Next-generation gene-editing technology: Path to a second Green Revolution?

One of the major limitations of the first-generation rDNA-based GM methods is the randomness of DNA insertions into plant genomes, just as the earlier mutagenesis methods introduced mutations randomly. The newer methods increase the specificity and precision with which genetic changes can be made. Known under the general rubric of “sequence-specific nuclease (SSN) technology” or gene/genome-editing, this approach uses proteins or protein-nucleic acid complexes that bind to and cut specific DNA sequences.[1] SSNs include transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and meganucleases.[2]

[This is part three of a four-part series on the progress of agricultural biotechnology. Read part one , part two and part four.]

The DNA cuts made by SSNs are repaired by cellular processes that often either change one to several base pairs or introduce deletions and/or insertions (aka indels) at the target site. Another recently added technology capable of editing gene sequences is termed oligonucleotide-directed mutagenesis (ODM) and uses short nucleic acid sequences to target mutations to selected sites.[3]

The hottest and the coolest

What is rapidly emerging as the most powerful of the SSN technologies is known by the uninformative acronym CRISPR/Cas, which contracts the unwieldy designation “clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein (Cas9)”. It’s based on a bacterial defense system against invading viruses and promises extraordinary versatility in the kinds of genome changes that it can make.[1,4]

CRISPR Cas infographic
Image Credit: SEARCA Biotechnology Information Center

The CRISPR/Cas editing “molecular machine” is comprised of an enzyme (Cas9 and other variants) that binds an RNA molecule (called the “guide” RNA or gRNA) whose sequence guides the complex to the matching genomic sequence, allowing the Cas9 enzyme to introduce a double-strand break within the matching sequence. The CRISPR/Cas system can be used to edit gene sequences, to introduce a gene or genes at a pre-identified site in the genome, and to edit multiple genes simultaneously, none of which could be done with rDNA methods.[1,5]

Many of the genetic changes created using either SSN or ODM are indistinguishable at the molecular level from those that occur in nature or are produced by mutation breeding. Since both spontaneous mutants and chemical- and radiation-induced mutants have been used in crop improvement without regulation, there is no scientific rationale for regulating mutants produced by the newer methods. In hopes of creating a distinction that will permit exemption of gene-edited crops from regulation, the newer methods are increasingly referred to as “new plant breeding techniques (NPBTs or just NBTs).”

Quick successes for NBTs?

Prime targets of gene editing are cellular proteins that are involved in pathogenesis.[6] Virus reproduction requires the recruitment of cellular proteins for replication, transcription and translation. There can be sufficient redundancy in the requisite protein infrastructure so that partial or complete virus resistance can be achieved by disrupting genes that code for proteins required for viral replication without damaging crop productivity.

For example, work with mutants of the model plant Arabidopsis identified translation initiation factor *eIF4E* as required for potyvirus translation. CRISPR/Cas-induced point mutations and deletions have recently been reported to enhance viral resistance not only in Arabidopsis, but in cucumber and cassava, as well.[7]

brown streaked l hires

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A farmer holds cassava affected by the brown streak virus, indicated by the brown splotches that make the root inedible

The many ways that plants and their bacterial and fungal pathogens interact offer opportunities to use gene editing to enhance plant disease resistance and reduce agriculture's dependence on chemical control agents.[6] The two main strategies are to inactivate genes whose products render the host plant sensitive to pathogen invasion and to enhance the ability of the host plant to resist invasion by providing functional resistance factors they lack.

