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Plant genome engineering from lab to field—a Keystone Symposia report

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Facing the challenges of the world's food sources posed by a growing global population and a warming climate will require improvements in plant breeding and technology. Enhancing crop resiliency and yield via genome engineering will undoubtedly be a key part of the solution. The advent of new tools, such as CRIPSR/Cas, has ushered in significant advances in plant genome engineering. However, several serious challenges remain in achieving this goal. Among them are efficient transformation and plant regeneration for most crop species, low frequency of some editing applications, and high attrition rates. On March 8 and 9, 2021, experts in plant genome engineering and breeding from academia and industry met virtually for the Keystone eSymposium "Plant Genome Engineering: From Lab to Field" to discuss advances in genome editing tools, plant transformation, plant breeding, and crop trait development, all vital for transferring the benefits of novel technologies to the field.

Keywords: CRISPR/Cas; NHEJ; HDR; MMEJ; DNA repair; multiplex genome editing; plant breeding; plant genome editing; plant genome engineering; prime editing; TALENs

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Introduction

Over the past few decades, plant genome-editing tools have undergone several iterations. In the late 1980s, meganucleases were extensively used in plant genetic engineering. In 1996, the first application of zinc-finger nucleases (ZFNs) to act as a hybrid restriction enzyme was reported. While ZFNs have been successful in generating herbicide-resistant plants and in achieving several types of targeted mutagenesis, they are no longer commonly used.[a] More recently, transcription activator-like effector nucleases (TALENs) have been developed based on the bacterial enzyme transcription activator-like effector (TALE) that targets specific DNA sequences in plant genomes and induces gene expression.[1,2]

The most recent addition to the genetic engineering toolbox is CRISPR/Cas. CRISPR/Cas technology is revolutionizing molecular biology. In addition to the exciting potential to treat genetic diseases,[3] it also has the potential to create a novel, sustainable agriculture. In brief, CRISPR/Cas systems consist of a guide RNA (gRNA) and a Cas nuclease. The gRNA chaperones Cas nuclease to a target sequence within the DNA, where it induces a double-strand break (DSB). In addition to the target sequence defined by the gRNA, Cas nucleases require a protospacer adjacent motif (PAM) near the DNA target sequence in order to recognize and cleave DNA. The position and sequence of the PAM varies according to the type of Cas nuclease used.

CRISPR/Cas systems can be used to achieve a variety of genetic modifications. In plants, initial applications consisted of generating gene knockout lines for functional studies and crop improvement. The technology has subsequently been improved and modified to achieve a variety of precise DNA modifications, including base editing, that is, a single base transition within the editing window; prime editing, that is, short indels, base transitions and transversions; and homology-directed repair (HDR), which can lead to all kinds of modifications, including the insertion of large DNA fragments/genes.[4]

CRISPR/Cas-mediated genome editing technologies enable precise modifications of DNA sequences *in vivo* and offer great promise for crop improvement. In particular, harnessing genetic diversity and introducing elite alleles from wild relatives or landraces into commercial cultivars has been a major goal in crop breeding programs. Compared with commercial breeding and traditional genetically modified organism trait development, gene editing for trait development is both time and cost-effective. However, key challenges remain in being able to use these tools efficiently in plants. Many of these challenges are not related to CRISPR/Cas itself but in delivering gene-editing reagents into a large variety of plant cells and in regenerating plants from transgenic cells grown in culture.[b]

Since the first gene-edited plants were developed over 10 years ago,[5–7] the field has seen enormous progress. On March 8 and 9, 2021, experts in plant genome engineering and breeding from academia and industry met virtually for the Keystone eSymposium "Plant Genome Engineering: From Lab to Field." Meeting organizers **Caixia Gao** from the

[a] All site-directed nucleases (SDNs) can have an off-target effect, which is very much related to the complexity of plant genomes, ploidy level, redundancy, and excess of repetitive sequences. ZFNs can be specific and very efficient in generating targeted DSBs; the main drawback is associated with the price tag and time needed to develop a good nuclease. By contrast, CRISPR/Cas technology is simple and very inexpensive.

[b] Notable exceptions include *Arabidopsis* and rice, excellent dicot and monocot model plants, respectively.

Chinese Academy of Sciences, **Daniel Voytas** from the University of Minnesota, and **Holger Puchta** from Karlsruhe Institute of Technology described the excitement at being at a point in the field where the technology has advanced enough to have the first international symposium dedicated to plant genome editing. Presenters described novel tools for gene and base editing, gene targeting by HDR, transcriptional regulation, epigenetic editing, and induced chromosomal rearrangements.

Keynote address

Enhancing food security through rice genetic improvement

Pamela C. Ronald from the University of California, Davis gave the keynote address that included three vignettes on genetic improvements in rice focused on disease resistance, flood tolerance, and nutritional quality. Ronald showed that the power of genetics can be harnessed to increase crop resiliency and improve food security to address the growing threats of climate change and increased population growth.

Ronald first focused on their lab's efforts to engineer and understand bacterial resistance in rice. The bacteria *Xanthomonas oryzae* pv. *oryzae* (Xoo) can reduce rice yields by up to 50%. Disease resistance is mediated by the gene *Xa21*, which was isolated in Ronald's lab.[8] Xa21 is representative of a large family of plant and animal receptor kinases that respond to microbial molecules.[8–10] In Xa21, the leucine-rich extracellular domain binds to the Xoo sulfated peptide RaxX, resulting in an immune response that includes the production of reactive oxygen species and ethylene production, and induction of defense genes.[11–14] RaxX is homologous to the endogenous plant peptide hormone PSY1. RaxX, but not PSY1, binds to Xa21. PSY is presumed to interact with an as yet unidentified PSY1 receptor to promote normal growth and development. Both PSY1 and RaxX promote root growth in Arabidopsis and rice seedlings, supporting the idea that RaxX is a molecular mimic of PSY1.[14] Ronald presented a model whereby RaxX facilitates infection by binding to the putative PSY1 receptor, which may trigger the formation of a niche for bacterial multiplication. In the presence of Xa21, however, binding of RaxX to XA21 initiates an immune response, preventing infection. Ronald's lab is working to understand how RaxX facilitates virulence and to identify and isolate the PSY1 receptor to better understand their interaction. This work provides insight into how hosts and microbe coevolve.

Another large threat for crop production is flooding, which the Intergovernmental Panel on Climate Change predicts will increase in both duration and intensity with climate changes. In South and Southeast Asia, 4 million tons of rice—enough to feed 30 million people—is lost each year due to flooding. In the 1980s, researchers at the International Rice Research Institute identified a variety of rice that can withstand 2 weeks of complete submergence. Conventional breeding efforts to introduce this trait resulted in low-yield varieties that were never widely adopted. Ronald and collaborators isolated the gene for submergence tolerance, *Sub1A*, that when introduced into rice via genetic engineering or marker-assisted breeding resulted in varieties that yielded 60% more than conventional varieties in flooded fields.[c] Last year, more than 6 million farmers grew *Sub1* rice. Field experiments in India have shown that Swarna-*Sub1* rice delivers yield advantages under flooding and disproportionately benefits the world's poorest farmers.[15,16]

Ronald also discussed improving the nutritional quality of rice, with genetically engineered golden rice as an example. First developed in 2005, golden rice was developed to address vitamin A deficiency (VAD) in countries where rice is a major food source; it is genetically engineered to contain two missing steps of beta-carotene synthesis.[17] Golden rice is approved for consumption in several countries and, in July 2021, was approved for commercial production in the Philippines with seed expected to be grown by Filipino farmers in communities with a high prevalence of VAD.

