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REVIEW

Hotter, drier, CRISPR: the latest edit on climate change

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Abstract

Key message **Integrating CRISPR/Cas9 genome editing into modern breeding programs for crop improvement in cereals.**

Abstract Global climate trends in many agricultural regions have been rapidly changing over the past decades, and major advances in global food systems are required to ensure food security in the face of these emerging challenges. With increasing climate instability due to warmer temperatures and rising CO_2 levels, the productivity of global agriculture will continue to be negatively impacted. To combat these growing concerns, creative approaches will be required, utilising all the tools available to produce more robust and tolerant crops with increased quality and yields under more extreme conditions. The integration of genome editing and transgenics into current breeding strategies is one promising solution to accelerate genetic gains through targeted genetic modifications, producing crops that can overcome the shifting climate realities. This review focuses on how revolutionary genome editing tools can be directly implemented into breeding programs for cereal crop improvement to rapidly counteract many of the issues affecting agriculture production in the years to come.

Introduction

The changing climate will continue to impact global agricultural productivity, farm incomes, and food security. World hunger continues to affect more than 800 million people worldwide, where approximately 11% of the population remain chronically hungry, and productivity is already predicted to be inadequate to meet the demands of the growing population (Dhankher and Foyer 2018; Ray et al. 2013). Climate trends such as increasing mean temperature, climate variability, and increase in extreme weather events will inevitably jeopardize global food security (Gornall et al. 2010).

Rising temperature and reduced and unpredictable precipitation are two of the major challenges caused by climate change and in many climate-vulnerable regions these two features are predicted to worsen simultaneously (Meehl et al. 2007). Even in the regions that are not predicted to have reduced precipitation are at risk for drought stress (Tebaldi et al. 2011), as the increasing temperature will reduce soil moisture, increase surface runoff from the intense storms and result in higher evapotranspiration (Dai 2011). Compounded by the rising CO_2 levels, especially around flowering, these features will have detrimental effects on grain set resulting in yield losses (Peng et al. 2004; Singh et al. 2015; Stratonovitch and Semenov 2015), effect the rate of development and senescence (Asseng et al. 2015; Challinor et al. 2016), as well as indirectly effect the prevalence of pests and diseases (Dawson et al. 2015). Thus, along with a push to reduce the agricultural footprint by limiting inputs of chemical fertilizers and pesticides, these realities highlight the importance of applying innovative yield-neutralising and yield-enhancing strategies to boost crop production under increased stress.

A key mechanism for adaptation of cropping systems to climate change is plant improvement, where current breeding practices rely on the long-term selection of rare, but naturally occurring genetic variation to select for favorable combinations. However, unwanted traits are often linked to beneficial ones, requiring several breeding cycles to replace these with the desired traits. Further, this approach requires many years to introduce desirable alleles via genetic recombination (Scheben et al. 2017). Significant parts of the genomes of major crops are fixed due to thousands of years of directed evolution, domestication, and breeding. Hence, genetic variability has been greatly reduced, limiting the

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capacity to improve many traits (Chen et al. 2019). Genetic variation can also be expanded by mutation breeding where random mutations are generated using chemical mutagens or physical irradiation (Pacher and Puchta 2017). However, generating and screening large numbers of mutants can be challenging and cannot match the demands for improved crop production, even when considering marker-assisted breeding approaches (Scheben et al. 2017). Although the generation of mutant populations is still used to this day, new technologies are available that enable targeted modifications within the genome.

Combining synthetic tools and conventional breeding into genomics-based breeding are a creative approach to overcome the hurdles of conventional breeding. Genome editing has the potential to accelerate fundamental research and plant breeding with improved qualities through the rapid, precise, and targeted modifications of genomes. These genome editing systems have created significant interest in plant science communities, as the outcomes are often indistinguishable from natural mutations or those from mutagenesis techniques. Since its advent, genome editing has led to significant agronomic improvements in a variety of crop species (Zhang et al. 2019b). There are, however, serious constraints to the commercialization of gene edited crops, primarily attributed to expensive regulatory evaluation processes and public concerns (Prado et al. 2014). Nonetheless, the incorporation of biotechnological tools into breeding programs can expedite the breeding of beneficial traits, break linkage drag associated with deleterious traits and develop novel combinations that are not found in nature.

This review aims to discuss the potential of gene editing tools and how it can be leveraged as a method to accelerate current breeding processes to satisfy global food demands. Limitations associated with gene editing, including the technical bottlenecks, current global regulations of gene edited products and modes of integration into breeding programs will also be discussed. The changing climate will impact a variety of abiotic stresses outside of the ones focused within this review and more information about biotechnological strategies to combat these issues can be found in other reviews (Borrelli et al. 2020; Zhang et al. 2019b; Jain et al. 2015; Joshi et al. 2020) This review outlines the major abiotic constraints of heat, drought, and salinity stress that negatively impact yields with the increasing unpredictability of climate and highlights the gene editing strategies in cereals that can be used to improve global agricultural systems.

Gene editing

The first gene editing systems involved zinc finger nucleases (ZFNs) and transcriptional activator-like effector nucleases (TALENs), where a DNA-binding domain targeting a specific location in the genome is fused to a FokI endonuclease (Christian et al. 2010; Kim et al. 1996). These techniques were followed by the advent of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas), leading to a simpler and cheaper alternative which has expanded its use in laboratories worldwide (Horvath and Barrangou 2010; Jinek et al. 2012; Ran et al. 2013). The key to genome editing is the generation of double-stranded breaks (DSB), forcing the plant to undergo endogenous repair mechanisms. The most common repair pathway in cereal crops is non-homologous end joining (NHEJ) where the DSB is repaired through an erroneous ligation. Alternatively, homology-directed repair (HR) reconstitutes the original sequence with the desired modifications, where the desired sequence is within a DNA template that has flanking regions that share homology to the cleavage site reconstitutes (Jasin and Rothstein 2013).

CRISPR/Cas genome editing technology involves a guide RNA (gRNA) that navigates the Cas endonuclease to a specific region(s) of the host DNA, cleaving strands of DNA and generating a DSB (Fig. 1). Any region of the genome can be targeted if it is upstream of a Cas-dependent protospacer adjacent motif (PAM), which makes this system versatile in terms of potential target sites (Cong et al. 2013; Wiedenheft et al. 2012). The most commonly utilised is the Cas9 protein from *Streptococcus pyogenes* which requires a 5′-NGG-3′, although other Cas proteins have been both discovered and synthetically developed that have alternative PAM recognition sequences and with increased specificities and editing efficiencies (Karvelis et al. 2015; Kleinstiver et al. 2015, 2016; Muller et al. 2016). Important criteria for design and implementation of CRISPR/Cas genome editing have been thoroughly described in a variety of reviews (Bortesi and Fischer 2015; Gerashchenkov et al. 2020; Ma et al. 2016; Yue et al. 2020). The ability to target multiple genes is also possible with genome editing through introducing multiple gRNAs into the host. This can be accomplished with each gRNA expressed under its own promoter, or via polycistronic cassette under the control of one promoter and processed post-transcriptionally (Xie et al. 2015). In sugarcane, genome editing was used to target 107 genes (Kannan et al. 2018), whereas in wheat a CRISPR/Cas9 system has reported and 35 genes were mutated simultaneously (Sánchez-León et al. 2018).