An example of the former is provided by the mildew resistance resulting from the inactivation of all three homeoalleles of the mildew resistance locus (MLO) of hexaploid wheat.[8] The efficiency of targeting both multiple alleles and multiple loci has taken a further jump with the development of "multiplexed" gene editing using vectors carrying several gRNA sequences capable of being processed by cellular enzymes to release all of them. This allows the gRNAs to edit multiple genes simultaneously.[9]

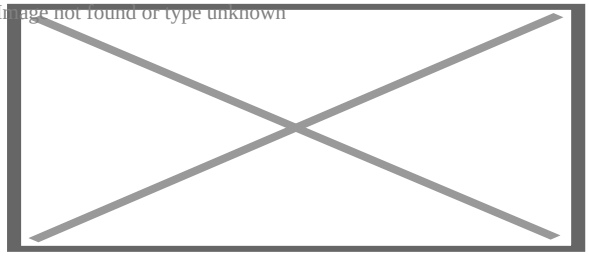
The second approach is to capitalize on the formidable arsenal of resistance genes residing in plant genomes.[10] Fungal resistance genes have long been a major target of breeders' efforts and have proved frustratingly short-lived, as pathogens rapidly evolve to evade recognition.[11] While desirable resistance

genes missing from domesticated crops still reside in wild relatives, extracting them by conventional breeding methods can be time-consuming or impossible.

European academic researchers created transgenic potatoes resistant to the late blight (*Phytophthora infestans*) that caused the Irish potato famine by inserting resistance (R) genes cloned from wild potato species into commercial potato varieties.[12] A blight-resistant variety, called the "Innate™ Generation 2" potato, is being commercialized by J.R. Simplot company in the U.S. and Canada and is already being marketed in the U.S. as the "White Russet" ™ Idaho potato.[13] Transgenic disease-resistance traits have been introduced in other crops, but have yet to be commercialized.[14]

Plant genomes contain hundreds to thousands of potential R genes, but it is not yet possible to determine whether a given one will confer resistance to a particular pathogen. Methods are currently being developed to accelerate the identification and cloning of active ones.[14] Once identified, CRISPR/Cas can be used to introduce cassettes carrying multiple R genes, making it possible to create more durable resistance than can be achieved by introducing a single R gene through conventional breeding[14]. Finally, direct editing of resident inactive R genes using a ribonucleoprotein (RNP) strategy that avoids creating a transgenic plant may prove useful, although no such products appear to be in the pipeline to commercialization at present.[15,16]

Multiplexed editing has proved particularly useful for editing genes in polyploid species. For example, Cas9/sgRNA-mediated knockouts of the six fatty acid desaturase 2 (*FAD2*) genes of allohexaploid *Camelina sativa* was reported to markedly improve the fatty acid composition of Camelina oil.[17] Using a different approach, Yield10 Biosciences is moving toward commercialization of a high-oil Camelina developed by editing a negative regulator of acetyl-CoA carboxylase.[18]



Gene-edited soybeans are used to make more healthful soybean oil.

As of this writing, the only gene-edited product that has been commercialized is a soybean oil with no trans-fat, trademarked Calyno™, developed by Calyxt.[19] Gene-edited crops that have been approved but not commercialized or are still in the regulatory pipeline include miniature tomatoes, high-fiber wheat, high-yield tomatoes, improved quality alfalfa, non-browning potatoes and mushrooms, as well as high starch-content and drought-resistant corn, most being developed by small biotech companies.[19]

Getting beyond the low-hanging fruit

It is becoming increasingly clear that yield increases in our major crops by traditional breeding approaches are not keeping pace with demand.[20] The gap is likely to widen as climate warming moves global temperatures farther from those prevailing when our crops were domesticated.

Overexpression of stress-related transcription factors has been reported to increase yields under water-stress conditions, but such increases are generally not maintained under optimal conditions.[21] Monsanto's drought-tolerant (Genuity DroughtGard™) corn hybrids are based on the introduction of bacterial chaperone genes.[22] Fortunately, research into drought stress tolerance in wheat and other grains continues apace, although no drought-tolerant varieties have yet reached farmers.[23]