In conventional genetic engineering, transgenes are inserted into the genome at random. This can have unexpected and deleterious effects, an outcome that slows down the process of creating a new plant with desired, but no undesired, traits. New technologies, such as CRISPR/Cas, allow targeted genetic insertion that, when combined with knowing the location of genomic safe harbors, that is, places where insertions will not affect yield but will also be well expressed, alleviates undesired outcomes.

[c] https://www.nature.com/articles/nature04920?proof=t

Because no safe harbors were known in rice, Ronald's group surveyed a collection of over 3000 rice mutants[18–20] and chose five morphologically normal mutants to target for insertion a 5.2-kb cassette from golden rice, successfully inserting the entire cassette at the designated target via nonhomologous DNA end joining (NHEJ). The resulting rice lines have grains that appear morphologically normal and accumulate beta carotene.[20] These studies demonstrated that large DNA fragments can be inserted into plant genomes in a targeted manner via CRISPR/Cas.

Plant genome-editing tools and technology development

Improving plant transformation via viral delivery

A major challenge for achieving precise gene editing in plants is reagent delivery to the cells. Currently, reagents are delivered via Agrobacterium or biolistics to plant cells grown in culture. The transgenic cells are then grown in culture and induced to form shoots and roots. Creating a transgenic plant this way is time consuming and often requires sophisticated techniques. In addition, methods for plant transformation and regeneration differ between species and varieties, and many plant varieties are not amenable to tissue culture and regeneration.

Daniel Voytas from the University of Minnesota discussed work on new methods to deliver reagents to plant cells, particularly the use of plus-strand RNA viruses. RNA viral vectors are widely used to deliver transgenes to mammalian cells; however, their use to edit plant cells is less developed, and they are limited by the size of the cargo they can carry. Voytas's group has also been experimenting with making transgenic plants that express activators, repressors, and base editors to achieve a variety of editing outcomes. They have also been exploring ways to increase the cargo capacity of RNA viruses.

Voytas described using an RNA virus to deliver gRNAs to Cas9 transgenic plants. As the plant is infected by the virus, cells that receive the gRNA are edited via the already-present Cas9. While this approach requires the initial generation of Cas9 transgenic plants, once developed, they can be used as a "platform" to create mutations with a wide variety of phenotypic variation. Voytas described that initial attempts at this strategy showed a low frequency of heritable mutations; less than 0.2% of seedlings carried the mutation.[21] Adding to the gRNA sequences that promote mobility achieved more efficient CRISPER/Cas9 editing, thereby increasing somatic editing to more than 75% and germline editing in seeds to 60% and higher. Voytas also showed that this approach can be used to edit multiple genes at once by delivering more than one gRNA via a single transcript.[22] While their work is primarily in tobacco (*Nicotiana benthamiana*), Voytas's group is investigating whether RNA virus delivery of gRNAs can induce heritable mutations in other species as well.

Another challenge facing viral delivery of reagents is achieving systemic distribution throughout the plant. Voytas showed that adding tRNA-like mobility motifs allows reagents to move uniformly throughout the plant.[22]

Improving plant transformation in a protoplast system

Feng Zhang from the University of Minnesota described work to achieve high-throughput, precise gene editing. Zhang's lab is interested in understanding the players that determine which DNA repair pathways are used. Several competing DNA repair pathways can be activated upon a DSB, including HDR, microhomology-mediated end joining (MMEJ), and NHEJ. The pathway that repairs the DSB can dictate gene-editing outcomes. Zhang discussed reagent delivery as a key bottleneck in plant gene editing, including using a protoplast system and electroporation.

A protoplast is plant cell in which the cell wall has been removed; among other things, this allows reagents like DNA, RNA, and proteins to be delivered into the cell using polyethylene glycol (PEG) or electroporation. Although protoplast technology is not new (having been around since the 1960s), Zhang is working to revive this technology for use in genome editing, as it is a good transient assay system before moving into a more labor-intensive transformation pipeline. Zhang's group has used protoplasts for editing a number of plant species, both dicots and monocots, in many cases consistently achieving 80–90% transformation efficiency.

Zhang showed that a protoplast system can achieve efficient multiplexed gene knockout in *N. benthamiana*. The system was used to deliver TALENs to target four genes involved in

glycosylation pathways. In this species, whole plants can be efficiently generated from single protoplasts. Screening over 100 regenerative plants without any selection demonstrated that over half of the plants had at least one mutation at one locus, while approximately 3% had all eight alleles knocked out.[23]

Zhang is interested in developing the protoplast system into a high-throughput plant genome-editing platform. Toward this goal, they are exploring ways to increase the mutation frequency in the NHEJ DNA repair pathway. In the model system *Setaria viridis*, protoplasts can be transformed with 80% efficiency, but the mutation frequency is only 50%. Zhang showed that codelivering CRISPR/Cas with the exonuclease Trex into protoplasts increased mutation efficiency up to approximately 75%.[24]

Zhang hopes that the protoplast system can be developed to the point where one typical protoplast isolation is sufficient for up to 100 treatments. Combining the system with flow cytometry or "omics analyses" would enable researchers to dissect different DNA repair pathways and optimize factors that impact gene-editing frequency and precision. Zhang is also currently working on leveraging MMEJ and HDR DNA repair pathways to achieve more precise outcomes, as well as exploring DNA-free gene editing.

Restructuring plant chromosomes via NHEJ

Holger Puchta from the Karlsruhe Institute of Technology described introducing large changes like restructuring chromosomes in plant genomes with CRISPR/Cas. Puchta's group was one of the first to use CRISPR/Cas in plants; in 2014, they showed that CRISPR/Cas9 can be used for genome engineering in *Arabidopsis thaliana*.[25] They also showed that CRISPR/Cas9 can achieve high editing rates in plants.[26] The success of the CRISPR/Cas system indicates that it might be useful in improving frequencies of inefficient reactions in plants, such as homologous recombination (HR), which typically has an efficiency of approximately 1%.[27]

Using the knowledge accumulated over the years, Puchta's group has been investigating the ability of CRISPR/Cas to do genomic restructuring and chromosomal engineering. Puchta hopes that chromosome engineering can overcome bottlenecks by breaking genetic lineages and restoring crossover rates in a previously crossover-dead region. For example, cereal genomes contain many inversions that are inaccessible for recombination. Inverting these regions with CRISPR/Cas can enable crossover and recombination to occur.

Puchta showed that by introducing two DSBs, CRISPR/Cas9 can facilitate inversions in somatic cells. In *Arabidopsis*, inversion frequency ranged from ∼0.5% to 2%, depending on the locus; most junctions in the inversion events were accurately ligated. Unexpectedly, Puchta found that classical NHEJ suppresses the formation of inversions. Most junctions were formed via microhomology-mediated NHEJ, which is highly mutagenic. Junctions formed in mutants deficient in classical NHEJ were more imprecise, making this approach unsuitable for practical applications.[28]

The most prominent example of an inversion in plants is the 1.2-Mb knob inversion in *Arabidopsis*. Puchta's group identified a line with the 1.2-Mb knob inversion and no deletions;[29] they showed that the inversion reactivates crossovers between two cultivars, indicating that chromosomal restructuring can be beneficial for plant breeding.

Puchta's group is also using CRIPSR/Cas-induced DSBs to facilitate chromosomal translocations in *Arabidopsis*. While translocations are typically very infrequent, Puchta showed that inhibiting classic NHEJ increases translocation efficiency approximately fivefold. They have been able to achieve heritable translocations, many of which were accurately ligated.[30]

Puchta hopes that large chromosomal restructuring will be useful in breaking and fixing genetic linkages for breeding, reconstructing genome evolution, and creating synthetic plant chromosomes.