The ability to specifically target regions of the genome has led to alternative strategies to manipulate the genome. The use of inactive or "dead" Cas nucleases (dCas9) that are fused to a variety of other enzymes are an emerging end use of this technology (Moradpour and Abdulah 2020). Base editing uses nickase Cas9 (nCas9) making a cut in one strand and facilitates transition mutations via cytosine or adenine deaminases (CBE, ABE) (Kang et al. 2018; Lu and Zhu 2017; Shimatani et al. 2017; Zong et al. 2017). Changes to expression patterns of native genes have also been

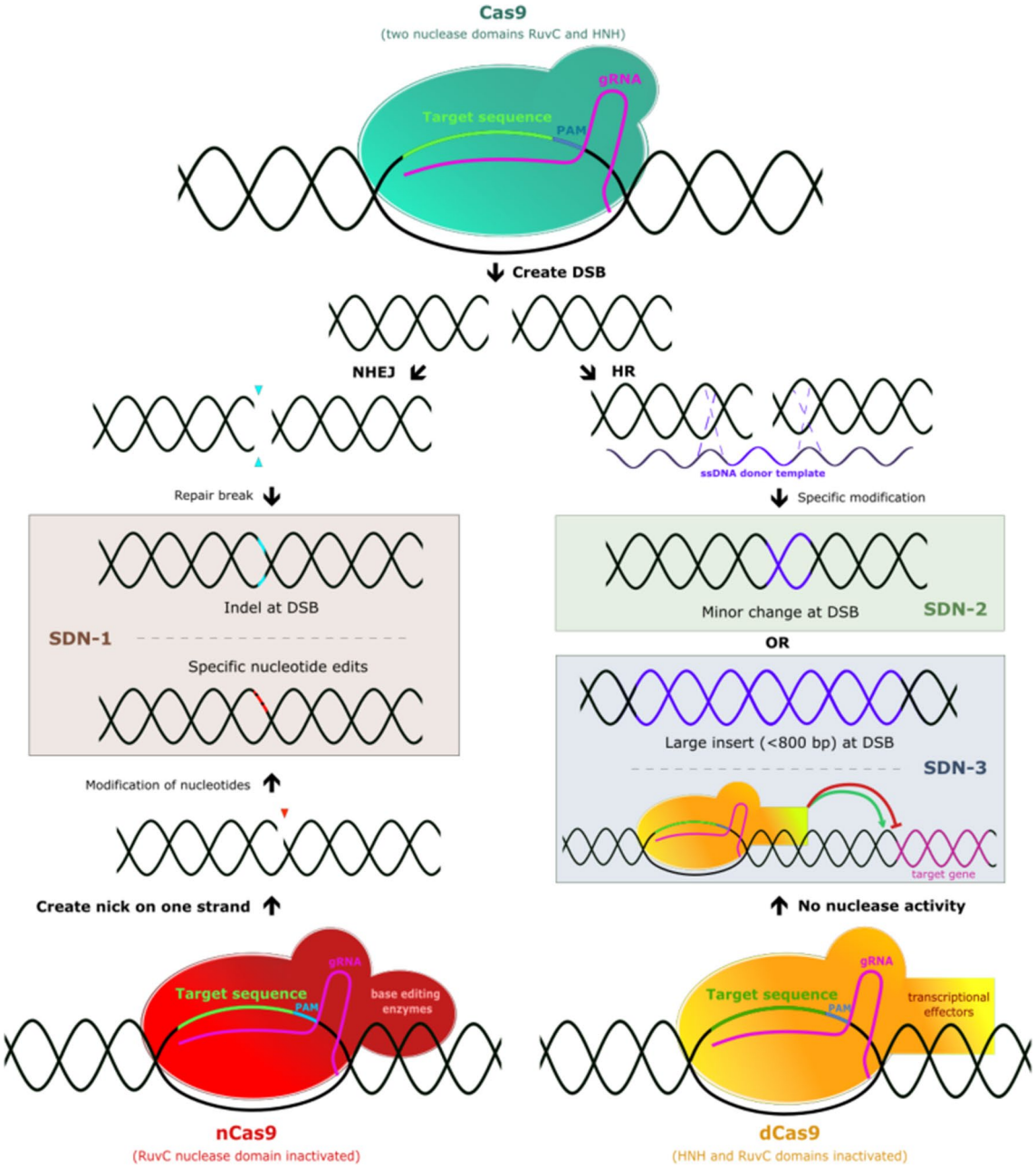

Fig. 1 Three potential applications of CRISPR/Cas9 genome editing, demonstrating their outcomes and classification within the context of site-directed nuclease (SDN) technology. The Cas9 protein contains two nuclease domains, HNH, and RuvC, which each cleave one strand of DNA to create a double-stranded break (DSB). The Cas9 nuclease is localised to the target sequence of the host genome via the guide RNA (gRNA) and can target any region of the genome as long as it is upstream of the protospacer adjacent motif (PAM). This DSB can be repaired through non-homologous end joining (NHEJ) or homology repair (HR). If the break is repaired via error prone NHEJ, an insertion or deletion (indel) will likely be made at the break site. In this scenario, the CRISPR machinery integrated in the host genome can be segregated away from the newly made edit and be classified as SDN-1. Whereas repair through HR requires the use of a single stranded DNA (ssDNA) template, resulting in either a minor change of a few nucleotides or a large change that will be regulated as SDN-2 or SDN-3, respectively. Cas9 with inactivated domain(s) such as nickase Cas9 (nCas9) or dead Cas9 (dCas9) can also be used to modify a target gene. The nCas9 fused with base editing enzymes can create a nick in the target sequence, where the enzymes fused to Cas9 are targeted and modify specific nucleotides. Once CRISPR machinery is segregated from the edited nucleotides, the outcomes are classified as SDN-1. There are a variety of enzymes that can be fused to dCas9, in this example, a transcriptional effector is fused which can increase or decrease the expression of a target gene. The outcomes of dCas9 are regulated as SDN-3 as it requires constant expression of the CRISPR/dCas9 system for the effect

accomplished through fusion of transcriptional effectors, CRISPR interference (CRISPRi), and epigenome modifications. Although relatively untested in plants, future strategies may employ the fusion of dCas9 to epigenetic modifiers such as DNA methyltransferases, methylcytosine, and histone acetyltransferases (Liu and Moschou 2018; Osakabe et al. 2016). Many of these strategies are promising because the outcomes will not be regulated as genetically modified once the transgenes are segregated out.

Genome editing could fundamentally transform agriculture, however early advancements have seen genome editing be viable in only a select few varieties of target crops. As it stands, to introgress current modifications into elite varieties would require too much time and money, hindering breeding programs. To see the full benefits of genome editing in tandem with breeding programs, the ability to transform commercial germplasm at high efficiencies will need to be further improved. Thus, genome editing offers a diverse and innovative toolbox of solutions to study gene function which may be expanded for crop improvement.

Regulation of genome-edited crops

The applications of gene editing techniques in the development of new crop varieties have the potential to increase yields, pest, and disease resistance, increase productivity with fewer inputs and produce altered and desirable product qualities and with potential novel end uses. Like any of the plant breeding technologies applied in the past, these new breeding techniques applied judiciously, will contribute to reducing poverty, obtaining food security for more of the world's population, and contributing to the need for greater environmental sustainability of agriculture. Nevertheless, it cannot be overlooked that there is considerable global uncertainty regarding the use and legal framework of gene editing techniques, including the difficulties of trade disruption and uncertainties.