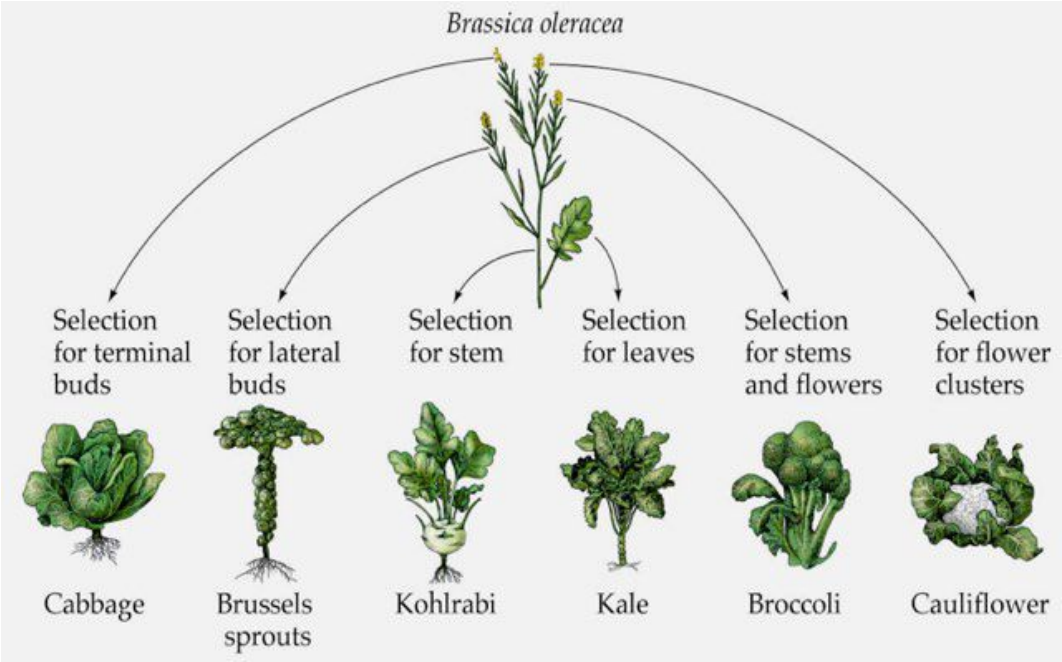
Real progress on crop yield is slow. What stands in the way is that we have so limited an understanding of how plants work at the molecular level. At every level of analysis, organisms are redundant networks of interconnected proteins that adjust their manifold physical and enzymatic interactions in response to internal signals and external stimuli, then send messages to the information storage facilities (DNA) to regulate their own production and destruction rates.

As well, many genes are present in families of between two and hundreds or thousands of similar members, making it difficult to determine either the function or the contribution of any given member to a complex trait such as stress tolerance or yield. That said, gene family functions are identifiable and some, such as transcription factor genes, encode proteins that influence multiple other genes, making them among the likeliest candidates for manipulation. Indeed, studies on the genetics of domestication often point to changes in transcription factor genes.[24]

But while there have been reports that constitutive overexpression of single transcription factor gene can increase grain yield in both wheat and maize, none appear to have been commercialized yet.[25]
The challenge of developing a yield-improved variety by simply overexpressing transcription factor genes is illustrated by a recent report from Corteva.[26] It describes a tour-de-force involving generation and testing of countless transgenic plants to identify a single transcription factor gene, *ZMM28*, that reproducibly increased yield when incorporated into 48 different hybrids and tested over a 4-year period in 58 locations.[26]

Getting there by a different route

Might gene-editing facilitate the task of generating and identifying yield-enhancing genetic variation? While the CRISPR/Cas toolkit is growing at dizzying speed, its utility in crop improvement has so far been limited to the simple traits controlled by individual genes, albeit including multiple alleles.[1,27]