Inducing homologous recombination in somatic cells

Avraham A. Levy from the Weizmann Institute of Science presented work on inducing crossover or gene conversion events in DSBs. Levy's goal is to generate a targeted DSB via CRISPR/Cas9 and induce recombination or gene conversion at the site. Recombination between homologous chromosomes typically occurs during meiosis. However, Levy argued that a DSB induced during meiosis would have to compete with the hundreds of naturally programmed DSBs. Therefore, their lab is developing systems to induce recombination in somatic cells.

There are conflicting reports on whether homologous chromosomes pair in somatic tissue; and somatic cells lack the machinery for recombination. Nevertheless, Levy showed that CRISPR/Cas9-targeted DSBs can be repaired via somatic HR using the homologous chromosome as a template in both tomato and *A. thaliana.* These changes could also be transmitted through the germline. For example, CRISPR/Cas9-mediated DSBs in *PYS1* in tomato, which affects fruit color, showed allele-dependent repair 14% of the time. Out of 10 events that were transmitted to the next generation in germinal tissues, four in *PSY1*[31] and six in *CRTISO*,[32] three were from crossover and seven to gene conversion.

Levy also described unpublished work on the kinetics of DSB repair to better understand what factors determine the fate of DSBs in somatic cells.

Epigenome engineering in plants

Steven E. Jacobsen from UCLA presented work on engineering the epigenome in plants, specifically patterns of DNA methylation and demethylation. In plants, epigenetic patterns can be stably inherited, giving rise to epialleles. Several epialleles have been identified in plants that affect traits, such as flower morphology, sex determination, fruit ripening, flowering time, and root length.[33–35]

To investigate methods for targeted methylation, Jacobsen's group has focused on the gene *FWA* in *A. thaliana.* In wild-type plants, *FWA* is methylated and silenced, resulting in the normal, early-flowering time. In mutants that have lost *FWA* methylation, the gene is overexpressed, and plants exhibit a late-flowering phenotype.

Jacobsen's lab has elucidated many of the mechanisms regulating the four interlinked methylation pathways in plants, each of which methylates in a specific sequence context.[36] Jacobsen showed that targeting the *FWA* promoter with an artificial ZFN fused to different components of the RNA-directed DNA methylation pathway can recruit the other components of the pathway and cause stable, heritable new methylation and gene silencing.[37,38] Using this approach and optimizing different promoter and terminator combinations, Jacobsen's group has achieved 90% silencing in the first generation.[39]

To increase specificity, Jacobsen's group has developed a CRISPR/Cas9 system using a SunTag approach in which catalytically dead Cas9 is fused to a chain of epitopes that recruits multiple copies of the DRM2 methyltransferase catalytic domain that has been optimized to reduce off-target methylation. This system produced heritable methylation, gene silencing, and delayed flowering time phenotype.[40] Jacobsen's group is targeting CG methylation, the most potent pathway for gene silencing and the most critical for inheriting methylation, using a bacterial DNA methyltransferase.[107]

Jacobsen also described work on targeted DNA demethylation in *A. thaliana* using a human TET1 catalytic domain. This system can heritably demethylate and reactivate *FWA* with almost no off-target effects.[41] Jacobsen's work shows that it is possible to stably engineer new, heritable epigenetic patterns without changing the DNA sequence. They hope to continue to test these tools at other genomic targets and in other plant species.

Finally, Jacobsen described viral delivery of epigenome editing reagents to achieve targeted and heritable DNA methylation in *A. thaliana.* Similar to Voytas's approach, Jacobsen's lab has used tobacco rattle virus (TRV) to deliver gRNA into transgenic plants expressing SunTag-TET1. The system achieved demethylation and reactivation of *FWA* expression, which results in a late-flowering phenotype by the third generation. These results show that gRNA delivery by TRV can be used for heritably targeted DNA demethylation.[42] Their group is working to improve the efficiency of TRV-mediated gRNA delivery and to engineer entire CRISPR systems into viruses for epigenome editing.

Enhancing gene targeting via homologous recombination

Seiichi Toki from the National Agriculture and Food Research Organization presented work on developing tools to improve the frequency of targeted mutagenesis and increase gene targeting via HR. The first part of Toki's talk focused on improving the frequency of targeted mutagenesis. Suppressing the NHEJ pathway can upregulate the frequency of targeted mutagenesis by shifting the repair pathway toward MMEJ. Approximately 10 years ago, Toki's group showed that targeted mutagenesis could be achieved in *A. thaliana* with ZFNs. Mutagenesis was enhanced by knocking out *ku80*, which encodes for a protein that protects the DSB during NHEJ.[7] Similarly, knocking out the gene for the DNA ligase involved in NHEJ can enhance the frequency of TALEN-mediated

targeted mutagenesis in rice.[43] More practically, small molecule inhibitors of NHEJ components may be able to achieve similar results.

Next, Toki discussed their group's efforts to improve gene targeting via HR. The frequency of HR-mediated gene targeting is extremely low in higher plants. Toki stressed that the selection step of gene-targeted cells is very important. In particular, during positive/negative selection–mediated gene targeting, it is important to eliminate the positive selection marker from the locus. One way to remove the positive selection marker is via the PiggyBac transposon, which integrates into and excises the plant genome without leaving a footprint.[44] Toki's group has developed a positive selection marker system using an I-SceI break and subsequent single-strand annealing (SSA) DNA repair system.[d] Similar to PiggyBac, this strategy leaves no footprint. Toki showed that this positive selection marker system can introduce mutations in the *MIR172* binding site in rice.[45] Positive/negative selection–mediated gene targeting and subsequent removal of the positive selection marker using either the piggyBAC transposon or break-induced SSA can be used to achieve any desired mutation without the need for sequence-specific nucleases.

Toki also described a break-induced gene targeting system that does not use a selection marker.[46] They showed that the system can achieve biallelic gene targeting in rice and hope that it will facilitate gene-targeting studies in vegetatively propagated crops.

Finally, Toki described how CRISPR-Cas9–mediated gene targeting can be achieved using an all-in-one vector in rice and tobacco.[47] Inducing HR by stimulating RAD51 increases gene-targeting frequency. Gene-targeting frequency can also be enhanced by promoting the resection step of HR via ectopic expression of OsRecQI4 and OsEXO-1 in rice.[48]

Developing a selectable marker-free genome editing system

Erika Toda from Tokyo Metropolitan University presented research on developing a DNA- and selectable-marker-free genome-editing system via direct delivery of Cas9-gRNA ribonucleoproteins into rice zygotes. Zygotes have several characteristics that make them suitable for genome editing. They are totipotent and capable of developing into an embryo. Genome editing in a single zygote cell can reduce the possibility of mosaics.

While genome editing in zygotes has been utilized in animals, a transfection system for plant zygotes has not been available. Toda's group has established a gene expression system in rice zygotes in which egg and sperm cells are isolated, and zygotes are produced via an *in vitro* fertilization system.[49] Expression vectors are delivered into the zygote via PEG-Ca^{2+}-mediated transfection. The system can achieve approximately 70–80% transfection efficiency with the fluorescent proteins pGFP-ER and pDsRED. Fluorescent signals were detectable in zygotes before the first division, suggesting that the delivered DNAs are transcribed and translated in the zygotes and that the chance of creating a mosaic is minimal.[50,51]

Toda's group has used this transfection system to deliver a CRISPR/Cas9 expression vector or Cas9-gRNA ribonucleoprotein into rice zygotes. They described a proof-of-principle study in which Cas9 was used to knock out *DsRed2* in a transgenic rice plant. Cas9-gRNA–treated plants had lower DsRed2 fluorescence than control plants; the intensity of the DsRed2 fluorescence in cell masses corresponded to the occurrence of the targeted mutation. Their group has also used this strategy to target endogenous genes. The efficiency of development and regeneration into plantlets was ∼60–70%, while the frequency of achieving the targeted mutation was 4–64%, depending on the locus.[51] Toda's group hopes that this efficient DNA- and selectable marker–free genome-editing system can be used to advance molecular breeding.