As with the rollout and commercialisation of GM technologies in crop improvement, government legislation has lagged in keeping up with the advancements in the applications of new techniques. Firstly, the patent landscape is difficult to navigate. Academic research institutions, government programs, and breeding and biotechnology companies are all faced with the ongoing complexities of the claims and counter-claims of the many players, such as the University of California and their opponents the Broad Institute and Massachusetts Institute of Technology. While this continues to play out in courts internationally, this may serve as a disincentive for governments and industry to invest in the technology (Ledford 2016). Even more damaging is the global inability to agree on what risks and benefits gene editing may deliver. The illegal and ill-advised use of gene editing, such as the "CRISPR baby" scandal in China in 2018–19 did little to raise public acceptance, where He Jiankui and some of his team were given prison sentences in late 2019 (Normile 2019; Normile and Cohen 2019).

Gene edited crops are regulated under site-directed nuclease technology (SDN), which describes biotechnological tools that rely on nucleases to generate the DSB, utilising the hosts natural repair mechanisms (Fig. 1). On a scientific basis, there are two major classes of gene editing: those which do not rely on a template to direct the edit and those that direct a specific edit with a template and rely on recombination. The first scenario describes SDN-1 where a DSB is created and repaired via NHEJ, leading to a simple mutation often targeted to genic regions to produce a knockout or change in function. For most seed-bearing crop species, the integrated gene editing machinery used to generate the DSB (i.e., plasmid DNA containing gRNA and Cas nuclease) can be segregated from the desired edit via crossing. Similarly, base editing approaches are viewed as SDN-1 repair since there is no repair template involved in the transition mutations made with the enzymes fused to the nCas9 or dCas9.

The second scenario utilises a DNA template that allows for HR repair at the DSB and can be classified as either SDN-2 or -3. The difference in classification is dependent upon the size of the mutation provided by the template. Outcomes from the SDN-2 mutations lead to only minor changes at the target site where only a few nucleotides are modified, inserted or deleted. Whereas in SDN-3, the repair template is a large genetic element usually more than 800 bp, and therefore the outcomes are viewed synonymously to inserted transgenes. Other uses of CRISPR/Cas9 such as the fusion to transcriptional effectors will continue to be regulated as genetically modified due to the presence of transgenes. This will also be the case for plant species that are clonally propagated as the inserted edit cannot be segregated from the inserted CRISPR machinery, or the technology requires a constant expression of the transgenes.

The way in which the different SDN classifications are regulated differs from jurisdiction to jurisdiction. The NHEJ-type edits rely on the natural error rate of DNA repairase mechanisms. In most circumstances, the double-stranded break is repaired to its original state. However, when a mistake is made this will result in small indel events which frequently knockout the gene's expression or result in a substitution or indel which change the activity of the encoded protein. As Custers et al. (2019) discussed, many of these types of genetic changes occur naturally, often at high frequency. They listed the following classes of genomic changes that regularly occur in natural systems: single nucleotide polymorphisms (SNPs), short deletions, short insertions, longer deletions (a few kbp), allele swaps, T-DNA insertions (although rarer but reported in *Nicotiana*, sweet potatoes, and *Linaria*), genome duplications, and transposon insertions and excisions (deletions).

They also pointed out that chemical and radiation mutations that have been regularly used in plant breeding programs led to > 20,000 sequence changes across the genome, but these have never been regulated, creating a vexing issue with regulators in the European Union. In a number of jurisdictions, such as Japan, USA, Australia, and numerous Latin American nations (Argentina, Brazil, Colombia, Chile, Guatemala, Honduras and Paraguay) (Gatica-Arias 2020), the NHEJ-type edits are not regarded as GM and are therefore not regulated as such. Many of the Latin American countries moved quite quickly to create a defined and operational legal framework for gene edited crops. Unsurprising, given that by 2017, over 40% of the worldwide area sown to GM crops was in Latin America.

The USDA-APHIS system has gone further, ruling that SNPs, deletions of any size, and substitutions with genes from compatible plant species would not be regulated. The US has led the world in area sown to GM crops for over 25 years, which in 2018 accounted for around 39% of total global area. In Australia, the Office of the Gene Technology Regulator ruled in 2019 that SDN-1 edited plants that did not contain any foreign DNA would not be regulated. Whereas SDN-2 edited organisms, even if the template was only used to deliver a single nucleotide substitution or insertion, would be regulated as containing foreign DNA and regarded as transgenic (Thygesen 2019). Japanese regulators also ruled that SDN-1 edited plants would not be regulated, deeming that they do not comply with the "Living Modified Organism" (LMO) as defined by the Cartagena Act (Tsuda et al. 2019). The same ruling deemed that SDN-2 organisms would be considered LMOs if they possess extracellularly processed nucleic acid, and therefore they are assessed on a case-by-case basis. Japan is the only country to date to have developed a policy for the regulatory oversight of gene edited plants and crops.

Given that the Asian continent has over half the world's population, and is the biggest consumer and producer of food, the situation does lead to uncertainty among research organisations and governments when considering trade markets. The two largest countries in the world, China and India, still do not have a clear pathway for the commercialisation of gene edited crops. Given that four of the five most populous countries on the planet are Asian (China, India, Indonesia, Pakistan), this is currently having a significant effect on the risk-appetite of markets for gene edited crops and food ingredients.

The European Union (EU), the largest market in the world with 500 million consumers, is arguably the biggest roadblock to the acceptance of GM and now gene edited crops for improving agricultural productivity and environmental sustainability. The Court of Justice of the EU ruled in 2018 that plants and animals derived from transgenesis, gene edited, or mutation breeding should be regulated under current legislation. This ruling was disappointing yet had been foreseen by several scientists who had been proposing a product-based rather than a process-based regulatory framework (Ricroch et al. 2016). They mentioned that two-point mutations which conferred herbicide tolerance in the weed, *Eleusine indica*, were deemed natural, yet the same targeted mutations made it a GMO under EU regulations when introduced in maize. The product versus process regulatory framework has been further examined and explored (Zhang et al. 2020) with the product-based regulations playing a major role in regulatory determinations in Canada, the United States and Argentina (Kleter et al. 2019; Schiemann et al. 2020), while in Australia the process-based system is used.

While policy and regulatory uncertainty remains, there are still considerable roadblocks to the way forward for new breeding technologies. The need for collective decision-making in the EU means that the majority required is very difficult to achieve when it comes to changing legislation (Casacuberta et al. 2017; Eriksson 2019). Thus, the future of gene editing lies not only in the science, but also in the politics.

Applications to breeding

For the first time in history, plant breeders have the extensive ability to control the specific introduction of targeted sequence variation, providing a game-changing technology to rapidly improve agricultural crops (Chen et al. 2019). Eliminating unwanted traits are now possible. Adding desired traits to elite varieties are now straightforward. The modification of crop traits can now be precisely altered in a single generation and the undesirable effects of linkage drag can be overcome. Gene editing will further our understanding of gene function and regulatory elements. Together, these possibilities will enhance the predictability of plant breeding outcomes thereby significantly contributing to food security.

Predicting the phenotypic consequences of a specific mutation in silico are rarely possible, since our mechanistic understanding of plant gene function and regulation is restricted (Rhee and Mutwil 2014). To overcome this limitation, a trial-and-error approach based on genome editing can be used to identify the optimal allele for a target trait. Generating an allelic series of individuals with mutations at different sites enables the identification of optimal alleles using a comparison of the gene expression and products, as well as phenotypes associated with each allele (Gilding et al. 2013; Ngangkham et al. 2018). Alternatively, qualitative traits can be achieved by varying the product of a gene, such as through altering the binding properties of a protein,

targeting the signal peptides, or altering active sites (Scheben and Edwards 2018).