Credit: Vox

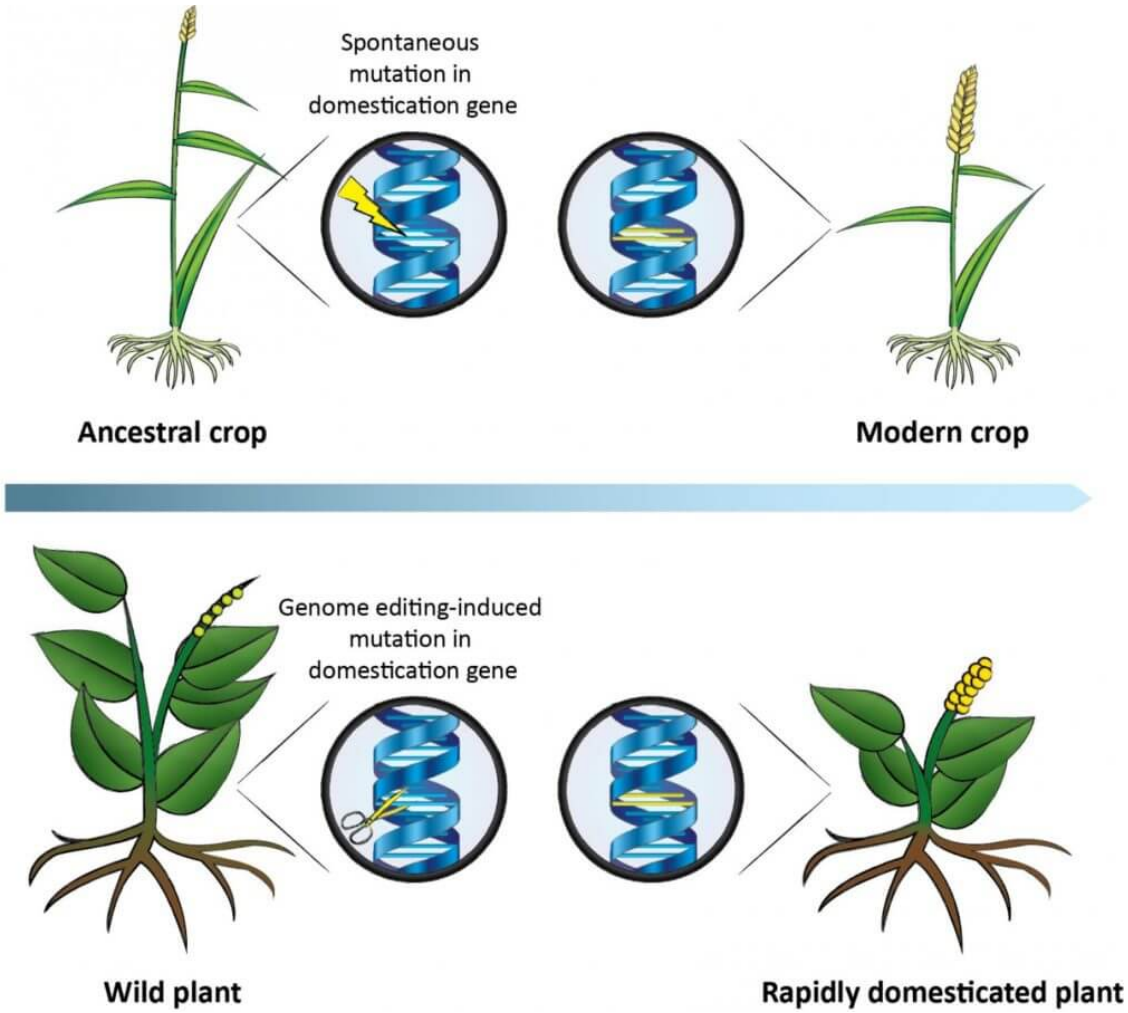
Crop domestication and plant breeding have vastly narrowed genetic diversity because the very process of selecting plants with enhanced traits imposes a bottleneck, assuring that only a fraction of the ancestral population's genetic diversity is represented in a new elite variety. This, in turn, limits what can be done by mutagenizing existing elite varieties, a process that is also burdened with the necessity to eliminate deleterious mutations through back-crossing.