Toda's group is working to apply this zygote transformation strategy to other crop species. Procedures to isolate gametes and zygotes for maize, wheat, and barley have been described. They are also working to adapt this strategy to achieve HR-mediated genome-editing by direct delivery of a DNA donor and Cas9-gRNA ribonucleoprotein (RNP) into zygotes and to develop functional analyses during zygotic development and embryogenesis.

Improving prime editing in plants

Caixia Gao from the Chinese Academy of Sciences discussed work on prime editing in plants. Prime editing has the potential to achieve a variety of

[d]http://doi.org/10.1111/pbi.13485

genomic mutations, including all 12 kinds of base substitutions and precise insertions and deletions.[52] Prime editors consist of two components: an engineered Cas9 nuclease fused to reverse transcriptase and a prime-editing guide RNA (pegRNA).[e] In brief, the Cas9 nuclease recognizes the target site and nicks the nontarget DNA strand. The single-strand DNA that is released pairs with the primer-binding site in the pegRNA, serving as a primer for reverse transcription. Through RT, the edit is transferred to the nontarget strand. The intended edit in the target site is then incorporated via DNA repair.[53,54]

Gao's group is adapting and optimizing prime editors for use in rice and wheat. They showed that optimizing the primer binding site (PBS), reverse transcription template, and nicking location can increase prime editing efficiency.[54] Another way to improve efficiency is to test a variety of pegRNAs; however, this can be laborious and time consuming. Gao showed that the melting temperature of the PBS sequence strongly affects prime editing efficiency. In rice, the optimal PBS melting temperature was approximately 30 °C. Prime editing was further optimized using a dual peg-RNA strategy that uses two separate pegRNAs *in trans* to encode the same edit on both DNA strands simultaneously. Dual pegRNAs increased prime editing efficiency by approximately fourfold compared with NGG-pegRNA, and twofold compared with CCN-pegRNAs, without increasing the production of undesired byproducts.[55]

To help researchers design efficient pegRNAs and other components for prime editing, Gao's group developed PlantPegDesigner, a user-friendly application available at www.plantgenomeediting.net. The application allows users to define specific parameters for their prime editing application and recommends spacer PAM sequences, PBS sequences, RT template sequences, and PCR primers for pegRNA construction. PlantPegDesigner RNAs achieved 3- to 17-fold higher prime editing efficiency than manually designed RNAs and 2- to 45-fold higher efficiency than RNAs designed via other web applications.[55]

Gao also showed research on the specificity of prime editors in plants. Recent work in their lab shows that pegRNA-dependent off-target editing in rice protoplasts is very low among predicted endogenous off-target sites, and that prime editing does not induce any pegRNA-independent off-target effects or interfere with endogenous reverse transcriptase mechanisms.[f]

Facilitating homologous-directed repair to achieve precision genome editing

Lan-Qin Xia from the Chinese Academy of Agricultural Sciences described work on CRISPR/Cas9-mediated HDR to achieve precision genome editing in rice. Achieving gene replacement or knockin in crops via HDR is challenging. HDR naturally occurs at very low frequency; most DSBs are repaired via NHEJ, which competes with HDR for DNA repair. In addition, the plant cell wall makes it difficult to deliver the donor repair template into the nucleus. Xia showed that CRISPER/Cas9-mediated HDR can be achieved in rice using double-stranded DNA as a donor repair template. Xia's group has used this system to edit genes that confer herbicide resistance and to transfer an allele from a wild rice variant into a cultivated variant to increase yield potential.[56–58]

Xia also showed that a different CRISPR-based system, CRISPER/Cpf1, can be used to mediate HDR for precision gene editing. Cpf1, also known as Cas12, is a small Cas protein that has low off-target effects and can induce long 5′ sticky ends, which may facilitate HDR. Xia's group has optimized gene editing with CRISPR/Cpf1 by incorporating flanking ribozyme units that undergo self-cleavage and release gRNA, and by codon optimization of *LBbCpf1* and the genes encoding U3 and U6.[59,60] Self-cleavage of the untranslated regions (UTRs) by ribozyme units enables transcripts to stay in the nucleus so that they can act as templates.[59] CRISPR/Cpf1-mediated gene replacement could be achieved by synthesis-dependent single strand annealing repair.[61] To further increase HR efficiency, Xia's group used an RNP-RNA donor repair template complex and showed that RNA transcripts-templated homology-directed repair of DSBs was achieved by LbCpf1 nuclease. Using this strategy, Xia was able to generate stable precisely edited rice plants.[62]

[e] https://doi.org/10.1038/s41586-019-1711-4

[f] https://doi.org/10.1038/s41587-021-00868-w

Xia's work demonstrates that precision gene editing for targeted gene or allele replacement in rice can be achieved through CRISPR/Cas9 and CRISPR/Cpf1 by using DNA and/or RNA donor repair templates. They hope that these gene-editing systems will expand researchers' abilities to modify agriculturally important genes to improve crops.

Improving gene targeting via tandem repeat-facilitated homology-directed repair

Jian-Kang Zhu from the Chinese Academy of Sciences presented work on precise gene targeting in plants. Zhu's group has conducted heritable gene targeting in Arabidopsis via a two-step transformation protocol to insert sequences. In this approach, Cas9 is expressed in the egg cell and gRNA and donor template are delivered via a second transformation. While the precise mechanism of gene targeting is unclear, Zhu noted that this strategy yields a relatively high frequency of HDR events, with efficiencies of up to 9% depending on the locus. Gene-targeting events were marker free, seamless, and identified via a simple PCR reaction.[63] Zhu's group developed an all-in-one strategy that expresses Cas9, gRNA, and donor template in one construct and includes a translational enhancer to boost expression of Cas9. This approach also achieved gene-targeting events without the need for two transformations.[64]

Zhu is interested in strategies to increase HDR efficiency. HDR efficiency can be fairly high in some contexts, such as between tandem-repeat sequences. Zhu's group has developed a strategy for gene targeting using tandem repeat-facilitated homology-directed repair. In this approach, a piece of DNA with the desired mutation and homologous to the neighboring sequence is inserted; this creates a new gRNA target at the junction between the inserted sequence and its neighboring homologous sequence. The DSB induced by CRISPR/Cas9 consequently creates two homologous fragments that can be repaired via HDR, and the mutation is incorporated. Zhu showed that this strategy can be used to replace or insert short sequences of 96-bp to 130-bp with up to approximately 10% frequency, depending on the length of insertion.[65]

The first step in this process is to insert a piece of DNA at a target site. Zhu showed that their group has been able to reliably insert target sequences in rice to achieve phenotypes, like increased salt tolerance and increased growth. They have also inserted promoter sequences upstream of genes that control plant height or salt tolerance. This results in a range of expression levels in the protein of interest and a corresponding range of phenotypes.[65] Zhu hopes that generating these expression-variation alleles via targeted insertion of transcriptional or translational regulatory elements can be instrumental in developing research materials, breeding materials, and in increasing crop genetic diversity.