The first approach of genome editing is to target the coding region of the gene which will likely generate a frameshift mutation leading to a gene knockout. This approach has proven to be highly useful in studying gene function and can be applied to remove deleterious traits. However, complete disruption of gene function can lead to extreme phenotypic effects which may not always be desirable for agronomic traits, thus editing regulatory elements to influence gene dosage and expression levels may be more beneficial for applied outcomes (Rodriguez-Leal et al. 2017; Wittkopp and Kalay 2011). Gene expression is regulated by the *cis*-regulatory elements (CREs) in the promoter sequences to control the timing and expression level of genes, whereby mutations can affect the patterned expression and/or levels of the genes (Wittkopp and Kalay 2011). One mode of modifying gene expression is to target the promoter and/or regulatory regions of a specific gene with more than one gRNA. This approach should lead to a variety of deletions between the target sites, removing key DNA elements and manipulate the expression of genes (Rodriguez-Leal et al. 2017). Further, there are approaches that one can modify the methylation patterns which will also be able to fine-tune the expression of desired genes.

The importance of genetic circuits is demonstrated by the high level of transcription factors throughout the genome comprising approximately 5% of flowering plant genes, and the discovery that over half of all variants found to influence traits are in *cis*-regulatory regions (Jin et al. 2014; Meyer and Purugganan 2013).

Creating new genetic diversity and removing genetic load

The evolution of natural QTL variants from spontaneous mutations has occurred over vast periods of time (thousands or even millions of years), resulting in only limited changes in agronomic traits. Current genetic improvement of agronomic traits is largely dependent on the slow and tedious selection and introgression of these rare natural mutations in QTLs that often occur in both coding and regulatory regions (Wittkopp and Kalay 2011). Gene editing systems will be particularly useful for creating novel allelic variations that are unfounded in nature, and in regions of low-recombination frequency that are likely linked to advantageous traits and therefore unlikely to be easily accomplished through conventional breeding.

Genome editing can also be used to recreate allelic variations from wild lines or other plant species into elite breeding lines (Belhaj et al. 2013). For example, these systems can be applied in polyploid species such as wheat, targeting all three homeologs simultaneously without combining mutations identified in different genomes through conventional crossing (Singh et al. 2018; Wang et al. 2014; Zhang et al. 2017b). Mutations can also be generated in three genomes of two different genotypes without the need for time-consuming and resource-intensive backcrossing (Singh et al. 2018). Thus, the creation of novel genetic diversity can easily and specifically be accomplished through genome editing approaches.

Genetic load is the unavoidable accumulation of deleterious mutations within a population. Due to the sizable implications of genetic load, a prospective avenue for crop improvement is to eliminate said load from breeding. Currently, the removal by selection-based breeding is impeded by several factors including the lack of recombination, drift, and the coupling with favorable loci. As an example, *Sorghum bicolor* (sorghum) is an important global crop that millions of people utilise for food, feed and fuel, where in biomass sorghums approximately 33% of nonsynonymous mutations are likely deleterious (Valluru et al. 2019). Furthermore, in *Zea mays* (maize) it is also known that deleterious mutations are enriched in low-recombination regions genome-wide, making it highly challenging to remove using conventional breeding strategies (Rodgers-Melnick et al. 2015). Theoretically, these issues can be overcome using targeted base editors to modify deleterious SNPs, whereas HR recombination can be used for modifications of larger deleterious traits. Testing the impact of these loci is not a trivial task, but there is potential for gene editing to be used to manipulate these loci to not only to create novel genetic variation, but also accelerate crop improvement through removal of deleterious mutations.

Modes of implementation

Genome editing is emerging as a powerful tool, but integrating the technology into crop breeding programs remains challenging because current methods: (1) require lengthy passages through tissue culture, (2) require specialised labs to undertake genetic manipulation with Cas9 components, and (3) are limited to a few amenable genotypes, which are generally non-elite (Hickey et al. 2019). Therefore, deployment of transgenic and genome-edited traits in new crop varieties is a doable but onerous task regardless of the breeding strategy.

Inbred systems, which are still utilised in some major cereal crops such as wheat and barley, propagate homozygotes plants via pedigree breeding. A high level of homozygosity is necessary to ensure limited segregation within the farmers field, which could expose deleterious recessive variants. Genetic gains are made in these systems through directed manual crossing, where those with the desired traits are continuously selected for. In theory, if diverse genotypes of lines within inbred breeding systems can be effectively

tissue-cultured, the desired manipulations with CRISPR/Cas9 can be applied directly. However, due the recalcitrant nature of many cereals in tissue culture, the manipulations are typically generated in specific transformable lines. Even if tissue culture protocols can be developed for elite varieties, the breeding population is constantly evolving which may not be adapted to the protocols. Currently, once the elite lines are identified late in the breeding cycle, a non-elite genotype is transformed and gene edited, and the desired edit (and preferably lacking the transgenes) can be introgressed into the elite line via repeated cycles of backcrossing. Once the trait is backcrossed, additional yield and quality evaluations are required to ensure the modified line maintains its original performance.

Hybrid technologies have numerous requirements for successful seed production. First, efficient cross-pollination between genetically distinct parental inbred lines must occur. Second, self-pollination of the female parent must be avoided, which can be difficult to achieve on a large scale due to a lack of reliable methods for separating the sexes and imposing outcrossing (Whitford et al. 2013). There are very few crop improvement technologies that offer rapid and substantial yield gains across multiple production environments, where capturing heterosis to harness vigor is one of them.

Based on the inheritance pattern, male sterility can be classified into cytoplasmic male sterility (CMS) and genic male sterility (GMS) (Wu et al. 2016). CMS is widely used in hybrid three-line seeds and stems from a mutation of a mitochondrial gene (Kim and Zhang 2018). The application of CMS is limited due to a variety of reasons, including the lack of stability in the CMS phenotype, insufficient restorer-line resources, and susceptibility of CMS-based hybrids to disease (Weider et al. 2009). Whereas genic male sterile lines can be used within a hybridization platform, using a two-line breeding system. The genic male sterile lines are either photoperiod-sensitive or thermo-sensitive, which ensures a wider genetic resource to better exploit heterosis (Yuan 1990, 2014; Zhang et al. 1994, 2013; Zhou et al. 2012, 2014). As the capacities of genome editing expands, transgene breeding could be implemented into these types of systems although it does require genome editing either directly into the male and female populations, or many rounds of backcrossing to integrate the edited traits into these populations.

Developing new seed production technology depends on the availability of more male sterile germplasm resources. Since the male sterility phenotype is generally regulated by the recessive mutation of a nuclear gene, it is possible to generate male sterile mutants using the CRISPR/Cas9 system. The identification and isolation of the rice male fertility gene OsNP1 extend this platform to rice (Chang et al. 2017). Additionally, the *male sterility 8* (*MS8*) was targeted with CRISPR/Cas9 producing novel ms8 mutant lines exhibiting a male sterile phenotype similar to the naturally occurring ms8 mutant (Chen et al. 2018; Wang et al. 2013). CRISPR/Cas9 was also used to target 10 sites within the coding regions of *TMS5* gene, inducing thermo-sensitive genic male sterile lines (Zhou et al. 2016). Similarly, in wheat cultivars Fielder and Gladius, biallelic frameshift mutations were introduced into *Ms1* resulting in complete male sterility (Okada et al. 2019). These studies highlight the potential of genome editing for quickly generating male sterile mutant lines that can be employed to create hybrid seed production on an industrial scale and accelerates the potential to exploit heterosis through breeding of sterile lines.