But to widen the genetic base and to modify genes that contribute to quantitative traits, it is still first necessary to identify the genes that contribute to agronomically important traits. Identifying such genes is currently a slow and tedious process of conventional and molecular mapping.[28] A recent report describes a method for combining pedigree analysis with targeted CRISPR/Cas-mediated knockouts that promises to markedly accelerate the identification of the individual contributing genes in the chromosomal regions that are associated with quantitative traits, technically known as quantitative trait loci (QTLs).[29]

Even as the QTL knowledge gap narrows, gRNA multiplexing is extending the power of SSNs to understanding and modifying complex traits in crop plants. For example, using multiplexed gRNAs, Cas nuclease was simultaneously targeted to three genes known to be negative regulators of grain weight in rice.[30] The triple mutants were reported to exhibit increases in the neighborhood of 25% in each of the three grain weight traits: length, width and thousand grain weight.

In another study, 8 different genes affecting rice agronomic traits were targeted with a single multiplexed gRNA construct and all showed high mutation efficiencies in the first generation.[31] Conversely, it has been reported that editing the same QTLs gives different outcomes in different elite varieties, improving yield in some but not other.[32]

Mutations affecting the expression of regulatory genes, such as transcription factors genes, account for a substantial fraction of the causative genetic changes during crop domestication.[33] Multiplexed gRNAs constructs targeting *cis*-regulatory elements (CREs) have been used to generate large numbers of allelic variants of genes affecting fruit size in tomato, mimicking some of the mutations accumulated during domestication and breeding of contemporary tomato varieties.[34]



Knowledge of domestication genes can also be used to accelerate domestication of wild plants that retain traits of value, such as salt tolerance, as reported for tomato.[35] This opens the possibility of rapidly domesticating wild species better adapted to the harsher climate conditions of the future.

While the above-described advances have been based on the CRISPR/Cas-mediated deletions, approaches to more precise sequence editing are developing as well. While Cas-generated cuts in the DNA are most commonly repaired by the non-homologous end joining pathway (NHEJ), the less frequent homology-directed repair pathway (HDR) has been shown to edit sequences at useful frequencies using Cas-gRNA ribonucleoprotein complexes.[15,36]

As well, mutant Cas9 proteins lacking nuclease activity have been fused with base-editing enzymes such as cytidine and adenosine deaminases to direct gene editing without DNA cleavage.[37,38] This approach can change single base pairs precisely in both coding and non-coding regions, as well alter mRNA precursor processing sites.[38] Finally, the sequence targeting properties of the CRISPR-Cas system can be used to deliver other types of hybrid proteins to target sequences to regulate gene expression and DNA methylation.[27]

In sum, the many variations on gene editing now developing hold the promise of revolutionizing crop breeding, prompting several colleagues to whimsically title a recent review of CRISPR/Cas-based methodology: "Plant breeding at the speed of light".[39] And indeed, the new methods make it possible to replace chemicals with biological mechanisms in protecting plants from pests and disease, as well as increase their resilience to stress.

That said, extraordinary progress in increasing grain yields has already been accomplished by what are now considered to be "traditional" breeding methods and increased fertilizer use. Further improvements continue, but will likely be harder won than the many-fold increases in corn, wheat and rice yields of the last century and its Green Revolution. But there is a persistent disconnect between what can be done to accelerate plant breeding using the new gene-editing toolkit and what is actually being done by both the public and private sectors to get varieties improved by these methods out to farmers.

[1]Zhang Y et al. (2019). The emerging and uncultivated potential of CRISPR technology in plant science. Nature Plants **5**:778-94.

[2]Podevin N et al. (2013). Site-directed nucleases: a paradigm shift in predictable, knowledge-based plant breeding. Trends Biotechnol **31**:375-83.

[3]Sauer NJ et al. (2016). Oligonucleotide?directed mutagenesis for precision gene editing. Plant Biotechnol J **14**:496-502.

[4]Zhang D et al. (2016). Targeted gene manipulation in plants using the CRISPR/Cas technology. J Genet Genomics **43**:251-62.

[5]Cong L et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science **339**:819-23.