Taking advantage of this ability to reliably insert DNA sequences into the rice genome, Zhu proposed a genome-wide editing project, the Rice Protein Tagging Project, in which N- or C-terminal tags are introduced near genes to create tagged proteins. These can be used for in-depth studies of protein function, including immunostaining, immunoblotting, immunoprecipitation, or chromatin immunoprecipitation.

Short talks

The Tools and Technology Development sections included three short talks on new plant genome-editing tools.

Two presenters discussed genome-editing tools for organelle genomes. **Ayumu Takatsuka** from Tohoku University presented unpublished work on mitochondrial-directed TALEN (mitoTALEN) used to understand the function of mitochondrial genes involved in cytoplasmic male sterility (CMS) in rice. This is one of the first studies to use mitoTALEN for gene identification in plants. In 2019, a similar study used mitoTALEN to knock out mitochondrial genes suspected to be involved in CMS in rice and rapeseed.[66] **Shin-ichi Arimura** from the University of Tokyo discussed unpublished work on modifying the plastid genome in *A. thaliana* using a plastid-targeted TALE cytidine deaminase.

Keishi Osakabe from Tokushima University described work characterizing and developing a novel CRISPR-based genome editing tool from CRISPR-Cas type I-D, dubbed TiD. Class 1 CRISPR-Cas systems, although more abundant, are less utilized for genome editing and less characterized than class 2 systems, which include Cas9, Cas12a, and Cas13. TiD consists of five Cas proteins and a gRNA. TiD targets to specific sequences that are different from those of Cas9 and Cas12a and utilizes a longer gRNA than does either Cas9 or

Cas12a, which could reduce the risk of off-target effects. TiD has a different DNA system than other CRISPR tools. Osakabe showed that the nuclease activity is present in Cas10d, which cleaves single-strand, but not double-stranded DNA. They showed that TiD can be used to incorporate both long-range deletions up-and downstream from the target site as well as short indel mutations at the target sequence in tomato plants. Somatic mutation rates were 20–100%, depending on the target. Using TiD, Osakabe was able to generate biallelic mutant tomato plants with no off-target mutations. The mutations were transmitted to the next generation. Similar results were seen in human cells.[67] Osakabe is working to improve the efficiency of TiD and to modify it for a variety of genome-editing applications, such as base editing, transcriptional control, and epigenome editing.

Applications of genome editing in agriculture

Engineering quantitative trait variation in crops

Zachary Lippman from Cold Spring Harbor Laboratory presented research on engineering quantitative trait variation in crops like the tomato. Research on pan genomes has revealed that there is much more genetic variation to discover. In collaboration with Mike Schatz at Johns Hopkins University, Lippman's group identified approximately 23,000 structural variants among 100 tomato genomes. Among these structural variants, 40% were within and nearby genes; 80% of those were in regulatory regions and 10% of were associated with expression changes. Lippman stressed that these data show that expression variation is perturbed because of structural variation in *cis*-regulatory regions.[68]

Lippman is interested in uncovering whether these structural variants in *cis*-regulatory elements result in phenotypic consequences. While there has been a lot of research on *cis*-regulatory control in animal systems, less is known about plants. It is known that *cis*-regulatory regions play a role in modifying quantitative trait variation in crops and wild species. For example, a natural inversion in the promoter of *CLV3*, which controls stem-cell proliferation in the meristem and was important in domestication, increases locules and fruit size in the tomato.[69] Lippman's group has used CRISPR/Cas9 to perturb *cis*-regulatory regions and create a range of quantitative trait variation in approximately 30 alleles in the tomato genome.[70,71] Their group has dissected the effects of different regions within *cis*-regulatory elements on quantitative trait variation. Lippman's work suggests that *cis*-regulatory regions are biased toward generating quantitative variation, whereas coding regions are biased toward qualitative variation.[71]

Lippman showed that pan-family genome analysis can identify noncoding regions important for quantitative trait variation. A pan-family analysis of Solanaceae plants identified approximately 90,000 conserved noncoding regions. Areas of highest conservation overlapped with regions of accessible chromatin, suggesting that sequence conservation among noncoding sequences within a family can be used as a proxy for *cis*-regulatory elements. Lippman's group used CRISPR/Cas9 to create a series of alleles in the conserved regions of the promoter of *WOX9*, which is involved in inflorescence. They noted that plants with 4 to 6 branches of inflorescence had mutations in the distal region of the promoter, while mutations in the proximal regions of the promoter were generally embryonic lethal. These data suggest that there are pleiotropic effects within the promoter; that is, different regions of the promoter can affect different aspects of the phenotype. For example, in *WOX9*, different regions of the promoter are responsible for embryonic, vegetative, and reproductive branching.[72] Lippman hopes that these insights will enable researchers to tune *cis*-regulatory regions to achieve quantitative trait variation.

Lippman's group is continuing their work in another Solanaceae family member, groundcherry, to see if the pleiotropic effects seen in the tomato are conserved. They hope that identifying shared *cis*-regulatory targets based on deep sequence conservation can allow researchers to achieve similar quantitative trait modifications across species.

Expanding the use of TALENs for genome editing

Jens Boch from Leibniz Universität Hannover presented work on expanding the use of TALENs for genome editing. TALENs were developed over 10 years ago, a few years before CRISPR/Cas tools became available.

TALENs consist of the natural bacterial protein TALE fused to the endonuclease *Fok*1. TALEs contain a repetitive DNA-binding domain that recognizes specific DNA sequences as well as a C-terminal transcriptional activation domain.[73] TALEs have been developed into versatile biotech tools, including gene activators and repressors, DNA de/methylases, histone modifiers, base editors, and nucleases. The modular DNA-binding domain of TALEs enables researchers to build in any DNA-binding specificity.[g] TALENs have had a lot of success in medical and commercial gene-editing applications, including in editing CAR T cells for cancer therapies and in developing genome-edited commercial livestock and crops.

Boch's lab uses the Golden Gate Cloning and Modular Cloning (MoClo) Toolkit developed by Sylvestre Marillonnet to design and create TALENs. In this system, genes are separated into functional modular domains, for example, promoters, ORFs, and terminator modules. Restriction enzyme overhangs at the 5′ and 3′ ends enable each module to be attached via ligation to form a single transcriptional unit. Several transcriptional units can be combined to create a multigene construct. Boch's lab has developed a large number of promoter and ORF modules and vectors that can be mixed and matched to develop new tools for genome editing.

While most of the presenters at the symposium described the use CRISPR/Cas for genome editing, Boch stressed that TALENs should not be dismissed as a powerful, versatile genome-editing tool. Their group is working to simplify TALEN protein purification and design to make this technology more accessible to researchers. Toward that goal, their group has optimized TALEN bacterial expression, purification, and storage parameters. They hope that these insights will enable high-throughput TALEN protein production for use in modifying plant genomes.

Boch presented research on comparing TALE gene activator activity to CRISPR-based tools and on modifying TALEs to serve as base editors. Normally, base editors require that the edited base is within a window for the enzyme's PAM. Finding such a sequence can be challenging. Recently, David Liu reported a fusion of an enzyme (DddA) fused to TALE to develop a TALE-based editor.[74] Boch presented unpublished research on using this TALE-based gene editor in plant protoplasts.