Major climate constraints to address with gene editing

The best protection from climate change for farmers is to have access to a steady stream of new cultivars bred in the current climate. This strategy requires access to elite global germplasm, reduced breeding cycles, and the capacity to test germplasm in a range of target environments. This is possible for farmers in many temperate regions due to competitive seed sectors, however those in climate-vulnerable areas are restricted to their access of elite germplasm. In these regions, enhancing food security in the face of climate change will require considerable investment in accelerated breeding and rapid varietal dissemination and should consider using all tools available to do so.

Breeding for climate change often focuses on genes with large effects on heat and drought tolerance, but phenology and stress tolerance are highly polygenic traits which can also be problematic in breeding (Atlin et al. 2017). Plant breeders have identified many underlying component traits that can be modulated to reduce the impacts of such abiotic stresses that have severe impacts to yields. These traits can be highly heritable, and even if they are underpinned by a variety of genes and can be manipulated in unison via a multiplex genomic editing approach.

Drought

Drought is the major abiotic stress affecting global crop production, as the unpredictable rainfall can severely affect yields. Despite the numerous studies on drought adaptation across plant species, it is a difficult trait to study as it is controlled by networks of genes which are interconnected to stimuli changes in the environment. Drought adaptation can be conferred by the following pathways: (1) Alterations in plant architecture to prolong survival by delaying senescence and avoiding moisture loss, (2) increased expressions of components to maintain cell turgor and prevent dehydration, and (3) acceleration of plant growth and life cycle to

avoid drought exposure. These pathways are often controlled by networks of regulatory genes and signaling factors, such as protein kinases and transcription factors, that are associated with plant architecture development, plant hormones signaling, chlorophyll retention, osmotic adjustments, and reactive oxygen species (ROS) scavenging (Hu and Xiong 2014). The breeding process requires some, if not many, of these key regulatory and signaling genes to be identified and tweaked to generate new alleles producing a larger and potentially synergistic downstream effects.

Plant hormones have been shown to play major roles in regulation of plant responses to biotic and abiotic stresses (Verma et al. 2016; Wani et al. 2016). Abscisic acid (ABA) signaling and response pathways are one of the most studied areas in plants responses to abiotic stresses such as drought, heat, and salinity (Lata and Prasad 2011; Zhang et al. 2006). Further, regulatory genes and transcription factors have been two of the major foci in studies for drought adaptation, as these components have the capability to regulate a group of genes in a stress-response pathway, thus making them potential targets to confer drought tolerance The promoter regions of ABA-responsive genes often contain the *cis* element known as ABA-responsive element (ABRE) and inducing gene expression often require the presence of multiple ABREs or combinations with other coupling elements (Fujita et al. 2011; Hattori et al. 2002; Hobo et al. 1999; Marcotte et al. 1989; Narusaka et al. 2003; Shen et al. 1996). Another *cis* element that regulates osmotic stress-responsive transcription, the dehydration-responsive element (DRE) requires the binding of DRE-binding proteins or transcription factors (DREBs) to activate downstream transcription (Agarwal et al. 2006; Nakashima et al. 2009). The expressions of *DREB* and *DREB-like* genes enhance drought tolerance in rice (Cui et al. 2011; Huang et al. 2018; Wang et al. 2008; Zhao et al. 2010), maize (Liu et al. 2013), foxtail millet (Li et al. 2014), barley, and wheat (Lucas et al. 2011; Morran et al. 2011).

Shoot and root traits are mostly positively correlated and coupled at the genetic level, and shoot traits are dependent upon root traits through resource allocation tradeoffs (Bouteillé et al. 2012; Enquist and Niklas 2002; Hammer et al. 2009). Responses to drought stress have also been shown to be influenced by cross-talks between the different plant hormones signaling pathways. Auxin has been implicated to enhance drought tolerance in rice, by affecting plant architecture in response to drought stress via the ABA-induced upregulation of the maize *TLD1/OsGH3.13*, which encodes indole-3-acetic acid (IAA)-amido synthetase and subsequent expression of putative osmoprotectant *LEA* (late embryogenesis abundant) genes (Zhang et al. 2009). The upregulation of *TLD1/OsGH3.13* affects auxin homeostasis by reducing free IAA, which conferred a wide leaf angle, high tillering, and dwarf phenotype.

A recent genome editing study of *ARGOS8* gene, a negative regulator of ethylene responses, has shown that single endogenous genes can be altered to create novel variants that positively effect drought tolerance (Shi et al. 2017). In this study, the genome-edited variant replaced the native promoter to a constitutive promoter, leading to increased grain yield under drought conditions. Although the outcome would be regulated as GM, it demonstrates the utility of the CRISPR/Cas9 genome editing system to confer tolerance of complex traits such as drought. Additionally, CRISPR/Cas9 genome editing on the rice drought and salt tolerance gene (OsDST) improved drought and salt tolerance by having broader leaf width and reduced stomatal density, in part due to downregulation of stomatal developmental genes (Santosh Kumar et al. 2020).

Deep rooting is a drought avoidance trait, which is associated with spatial root distribution, determined by components such as root growth angle and root length (Abe and Morita 1994; Araki et al. 2002; Fukai and Cooper 1995). The *deep rooting* loci in rice are characterized by QTLs for root growth angle (e.g., DRO1, DRO2, DRO3, DRO4, and DRO5) (Kitomi et al. 2015; Uga et al. 2013a, b) and genes associated to these QTLs would be potential targets for genome editing to produce deep rooting phenotype in shallow-rooting varieties. *DRO*1, a gene found to affect root angle growth independent of shoot and root biomass, is negatively regulated by auxin and at least 3 auxin response elements (AuxREs). AuxREs, which are binding sites for auxin response factors (ARFs) that regulate transcription of the gene by auxin, are found in the upstream promoter region of *DRO1* in rice (Uga et al. 2013a). This suggests that expression of DRO1 can also be altered by editing AuxREs in the promoter region. Orthologues of *DRO1* are present in other cereal crops such as foxtail millet and sorghum, thus functional studies on these orthologues would also highlight them as potential targets for breeding and genome editing in these crops.

All the above-mentioned studies have shown that increasing the expressions of these transcription factors positively enhance drought tolerance in plants. Constitutive expression of these transcription factors in the native plant could potentially be achieved by utilizing the remodeled CRISPR mechanism with the ability to activate or repress transcription of target genes, via the use of dCas9 fused with a transcription activator or inhibitor (Chavez et al. 2016; Lowder et al. 2015; Piatek et al. 2015). A recent study in transgenic *Arabidopsis* has shown that the construct of dCas9 fused with histone acetyltransferase (HAT) with a sgRNA targeting an AREB1 promoter region, positively enhance the transcription of *AREB1* gene and conferring enhanced drought stress response (Roca Paixão et al. 2019). Further, downstream targets of the transcription factors may be useful targets for gene

knockout that may confer some of the beneficial drought-tolerant phenotypes.

