997, and 2005, to institute exemptions from permitting for certain microorganisms and Arabidopsis, to institute the notification, petition, and extension procedures referenced above, and to exclude plants engineered to produce industrial compounds from the notification process.

Although, as discussed above, the current regulations have various functions, their primary function to date has been as a means for APHIS to authorize the importation, interstate movement, and introduction of certain GE organisms via the permit and notification procedures referred to above. Permits and notifications are collectively known as “authorizations.” To date, APHIS has issued more than 18,000 authorizations for the environmental release of GE organisms in multiple sites, primarily for research and development of improved crop varieties for agriculture. Additionally, APHIS has issued more than 12,000...
Gene editing is GE...

• “For the purposes of this rule, APHIS is proposing to consider genome editing to be within the definition of genetic engineering. “

• Does that mean every editing line must come before USDA?

• How much data and confinement is required during research and breeding?

• Is it essentially a GMO?
But.....the definition of GE is further restricted...

• ... an organism will not be considered a genetically engineered organism if:

• (1) The genetic modification to the organism is solely a deletion of any size or a single base pair substitution which could otherwise be obtained through the use of chemical- or radiation-based mutagenesis; or
But.....the definition of GE is further restricted...

• ... an organism will not be considered a genetically engineered organism if:

• (2) The genetic modification to the organism is solely introducing only naturally occurring nucleic acid sequences from a sexually compatible relative that could otherwise cross with the recipient organism and produce viable progeny through traditional breeding...or
But.....the definition of GE is complex

• ... an organism will not be considered a genetically engineered organism if:

• (3) The organism is a “null segregant,” that is, the progeny of a GE organism where the only genetic modification was the insertion of donor nucleic acid into the recipient’s genome, but the donor nucleic acid is not passed to the recipient organism’s progeny
What does this mean?

• “For the purposes of this rule, APHIS is proposing to consider genome editing to be within the definition of genetic engineering. “

• Does that mean every editing line must come before USDA?

• How much data and confinement is required during research and breeding?

• Is it essentially a GMO or not?
ASTA's overarching policy is that plant varieties developed through the latest breeding methods should not be differentially regulated if they are similar to or indistinguishable from varieties that could have been produced through earlier breeding methods.
Regulation of gene editing depends on decisions of three agencies – coordinated but distinct

**EPA** - Environmental Protection Agency: Biopesticides

**USDA** – US Department of Agriculture:
Agricultural products of plant or animal origin

**FDA** - Food & Drug Administration:
Food consumed by humans or animals
FDA is proposing to regulate gene edited animals as animal “drugs”
Markets are another thing....

The National Organic Standard Boards has banned gene editing technologies

“Every organic stakeholder is clear that genetic engineering is an imminent threat to organic integrity. Every effort must be made to protect that integrity,”
Proliferation of self-defined no-GMO labels likely to exclude gene editing?

Summary

• Gene editing works well everywhere it's been tried
  – This one is not hype!
• Still depends on capability for GE
  – Difficult in many species and genotypes
• Significant social issues and uncertainties
  – Ethics, regulation, market exclusions, labeling, and patents
  – Key determinants of whether this technology will matter a lot or not so much
• Could be a powerful tool for crops, including mint?
  – Key constraints are science knowledge, genetic engineering capability, and regulation/markets
Thanks

Cathleen Ma

Stef Elorriaga

Amy Klocko

Haiwei Lu

Flavia Tussulini

Clark Embleton, High School intern