Elucidating insect–plant interactions with CRISPR/Cas

Joyce Van Eck from the Boyce Thompson Institute presented research on understanding insect interactions in two Physalis species, groundcherry and goldenberry. Van Eck's group is interested in using genome-editing tools like CRISPR/Cas to improve and domesticate underutilized species like the groundcherry and goldenberry. Both species are in the Solanaceae family; unlike other members, such as the tomato and peppers, little crop improvement has been done in these species. Van Eck believes that both the groundcherry and goldenberry have the potential to become specialty fruits in the United States because of their good nutritional content, flavor, and anti-inflammatory and antioxidant properties. Improving characteristics like growth habit and fruit dropping, which is a problem with the groundcherry, could make these plants more attractive for commercial uses.

Van Eck's group has been working with farmers, home gardeners, and consumers across the United States to understand how to improve the groundcherry and goldenberry. During these exchanges, they realized that there were differences in interactions between the groundcherry and goldenberry and the three-lined potato beetle and *Chloridea subflexa*. While adult insects visit and lay eggs on both goldenberry and groundcherry, larvae only fully develop on the goldenberry.

To understand these differences, Van Eck's group is conducting unbiased metabolic profiling of leaves, husks, and fruit of three accessions each of the groundcherry and goldenberry. They showed that there are differences in leaf metabolites both within and between species. Their group is focusing on a class of steroidal compounds called *withanolides* that have shown insect-feeding deterrent properties. To understand whether withanolides affect interactions between plants and insects, Van Eck's group is characterizing the phenotypes and metabolic profiles of CRISPR/Cas-generated mutant plants targeting the promoters or coding sequences of genes involved in withanolide synthesis.

[g]https://doi.org/10.1016/j.ggedit.2021.100007

Viral interference in plants with CRISPR/Cas

Magdy Mahfouz from King Abdullah University of Science and Technology discussed work using CRISPR/Cas systems to generate viral immunity in plants. CRISPR/Cas systems are natural immune systems in bacteria; they enable bacteria to recognize and attack invading viruses. Mahfouz showed that CRISPR/Cas systems could be transferred to plants and elicit an immune response against viruses to protect the plant from infection. TRV-mediated delivery of crRNAs resulted in tomato yellow leaf curl virus (TYLCV) interference in Cas9-expressing tomato plants. Using an sgRNA against an intergenic region or coding protein of TYLCV resulted in reduced virus titers after infection as well as phenotypic resistance.[21,75]

While these initial studies were conducted with CRISPR/Cas9, Mahfouz's group has more recently been investigating the potential of CRISPR/Cas13 to mediate viral interference. CRISPR/Cas13 has been shown to be useful to confer interference and can be engineered for a variety of RNA manipulations that may be useful not only in engineering plant immunity but also in functional genomic applications.[76]

Similar to the CRISRP/Cas9 results, Mahfouz showed that CRISPR/Cas13a could mediate viral immunity in plants. In this study, three crRNAs targeting GFP-expressing turnip mosaic virus (TuMV) were delivered via TRV in a Cas13a-expressing line. Mahfouz showed that this interferes with TuMV-GFP fluorescence and viral transcript expression *in planta*.[76] Similar results were seen in *Arabidopsis*.[77] While these studies show that CRISPR/Cas13 can be transferred to different plant species for RNA virus interference, the efficiency of viral interference was modest.

Mahfouz's group has tested the efficiency of four Cas13 variants, Cas13a, b, and d, in addition to testing the effect of nuclear localization (NLS) and nuclear export signals (NES). They showed that different Cas13 variants have different viral interference efficiencies, with CasR × NLS and CasR × NES demonstrating robust efficiency against TRBO-GFP and TuMV-GFP. Including multiple crRNAs for the two viruses resulted in multiplex targeting and interference against both viruses.[78]

Mahfouz's work shows that CRISPR/Cas systems can be used to boost plant immunity by directly targeting the viral DNA or RNA genomes and have the potential to provide resistance against multiple pathogenic viruses. Future work will consist of translating these results to the field, for example, by investigating whether CRISPR/Cas systems can be delivered into mature plants as a type of vaccination and to determine the best CRISPR/Cas systems to provide efficient, broad-spectrum viral resistance.

Improving potato traits with CRISPR/Cas

Mariette Andersson from the Swedish University of Agricultural Sciences presented research on using genome editing for improving traits in the potato (*Solanum tuberosum* L.). The potato is the third most important food crop and is also grown for starch production. Traditional crossbreeding can be challenging owing to inbreeding depression; therefore, Andersson stressed that there is a need for tools to add desirable traits to elite genotypes that do not require crossings.

Andersson's group has used CRISPR/Cas9 to introduce desirable traits into the potato, which is especially amenable to CRISPR/Cas modifications. Potato protoplasts can be induced to regenerate, and PEG-mediated transfection of protoplats is very efficient. Mutation frequency is fairly high at approximately 40–90%. Andersson's group has produced full knockouts of at least four genes in one round of transfection. One caveat is that integration of vector or endogenous DNA is common; therefore, Andersson's group prefers to use RNP for transgene-free potato development.

Andersson described three examples of how their lab has introduced traits into potato CRISPR/Cas9: developing an amylose potato, a short-chain amylopectin starch potato, and a nonbrowning potato.

High amylose potatoes have a low glycemic index, which reduces spikes in blood sugar and can be advantageous for diabetics and weight loss. Andersson's group used CRISPR/Cas9 to target the starch-branching enzyme (SBE) genes involved in amylopectin synthesis. This resulted in two groups of potato lines: intermediate amylose lines with *Sbe1* knocked out and 2–3 alleles of *Sbe2* knocked out, as well as amylose-only lines that have mutations in all eight alleles of *Sbe1* and *Sbe2*, though at least one allele has an in-frame mutation. Intermediate lines have higher amylose levels and longer amylopectin chains with a comparable tuber and starch yield to wild-type plants. Amylose-only

lines had a significant increase in amylose, with a preference toward long amylose chains, and no amylopectin. The yield of amylose-only lines was significantly lower than that of wild-type plants.[79]

Andersson described a second project to produce an amylopectin starch-producing potato. Potato starch is commonly used in food products; however, unprocessed potato starch loses stability with freezing and freeze-thaw cycles. Currently, potato starch is chemically modified to increase stability. Andersson's group is using CRISRP/Cas9 to target the genes responsible in amylose synthesis and amylopectin chain length with a goal of creating a potato that produces amylopectin starch that is stable and will not require chemical processing to be used in food products.

In their third example, Andersson described a collaborative project with Sergio Feingold's research group at the Laboratorio de Agrobiotecnología in Argentina to reduce browning in potatoes. Browning can be caused by damage during harvest and postharvest procedures. It is a result of polyphenol oxidase (PPO) activity, which catalyzes the conversion of phenolic compounds to brown pigments in the presence of oxygen. By using CRISPR/Cas9, they knocked out *PPO2*, which is responsible for more than 50% of PPO activity in potato tubers. Lines in which all four alleles were mutated had decreased PPO activity and reduced browning compared with the wild type. Andersson showed that allele dosage is important for the nonbrowning phenotype, as lines with at least one wild-type allele had similar browning activity as wild-type plants.[80]

Mini-maize: improving plant morphology for genetic editing studies

Kan Wang from Iowa State University presented work on building tools to alleviate bottlenecks in plant genome editing. Some of the biggest challenges in CRISPR/Cas-mediated plant genome editing involve plant transformation and regeneration. Wang's group is developing maize plant lines that are amenable to transformation and regeneration, can be grown at scale in greenhouses, and have short generation times. Previous work by Morgan McCaw in James Birchler's lab at the University of Missouri resulted in a fast-flowering mini-maize that performs well in greenhouse spaces and has a generation time of approximately half that of traditional maize.[81] McCaw has continued to develop this mini-maize variety in Wang's lab to create an inbred line that produces 100% regenerable calli that can be transformed using standard *Agrobacterium* methods. They also were amenable to CRISPR/Cas-mediated mutagenesis. Using this mini-maize variety can reduce total transformation time from approximately 44 weeks for traditional maize to 22 weeks.[82]

Wang hopes that mini-maize and other mini-plants, such as the mini-rice variety Xiaowei,[83] will accelerate genomic research by facilitating large-scale studies and reducing turnaround time.