[6]Borrelli VM et al. (2018). The enhancement of plant disease resistance using CRISPR/Cas9 technology. Frontiers Plant Sci **9**:Article 1245.

[7]Chandrasekaran J et al. (2016). Development of broad virus resistance in non?transgenic cucumber using CRISPR/Cas9 technology. Molec Plant Pathol **17**:1140-53; Pyott DE et al. (2016). Engineering of CRISPR/Cas9?mediated potyvirus resistance in transgene?free Arabidopsis plants. Molec Plant Pathol **17**:1276-88; Gomez MA et al. (2019). Simultaneous CRISPR/Cas9?mediated editing of cassava eIF 4E isoforms nCBP?1 and nCBP?2 reduces cassava brown streak disease symptom severity and incidence.

Plant Biotechnol J **17**:421-34.

[8]Wang Y et al. (2014). Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nature Biotechnol **32**:947.

[9]Xie K et al. (2015). Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc Natl Acad Sci **112**:3570-5; Wang W et al. (2018). Transgenerational CRISPR-Cas9 activity facilitates multiplex gene editing in allopolyploid wheat. The CRISPR J **1**:65-74.

[10]Petit-Houdenot Y and Fudal I (2017). Complex interactions between fungal avirulence genes and their corresponding plant resistance genes and consequences for disease resistance management. Frontiers Plant Sci **8**:1072.

[11]Bebber DP and Gurr S (2015). Crop-destroying fungal and oomycete pathogens challenge food security. Fungal Genet Biol **74**:62-4; van Esse HP et al. (2020). Genetic modification to improve disease resistance in crops. New Phytol **225**:70-86.

[12]Jones JD et al. (2014). Elevating crop disease resistance with cloned genes. Phil Trans Royal Soc B: Biol Sci **369**:20130087; Haesaert G et al. (2015). Transformation of the potato variety Desiree with single or multiple resistance genes increases resistance to late blight under field conditions. Crop Protection **77** :163-75.

[13]Halsall M. Innate outlook. Spudsmart, 24 April 2019 https://spudsmart.com/innate-outlook/

[14]Dong OX and Ronald PC (2019). Genetic engineering for disease resistance in plants: recent progress and future perspectives. Plant Physiol **180**:26-38.

[15]Svitashev S et al. (2016). Genome editing in maize directed by CRISPR–Cas9 ribonucleoprotein complexes. Nature Communications **7**:1-7.

[16]Mao Y et al. (2019). Gene editing in plants: progress and challenges. Nat Sci Rev **6**:421-37.

[17]Morineau C et al. (2017). Selective gene dosage by CRISPR?Cas9 genome editing in hexaploid Camelina sativa. Plant Biotechnol J **15**:729-39; Jiang WZ et al. (2017). Significant enhancement of fatty acid composition in seeds of the allohexaploid, Camelina sativa, using CRISPR/Cas9 gene editing. Plant Biotechnol J **15**:648-57.

[18]Yield10 Bioscience (Jan 16, 2020). Yield10 Bioscience submits “Am I Regulated?” letter to USDA-APHIS BRS for CRISPR genome-edited C3007 in Camelina to pave the way for U.S. field tests. https://www.globenewswire.com/news-release/2020/01/16/1971418/0/en/Yield10-Bioscience-Submits-Am-I-Regulated-Letter-to-USDA-APHIS-BRS-for-CRISPR-Genome-Edited-C3007-in-Camelina-to-Pave-the-Way-for-U-S-Field-Tests.html

[19]Genetic Literacy Project (2020). Global Gene Editing Regulation Tracker. https://crispr-gene-editing-regs-tracker.geneticliteracyproject.org/united-states-crops-food/

[20]Ray DK et al. (2013). Yield trends are insufficient to double global crop production by 2050. PloS One **8**:e66428.

[21]Rice EA et al. (2014). Expression of a truncated ATHB17 protein in maize increases ear weight at silking. PLoS One **9**:e94238; Araus JL et al. (2019). Transgenic solutions to increase yield and stability in wheat: shining hope or flash in the pan? J Experimental Bot **70**:1419-24.