Thermo tolerance

Both heat and cold stress are major concerns for food security due to the instability of climate. As sessile organisms, crops must adapt to these changes through molecular signaling cascades of heat- and cold stress pathways. Unfortunately, the expression of these genes may have negative pleiotropic effects on the quality and yield of the crops throughout development. Extreme heat can further exacerbate the issues surrounding drought, as it leads to increased demand for water used for transpiration (Lobell et al. 2013, 2012).

Diversity in heat stress-linked and -responsive genes has been discovered using GWAS and transcriptomic studies (Singh et al. 2019). Modification of the expression of these genes may provide tolerance, as demonstrated through studies overexpressing the heat shock protein 101 (*HSP101*), providing heat-tolerance in both Arabidopsis and rice which lack any pleiotropic negative effects on growth or yield (Katiyar-Agarwal et al. 2003; Queitsch et al. 2000). Other validated heat-responsive genes such as the spotted-leaf gene (*SPL7*) in rice (Yamanouchi et al. 2002), a peroximal type ascorbate peroxidase (HvAPX1) gene in barley (Shi et al. 2001), and both a MYB transcription factor (*TaGAMYB*) and a gibberellic acid-stimulated transcript (*TaGASR1*) in wheat (Wang et al. 2012; Zhang et al. 2017a) are potential targets for genome editing to further understand their function in thermotolerance response.

Targeting an auxin efflux carrier-like gene *OsPIN5b* in rice using RNAi resulted in high tillering and a more robust root system phenotype (Lu et al. 2015). Later, along with two other gene candidates including a gamma-subunit G-protein receptor kinase (*GS3*) and a *MYB* transcription factor (*OsMYB30*), triple edited lines led to increased grain yield and cold tolerance (Zeng et al. 2020). Orthologues of these candidate genes in other cereal crops could also be similarly altered to produce a more robust root system, providing yield increases, and generating improved cold tolerance through the generation of targeted knockouts, potentially to improve drought adaptation. Conversely, a cold tolerance regulatory gene annexin (*OsAnn3*) was successfully targeted and demonstrated significant reduction of cold tolerance, demonstrating the importance of annexin genes for cold adaptation (Shen et al. 2017). These studies demonstrate that thermotolerance can be modified through monogenic approaches, but also the importance of considering using multiplexed genome editing to provide yield stability traits that may be affected through gene disruption that provide tolerance.

Salinity and soil nutrient deficiencies

Breeding for salinity tolerance has been attempted using conventional rice breeding, however, the lack of screening techniques and resources and the high polygenic control of this abiotic stress demonstrates the need for modern tools to combat salinity stress (Farhat et al. 2019; Hoang et al. 2016). In rice, transgenic plants have shown positive outcomes for salinity stress. The knockdown of OsRMC which is a negative regulator of salinity stress demonstrated improved tolerance (Zhang et al. 2019a). CRISPR/Cas9 systems have also been used to improve salinity tolerance in elite rice cultivars by targeting the OsRR22 gene (Zhang et al. 2019a). In this study, only one year was required to improve the salt tolerance of WPB106 via CRISPR/Cas9 technology, indicating that genome editing tools are an important resource for enhancing salinity tolerance.

Plants have highly sophisticated mechanisms and regulatory networks to control nutrient homeostasis, but because conventional breeding is often performed with ample fertilisation, the necessary genetic diversity for genes to improve the nutrient use efficiency is lacking in most breeding programs. These are further complicated by the diversity of agro-environments, soil properties, and co-occurrence of multiple stresses. Therefore, it seems unlikely that a monogenic approach will be enough to have a significant beneficial effect. Although a gene disruption approach may not be required, base editing and modification to *cis*-elements may provide the necessary allelic diversity changes to generate improved lines. Thus, further research into studying allelic variation within pathways is required to generate key targets for CRISPR/Cas9 genome editing approaches (Hawkesford and Griffiths 2019; Massel et al. 2016).

Photosynthesis and biomass

Various C_3 cereal crops such as wheat, barley, and rice can experience a decrease in photosynthetic conversion efficiency of up to 50%, with the largest losses apparent in hot and dry climates (South et al. 2019). Given the rate of climate change, maximizing photosynthetic efficiencies will be integral for future crop development. Through the Green Revolution, yield potential has seen exponential increases by selecting for genotypes (i.e., dwarf genotypes) where the biomass partitioning has favored the harvested portion of the crop. This proportioning of biomass is referred to as the harvest index or portioning efficiency (E_p) and is one variable in the yield potential (Yp) equation (Long et al. 2015):

$Yp = Q \bullet E_i \bullet E_C \bullet E_p \ldots$

Q: product of solar radiation received over the growing season by a unit area of land.

E_i: efficiency that the crop intercepts that radiation.

E_c: conversion of intercepted radiation into biomass energy.

E_p: amount of biomass partitioned into harvested part of plant.

Both E_i and E_p have been nearly maximized through the conventional breeding methods used during the Green Revolution (Long et al. 2015; Zhu et al. 2010). However, E_c, which is highly dependent on photosynthetic efficiency, currently stands at 0.1 for C_3 crops and 0.13 in C_4 crop (Zhu et al. 2010). The lack of improvements made to photosynthetic efficiency can be attributed to the high conservation of E_c across all plants, whereas the other two variables E_i and E_p exhibit high variation. Much of the photosynthesis-related genetic modifications have relied on inserting synthetic or non-native genes or down-regulating native genes in the host species, thus minimal gene editing has been done to improve photosynthetic efficiencies.

One study, using tobacco as a model species, expressed alternative pathway genes of bacterial or algal origin while simultaneously knocking-down a glycolate transporter (*PLGG1)* to minimize native photorespiration (South et al. 2019). The overexpression of these alternative pathway genes combined with the PLGG1 knockdown showed increases in biomass compared to both the wildtype under an environment with increased CO_2. Given that the knockdown was only 80% efficient, a CRISPR-targeted knockout of *PLGG1* could further increase the biomass growth from the reported 19–37%. However, there are other targets within this pathway to reduce photorespiration that could prove to be another potential target to enhance the photosynthetic capacity of critical C3 crops by reducing the inefficiencies of photorespiration (South et al. 2019; South et al. 2017). Loss-of-function mutants in rice show a reduction in photorespiration, but also a severe loss of biomass (Shim et al. 2020). Thus, modification to key genes in photosynthesis may require a finessed approach and further understanding of the complex regulatory circuits of these biochemical networks. Additionally, dissimilar approaches may be required to improve the photosynthetic capacity in C_3 crops, such as wheat, compared to C_4 crops like maize.

Phenology

In addition to the increase in temperatures, the window for optimal plant development has become more erratic causing yield losses in several key cereal crops. The phenology of a plant is highly plastic since the molecular and epigenetic mechanisms help respond to both environmental and endogenous cues to regulate flowering behavior (Hills and Li 2016). Phenology, in relation to plant development, plays a critical role in determining the success (i.e., maximum yield) of the crop (Hyles et al. 2020; Trethowan 2014). This is particularly true for winter crops grown in areas with frost incidences, where frost damage and lack of sunlight may damage reproductive structures and impact yield. Conversely, flowering too late when conditions are hotter and drier may risk heat damage and water limitations (Flohr et al. 2017). To avoid these environmental constraints that are being amplified due to climate change, flowering at optimal times and conditions are critical to a plant's adaptability under volatile environments.