Extending the base editing capabilities of CRISPR/Cas

Yiping Qi from the University of Maryland presented research on expanding the targeting range of CRISPR/Cas systems in plants. Qi focused on cytosine base editing functions of CRISPR/Cas9. There are two main CRISPR/Cas9-based base editors being developed: cytosine base editor and adenine base editor. In both, a deaminase is fused to a Cas9 nickase. Guide RNAs are designed that target the nonediting strand, and the desired base edit from C to G or A to G is accomplished via DNA replication or repair. Qi stressed that the activity and editing windows of Cas9 matter for base editing. Their lab has been able to achieve highly efficient editing in rice protoplasts as well as other dicots like tomato and poplar.[h] Qi's lab has characterized different Cas9 variants to determine their PAM sequences, specificity, and activity in plants. They showed that PAM sequences can differ between human and rice cells and that Cas9 variants have different specificity and activity than wild type.[84–86] Qi hopes that Cas9 variants that target alternate PAMs, and therefore different sequences, will expand the number of sites available for base editing with CRISPR/Cas.

The second part of Qi's talk focused on CRISPR/Cas12a. In contrast to Cas9, which creates blunt ends at DNA DSBs, Cas12a creates staggered ends, potentially increasing the likelihood of NHEJ repair-based insertion of heterologous genes.[87,88] Qi's group has been able to achieve frequency biallelic editing in rice and maize using CRISPR/Cas12a.[89,90]

[h]https://doi.org/10.1111/pbi.13635; https://doi.org/10.1093/plphys/kiab264; https://doi.org/10.1111/pbi.13581.

One of the goals of Qi's lab has been to improve Cas12a genome-editing tools. Normally, Cas12a recognizes the PAM TTTV (V = A, C, G), which can limit the sequences available to edit with Cas12a. To create a Cas12a that recognizes more relaxed PAMs, Qi's group characterized several Cas12a orthologs that recognize relaxed PAMs in bacteria. Mb2Cas12a was able to recognize relaxed PAMs. Introducing several point mutations in Mb2Cas12a relaxed the PAM requirements even more. Qi noted that this variant, named Mb2Cas12a-RVRR, can target nearly double the number of sites in the rice and maize genomes as wild-type Cas12a.[91]

They have also developed a highly multiplexable Cas12a system that can target at least 16 sites across nine chromosomes with efficient editing.[91]

Designing and targeting cis*-regulatory regions in plants*

Tobias Jores from the University of Washington presented work on identifying and characterizing *cis*-regulatory regions in plant genomes. Compared with coding sequences, *cis*-regulatory regions are often poorly annotated and the genetic code in these regions is largely unknown. This can make it difficult to target these sites for genome-editing application. However, *cis*-regulatory regions are important for many plant characteristics. For example, as Lippman showed in his presentation, mutations in the *cis*-regulatory regions of the genes *SlCLV3* and/or *SlWUS* affect the number of locules in tomato fruit and affect fruit size.[92,93]

Jores focused on a project of a comprehensive analysis of plant core promoters (see Fig. 1). Jores and colleagues synthesized the putative promoter region for every protein-coding and miRNA gene in the *Arabidopsis*, maize, and sorghum genomes. The strength of more than 75,000 promoters was assessed in a massively parallel reporter assay conducted in both tobacco leaves and maize protoplasts. There was a greater than 250-fold difference in activity of promoters within the three species. Jores noted that there were species-specific differences in promoter nucleotide frequency and TATA box distribution. For example, *Arabidopsis* promoters, which were generally AT rich, were stronger than maize or sorghum promoters in the tobacco leaf reporter assay, whereas maize and sorghum promoters, which were generally GC rich, were stronger in the maize protoplast reporter assay. Core promoter elements like a TATA box, initiator, Y patch, BREu, BREd, or TCT initiator could affect promoter strength; some elements were associated with strong promoters in both tobacco and maize, while some had differential effects on promoter strength between tobacco and maize.[94]

Jores and colleagues have used these insights on promoter strength to design synthetic promoters. Using randomized sequences with nucleotide frequencies derived from *Arabidopsis* (AT rich) or maize (GC rich) promoters and different combinations of core elements and transcription factor binding sites, they were able to design promoters that largely recapitulated their data with natural promoters. They have further expanded on this by

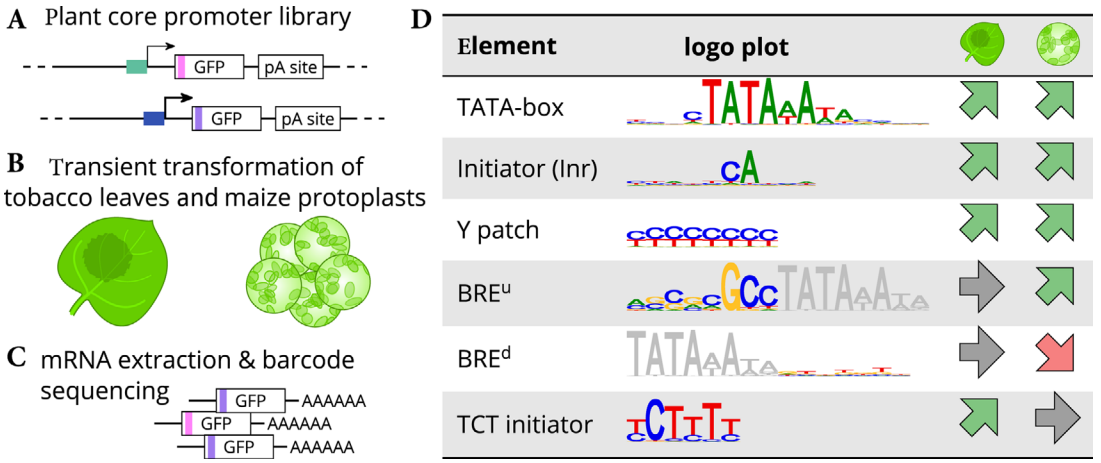

Figure 1. A comprehensive analysis of plant core promoters.

developing a computational model that predicts promoter strength in collaboration with Travis Wrightsman and Ed Buckler. Jores showed that the model can be used to develop stronger promoter sequences via *in silico* evolution.[94] Jores hopes that these results will not only enable researchers to design strong promoters but also help them identify relevant target sites within promoters to edit promoter strength and consequently gene expression.

Improving cereal crop transformation

William Gordon-Kamm from Corteva Agriscience described work on developing a variety of morphogenic gene systems to aid in maize transformation and genome editing. As Kan Wang discussed in her talk, one of the major bottlenecks in genome editing in plants remains the processes of transformation and regeneration. Gordon-Kamm described how two morphogenic genes (*Wus2* and *Bbm*) could stimulate rapid division of the transformed cells *in vitro* and improve transformation and regeneration frequencies in maize.[95] The original system required excision of *Wus2* and *Bbm* prior to regeneration. An improved version of this system was developed in which *Wus2* and *Bbm* expression were under the control of promoters active in plant embryos but inactive in roots, ears, and leaves. Using these two genes allowed not only significantly enhanced maize transformation and regeneration but also considerably shortened regeneration time. This QuickCorn transformation method results in the formation of somatic embryos within days of transformation; T0 plantlets can be produced in as little as 3.5 weeks.[96] Gordon-Kamm showed that the QuickCorn transformation protocol can be combined with the fast-flowering mini-maize system to achieve a very rapid generation time; seeds can be harvested approximately 3 months after *Agrobacterium* infection.