[22]Castiglioni P et al. (2008). Bacterial RNA chaperones confer abiotic stress tolerance in plants and improved grain yield in maize under water-limited conditions. Plant Physiol **147**:446-55.

[23]Mwadzingeni L et al. (2016). Breeding wheat for drought tolerance: Progress and technologies. J Integrative Agricult **15**:935-43; Sallam A et al. (2019). Drought stress tolerance in wheat and barley: Advances in physiology, breeding and genetics research. Internat J Mol Sci **20**:3137.

[24]Swinnen G et al. (2016). Lessons from domestication: targeting cis-regulatory elements for crop improvement. Trends Plant Sci **21**:506-15.

[25]Nelson DE et al. (2007). Plant nuclear factor Y (NF-Y) B subunits confer drought tolerance and lead to improved corn yields on water-limited acres. Proc Natl Acad Sci **104**:16450-5; Qu B et al. (2015). A wheat CCAAT box-binding transcription factor increases the grain yield of wheat with less fertilizer input. Plant Physiol **167**:411-23; Yadav D et al. (2015). Constitutive overexpression of the TaNF-YB4 gene in transgenic wheat significantly improves grain yield. J Experiment Bot **66**:6635-50.

[26]Wu J et al. (2019). Overexpression of zmm28 increases maize grain yield in the field. Proc Natl Acad Sci **116**:23850-8.

[27]Chen K et al. (2019). CRISPR/Cas genome editing and precision plant breeding in agriculture. Annu Rev Plant Biol **70**:667-97.

[28]Cavanagh C et al. (2008). From mutations to MAGIC: resources for gene discovery, validation and delivery in crop plants. Curr Opin Plant Biol **11**:215-21.

[29]Huang J et al. (2018). Identifying a large number of high-yield genes in rice by pedigree analysis, whole-genome sequencing, and CRISPR-Cas9 gene knockout. Proc Natl Acad Sci **115**:E7559-E67.

[30]Xu R et al. (2016). Rapid improvement of grain weight via highly efficient CRISPR/Cas9-mediated multiplex genome editing in rice. J Genet Genom **43**:529.

[31]Shen L et al. (2017). Rapid generation of genetic diversity by multiplex CRISPR/Cas9 genome editing in rice. China Sci Life Sci **60**:506-15.

[32]Shen L et al. (2018). QTL editing confers opposing yield performance in different rice varieties. J Integrative Plant Biol **60**:89-93; Zhou J et al. (2019). Multiplex QTL editing of grain-related genes improves yield in elite rice varieties. Plant Cell Rep **38**:475-85.

[33]Meyer RS and Purugganan MD (2013). Evolution of crop species: genetics of domestication and diversification. Nature Rev Genet **14**:840-52.

[34]Rodríguez-Leal D et al. (2017). Engineering quantitative trait variation for crop improvement by genome editing. Cell **171**:470-80. e8.

[35]Li T et al. (2018). Domestication of wild tomato is accelerated by genome editing. Nature Biotechnol **36** :1160-3; Zsögön A et al. (2018). De novo domestication of wild tomato using genome editing. Nature Biotechnol **36**:1211-6.

[36]Puchta H et al. (1996). Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination. Proc Natl Acad Sci **93**:5055-60; Zhang Y et al. (2016). Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nature Communications **7**:1-8.

[37]Komor AC et al. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature **533**:420-4; Hua K et al. (2019). Expanding the base editing scope in rice by using Cas9 variants. Plant Biotechnol J **17**:499-504.

[38]Kang B-C et al. (2018). Precision genome engineering through adenine base editing in plants. Nature Plants **4**:427-31.

[39]Wolter F et al. (2019). Plant breeding at the speed of light: the power of CRISPR/Cas to generate directed genetic diversity at multiple sites. BMC Plant Biol **19**:176.

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