Flowering time, heading date, and photoperiod-sensitive regulating genes play a fundamental role in determining the adaptive capacity of the crop in relation to the geographic environment (Andrés and Coupland 2012; Hills and Li 2016). Many gene editing studies have produced phenological modified mutants in key crops such as rice, wheat, and sorghum to maximize the yields under the changing day lengths. The flowering process of many cereals is critical in determining both the quality and quantity of yield, thus flowering under sub-optimal conditions can be detrimental for both crop and farmers (Reynolds et al. 2016). In sorghum, the flowering time gene *FLOWERING LOCUS T (FT)* resides within both flowering time and plant height QTL's. When knocked out using CRISPR/Cas9, the T_1 mutants were found to flower 10 day earlier, on average, compared to the wildtype (Char et al. 2020). This would be advantageous in environmental scenarios where days are shorter and decrease in solar irradiance, reducing the level of potential photosynthesis. In the model species *Arabidopsis*, it has been shown that accelerated flowering could reduce the lifetime water usage without negatively impacting the reproductive performance of the plant, indicating that this pathway may provide a suitable relief against shorter day lengths without sacrificing crop yields (Ferguson John et al. 2019).

The heading date family is a key group of genes that, in addition to regulating flowering time, have exhibited pleiotropic effects on grain yield in numerous cereals, including the staple food crop rice (*Oryza sativa* L). In rice, the determination and timing of head development is regulated by a network of various heading time genes including early heading date 1 (*Ehd1*), grain number, plant height and heading date 7 (*Ghd7*), and heading date 3a (*Hd3a*) (Cui et al. 2019). One study utilized CRIPSR/Cas9 to target ten key heading time genes and demonstrated a correlation between biomass accumulation and yield, where certain early heading mutants experienced a decrease in yield, while mutants that flowered too late also experienced a decrease in yield (Cui et al. 2019). A similar study targeted three flowering suppressors (*Hd2, Hd4,* and *Hd5*) in the rice photoperiodic flowering pathway to develop early maturing rice varieties. The mutant lines were shown to benefit greatly in the more northern cultivation zones where some of the corresponding parents were unable to flower at all (Li et al. 2017).

Developmental genes for adaptation/yield

Developmental genes establish the growth of various plant structures, thus are key to improving and maintaining yield gains. In addition, these developmental genes are tightly linked to various regulatory processes, resulting in downstream regulation of genes linked to stress response (Phukan et al. 2017). With the state of the current and projected climates, the ability to regulate plant development that will be critical for food security. Many of the developmental genes are required for the initiation of growth and development, thus the downregulation of them would most likely produce negative effects. To capitalize on the benefits of these developmental genes using modern gene editing tools, edits targeting negative regulators of these genes would be beneficial. Further, when considering all the aforementioned traits that require improvements to combat climate change, the maintenance and improvement of grain yield is required above all.

An important gene family for development and yield is the *APETALA* family, due to the central role they play in various aspects of plant development. The *APETALA* 1-like family (AP1) is critical for inflorescence meristem development but have been implicated to have downstream effects on plant architecture (Debernardi et al. 2020; Li et al. 2019; Theißen et al. 2016; Voss-Fels et al. 2018a, b). The *APETALA* 2-like (AP2) family is believed to regulate the transition of the spikelet meristem from sterile glume production to the production of florets. Genes belonging to this family have been found to collocate with a grain weight QTL (GW7) and using genome editing techniques demonstrated a considerable increase in yield by improving grain weights (Ma et al. 2019). Further, the AP2 genes also belong to a superfamily of ethylene responsive factors (AP2/EFF) which are transcription factors known to participate in abiotic stresses (Debbarma et al. 2019). Although there is a limited number that provide a negative regulation to abiotic stresses, modifications of proteins within this superfamily may be a key to multi-stress tolerance responses across cereal crops.

Grain yield is one of the most important and complex traits for genetic improvement in crops. It is an important quantitative crop trait for plant breeding and previous gene disruption approaches have been shown to be successful. Variations in gene dosage and expression patterns can also cause phenotypic changes in crops (Meyer and Purugganan 2013). In barley, grain yield has been improved by increasing the size of grain in the central spikelets through a single amino acid substitution in the *VRS1* gene encoding a homeodomain leucine zipper protein (Sakuma et al. 2017). This mutation leads to suppression of floret development in the lateral spikelets. It remains unclear, however, the extent to which such QTLs confer yield performance in different genetic backgrounds. Two genes in rice, *GS3,* and *Gn1a*, were knocked out using CRISPR/Cas 9 technology to generate different effects on variations of grain size, grain number, and tiller number in different genetic backgrounds (Shen et al. 2018). This CRISPR/Cas9-mediated QTL editing in five broadly cultivated rice varieties showed that the same QTL can have multiple, even opposing, effects on grain yield in different genetic backgrounds.

Future prospects for trait dissection and integration of gene editing into breeding programs

Targeting extranuclear genetic material

The ability to target extranuclear regions of the host genome is an attractive approach as they play essential roles in energy metabolism, photosynthesis, and development. Additionally, there are considerable benefits associated with placing transgenes within plastid genomes over the nuclear genomes, including the lack of gene silencing and the ability to express genes as an artificial multigene operon (Bock 2015). However, there are numerous complications to target and adapt CRISPR/Cas9 genome editing systems to function within plastids (Gammage et al. 2018).

Chloroplast transformation protocols have been developed in some plant species which could be used to integrate machinery into these organelles (Day and Goldschmidt-Clermont 2011). Chloroplasts are important targets, as they could be manipulated to improve photosynthetic activity in crops, for example, by increasing Rubisco catalysis efficiency (Sharwood 2017). One successful example of chloroplast editing in plants was performed in tobacco using a two-step editing system, targeting the ribulose-bisphosphate carboxylase (*rbcL)* gene and resulting in reduced accumulation of the Rubisco large subunit in transplastomic derivatives (Avila et al. 2016).

Current genome editing in plant mitochondria is also limited, although the use of transcription activator-like effector nucleases (TALENs) with mitochondria localization signals (mitoTALENs) has been shown to work in studying gene function in mitochondria (Kazama et al. 2019). Transcriptome studies of deep and shallow-rooting rice varieties have revealed genes that are involved in energy metabolism and DNA processing is differentially expressed (Lou et al. 2017). Thus, there is great potential for gene editing in plastids to benefit food security if the protocols can be adapted to specifically and efficiently target extranuclear genomes.

High-throughput phenotyping

Throughput may be further increased by automation of breeding pipelines which should streamline target

identification, mutation tracking, and plant phenotyping. Plant breeders build on physiological traits to inform their selection of material for crossing and genetic gain, with plant phenotyping at the core of crop breeding (Furbank et al. 2019). More recently, crop physiologists and breeders have been able to quantitatively measure complex and previously intractable traits by utilising high-throughput techniques based on machine vision, robotics, and computing. Breeders have an opportunity to make rapid genetic progress by bringing together these methods with affordable genomic sequencing and genotyping, genome selection, and transgenic approaches.