Moreover, Gordon-Kamm showed that the QuickCorn technology improves not only transformation frequency but also genotype range among maize inbreds and works well in sorghum and wheat. Corteva Agriscience uses this technology for all *Agrobacterium* and particle gun–mediated transformations as well as for CRISPR/Cas-mediated genome modifications. Gordon-Kamm showed an example in which the QuickCorn protocol was used to drop out the gene *WAXY* in several maize inbreds. While the transformation efficiency varied across lines, all lines were amenable to QuickCorn-mediated transformation.[97] The QuickCorn construct has also been used to mediate HDR, with efficiencies ranging from approximately 2% to 10%.

Gordon-Kamm also showed that constructs containing inducible expression of *Wus2* alone can result in rapid development of somatic embryos and T0 plant production from immature embryos in maize and sorghum.[98]

Finally, Gordon-Kamm showed that using constitutive promoters for *Wus2* and *Bbm* can improve the efficiency of leaf transformation in several cereal plants, including corn, sugarcane, millet, rice, and wheat. While leaf transformation is typically inefficient, recent improvements have achieved leaf transformation efficiencies that are nearly equal to immature embryo methods in corn.

Increasing fruit and vegetable consumption with genome editing

Tom Adams from Pairwise discussed the company's work to use CRISPR technology to improve fruits and vegetables and break down barriers that prevent people from eating produce. Pairwise is building a diverse gene-editing platform in food and agriculture and to leverage that platform to develop consumer-facing traits, such as seedlessness and off-season availability.

While innovations in produce are relatively rare compared with snack foods, Adams described several examples that illustrate how improving produce characteristics can drive consumer behavior. For example, the introduction of baby carrots increased U.S. fresh carrot consumption by 30% after 1 year. Halos® mandarin seedless snack-size oranges increased total citrus consumption by 30% and the availability of year-round blueberries increased the blueberry market fourfold.

Adams described Pairwise's efforts to develop a high-nutrient leafy green dubbed Veridi®. While many people recognize that greens like kale and arugula have high nutrient quantities, people tend to eat less nutrient-dense greens like romaine and iceberg lettuce. Veridi is a nutrient-dense green with sturdy, supple leaves that will stand up to dressings and toppings. Veridi was developed from the mustard green *Brassica juncea*. While *B. juncea* has a desirable leafy texture, it has a strong, pungent flavor. To improve the flavor

profile, CRISPR/Cas12 was used to simultaneously engineer loss-of-function mutations in all 16 copies of the enzyme responsible for catalyzing the production of compounds with pungent flavor. Pairwise has developed true breeding lines of Veridi and is in the process of generating seeds for sale and optimizing commercial production.

Adams also described the company's efforts to produce a sweeter, seedless blackberry that is available year-round. The company is currently developing a transformation system for the blackberry. Additional efforts are ongoing to produce pitless stone fruits like cherries, peaches, and plums.

Short talks

Eyal Fridman from the Volcani Agricultural Research Organization presented their work in using RECAS9 to map QTL in barley. Fridman is interested in understanding the evolutionary and genetic bases for adaptation in wild crops and cultivars. This is especially pertinent as climate change is expected to decrease cereal crop yields in many regions. During field trials, Fridman's group identified a locus associated with drought resistance, *HsDry2.2*.[99] Given the size of the locus (>400 million bp), it was difficult to clone the gene. Work in yeast had shown that Cas9 can be used to map traits.[100] Fridman's group used a similar strategy using RECAS9 to recombine the drought resistance-associated QTL *HsDry2.2* and a heat resistance-associated QTL, *HsHeat3.1*.[101] They showed that for the *HsHeat3.1* locus, recombination was more efficient with Agrobacterium than with RNP, while no recombination was observed for the *HsDry2.2* locus.[102]

Sergei Svitashev from Corteva Agriscience described work to induce a 75-Mb inversion in maize. A pangenome project by Corteva of 66 maize genotypes identified large rearrangements (≥100 kilobases) in every chromosome, the largest of which was a 75-Mb inversion in chromosome 2 present in three lines. Inversions prevent recombination and therefore exclude the corresponding regions from breeding projects. Svitashev's group was interested in inverting this region to make it available for recombination. They reasoned that inducing DSBs at the inversion borders could potentially lead to an event in which the excised 75-Mb fragment would pair with the opposite ends and be repaired via NHEJ, essentially inverting the region via intrachromosomal translocation. They were able to generate such an inversion in the elite maize genotype PH1V5T, using two gRNA/Ca9 RNP complexes. Out of 1500 T0 plants analyzed, the desired inversion was detected in two plants.[103] Svitashev's group has generated transgene-free plants that are homozygous for the inversion and are available for crossing experiments and agronomic evaluation. They hope that chromosomal engineering projects like this will open up new opportunities for crop breeding.

Trevor Weiss from the University of Minnesota presented work aimed at understanding how chromosomal context affects CRISPR/Cas9 efficiency and editing outcomes. Previous studies have shown that not all gRNAs are equally effective. While sequence features undoubtedly play a role,[i] other features like nucleosome occupancy and chromosomal accessibility also play a role.[104,105] It can be challenging to compare the cutting efficiency and outcomes between different gRNAs. To remove this complication, Weiss investigated the efficiency and editing outcomes of a single gRNA that targets multiple loci in the Arabidopsis genome. They presented unpublished work looking at the effects of chromosomal accessibility and context on cutting efficiency and editing outcomes. Weiss hopes that this work can shed light on the rules governing CRISPR/Cas9 editing, enabling new strategies to further optimize genome editing at refractory sites.

Rammyani Bagchi from the University of North Carolina, Greensboro presented work on developing a gRNA-based strategy for CRSIPR antiviral applications. In plants, CRISPR/Cas has mainly been used for precision breeding; however, as Mahfouz also described earlier in the meeting, CRISPR/Cas also has potential for applications as antivirals.

Viral genomes are highly polymorphic, and viruses often developing mutations that enable them to evade antivirals. To address these challenges, current CRISPR-based antiviral strategies have focused on conserved regions of the genome

[i] https://doi.org/10.1038/s41587-020-0555-7; https://doi.org/10.1038/nbt.4317

that are unlikely to diverge between viruses and/or incorporate more than one gRNA to limit mutagenic escape.

Algorithms to design gRNAs for viral detection are typically based on those for genome editing. In this case, precision is key. The algorithms are optimized to design a single gRNA that recognizes a single target with no off-target activity. Bagchi proposed an alternative CRISPR gRNA-based antiviral strategy that exploits the natural tolerance for mismatches between the gRNA and target sequence. They have developed an algorithm that identifies homologous regions in the viral genome and designs a single polyvalent guide that can target multiple sites.

This approach was validated *in vitro* using a polyvalent gRNA (pgRNA) that targets two homologous targets on TRV genome. Bagchi showed that while monovalent gRNAs only targeted protospacers that resembled their own sequence, the pgRNA was highly active at both sites. In *in vivo* experiments, pgRNAs were able to robustly suppress TMV spread in *N. benthamiana* and were more effective than monovalent guides.[106]

Targeting multiple sites with a single gRNA increases the probability of suppressing mutagenic escape and improves the sensitivity and robustness of viral detection in diagnostics. Bagchi is working to transfer this technique to crops to see if it can suppress viral propagation and prevent mutagenic escape.

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