In future, it is likely that CRISPR/Cas mutation panels will be generated at an industrial scale by breeding companies. If this does eventuate, high-throughput precision phenotyping will be crucial to save time and money. Traits such as biomass, yield, water use, and photosynthetic efficiency can already be measured with mobile and static sensors (Singh et al. 2016), including studies on biomass dynamics in maize (Muraya et al. 2017). As the scale and complexity increases, the importance of high-throughput phenotyping will increase. Machine learning algorithms can search for patterns in the large, complex datasets generated by sensors and cameras. Rapidly selecting mutants that are candidates for breeding would be enhanced by integrating the phenotypic data automatically with the mutation tracking platform. More complex traits will be engineered using allelic series with multiple combined edits in line with increasing scales of editing, enabled by automation of breeding workflows. A very different future is almost upon us. In addition to scanning their field plots with their human spectrometers, plant breeders will also identify new sets of targets for genome engineering and use phenome/genome data to refine genome selection models (Feng et al. 2017; Furbank et al. 2019; Ricroch et al. 2016).

Enhanced efficiency with the CRISPR/Cas plant breeding pipeline

Our ability to engineer crops is limited by our inadequate understanding of gene-to-phenotype interactions (Scheben and Edwards 2018). This then requires us to use guided 'trial-and-error' approaches in breeding such as parental crosses or CRISPR/Cas-induced allelic series. However, the number of mutations required in allelic series is increased because many important agronomic traits such as plant architecture are controlled by large numbers of minor-effect QTLs (Tian et al. 2011). To enhance genetic gains in crop improvement, researchers could target sets of alleles throughout the genome. The multiplexing capability of CRISPR/Cas systems can be scaled up to target editing of multiple alleles for a high-throughput approach, where they have already been used to target eight sites simultaneously with the possibility of greater multiplexing in future (Xie et al. 2015).

Breeding methods such as double haploid (DH) production (Shen et al. 2015) and speed breeding (Watson et al. 2018) should integrate well with highly multiplexed genome editing. Homozygotes for genotype–phenotype validation can be produced in a single generation with DH lines, where the gametic haploid chromosome set has been doubled. This is particularly helpful when multiple alleles have been edited because fixation of all desired alleles would otherwise take several generations of selfing. Together, these practises should help to decrease the number of breeding generations required to fix traits.

Many private breeding programs and some public programs already adopt a predictive breeding framework that uses genomic selection (GS) to improve breeding efficiency. Key benefits include a shorter and more efficient breeding cycle, achieved by predicting the genetic merit of untested lines, which enables more targeted field evaluation and earlier selection of parents for the next breeding cycle (Maher et al. 2020; Voss-Fels et al. 2019). Another powerful tool to reduce the length of the breeding cycle is 'speed breeding', which uses controlled photoperiod and temperature to reduce generation time (Watson et al. 2018). Protocols enable growing up to 4–6 generations of most crops, including day-neutral, long-day and short-day species (Ghosh et al. 2018; Jahne et al. 2020). This enables rapid development of inbred lines following crossing. Combining GS and speed breeding provides a dual benefit to reduce the length of the breeding cycle and substantially accelerates genetic gain per unit time (Voss-Fels et al. 2018a, b).

There are many ways that genome editing could be integrated into a breeding scheme, but the efficiency and flexibility of the protocol will determine the stage and scale that it is applied. Genome editing protocols that avoids the lab and could be applied to any plant would be highly desirable for plant breeding. It's been proposed that "ExpressEdit" approaches could bypass the bottleneck of regeneration and instead perform genome editing in a contained glasshouse or growth room where plants can be rapidly cycled under speed breeding conditions all-year-round (Hickey et al. 2019). Soon this could be possible, as several tissue culture-free techniques have already been developed, including ribonucleoprotein complexes (Liang et al. 2017), viral vectors (Wang et al. 2017) and most recently, de novo induction of meristems (Maher et al. 2020). Ultimately, multiple breeding technologies must be integrated to breed climate resilient crops and simulations can be performed to determine the most optimal breeding scheme given the available budget.

This provides an example how lab-free genome editing could be integrated into a modern breeding scheme that uses GS and speed breeding (Fig. 2). To minimise the number of plants for editing, parental lines (identified using GS) could

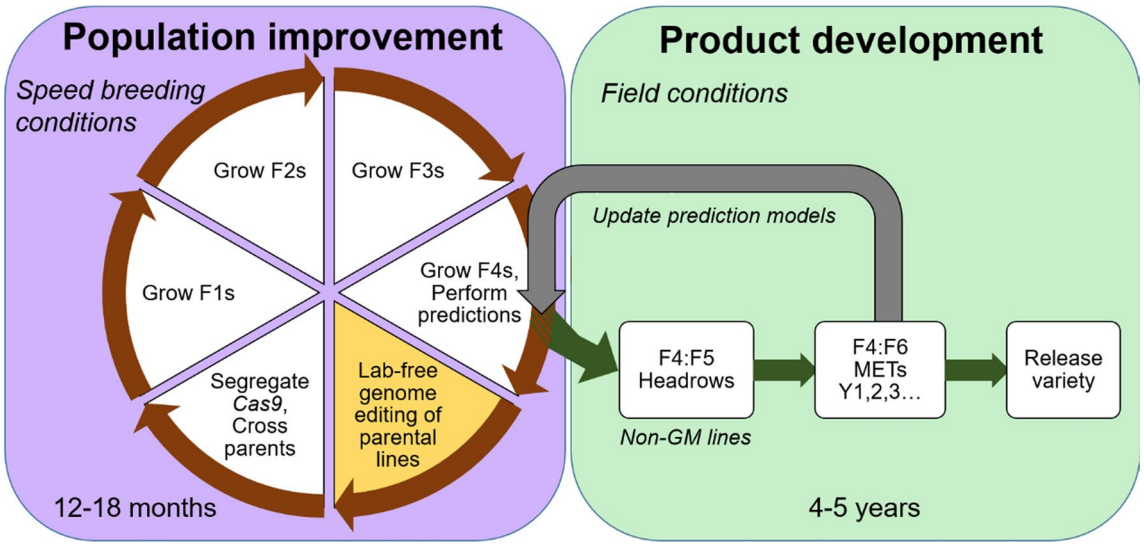

Fig. 2 Conceptual model of a futuristic inbreeding program that integrates lab-free genome editing, speed breeding, and genomic selection to fast-track climate resilient inbred crops

be edited prior to crossing. The "Population Improvement" phase uses speed breeding to accelerate editing, crossing, and selfing generations and can be performed within just 12–18 months in a PC2 contained facility. Prior to crossing the edited parental lines, CRISPR machinery can be segregated out, to generate non-GM elite lines based on some country's regulation. Based on genomic predictions performed on F4 plants, the most promising lines can be progressed to the "Product Development" phase for bulking up seed and subsequent multi-environment trials (METs) across multiple years. Field-based phenotyping is essential to update prediction models and for identifying superior lines for commercialisation. For crops that require hybrid breeding, generating genome-edited female and male lines separately are required, and additional test crosses will be required but should otherwise follow a similar pathway for product development. Additionally, for traits that have heterozygous advantage, this can be more easily produced in these systems.

Genome editing is already benefiting the agricultural industry and will continue to push how we can manipulate crop genetics for both fundamental and applied research practices. Especially in the context of changing global climate, there is a push to rapidly develop new crop varieties that can withstand the growing challenges to produce greater yields with enriched qualities. Genome editing has already been successfully applied in a variety of crops, providing outcomes that can be integrated into breeding programs (Zhang et al. 2019b). As transformation protocols continue to improve and new technologies are developed for lab-free genome editing in diverse crop genotypes, it will allow for a more seamless integration of edited traits into breeding programs to providing fast-tracked resilient crops.

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Compliance with ethical standards

Conflict of interest There are no conflicts of interest or competing interests.

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