Chapter 1
Genome Editing of Gene Families for Crop Improvement

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Abstract Crop improvement has been a long-standing focus of agricultural research, aiming to enhance nutritional richness, aroma, visual appeal, and yield to meet the growing global food demand. Recent advances in molecular biology and genetic engineering, particularly genome editing, offer precise and targeted tools for modifying crop genomes. Traditional plant breeding methods, while successful in the past, are time-consuming, and techniques like mutagenesis and transgenesis have limitations. Genome editing techniques provide unprecedented precision and enable scientists to make desired modifications to a plant's DNA. This chapter explores the role of genome editing, specifically in gene families, for crop improvement, highlighting its potential benefits and challenges.

Gene families are crucial for important crop traits like yield, disease resistance, and environmental adaptation. However, conventional breeding methods often struggle to effectively manipulate gene families due to their complex nature. Genome editing offers a promising solution by allowing targeted modifications to specific gene family members. The precision of genome editing tools can help unravel the functions of gene family members in diverse plant species.

With the challenges posed by climate change, global conflicts, and population growth, the conventional food system falls short of meeting future demands sustainably. Genome-edited crops hold promise in obtaining elite genotypes with desirable traits, contributing to a resilient and sustainable agriculture and food system. Moreover, genome editing facilitates the study of genetic diversity that governs desirable crop characteristics, benefiting both genome-edited and conventionally bred crops.

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1 Introduction

Crop improvement has been the main target of agricultural research for centuries in comprehensive areas such as increasing the nutritional richness of foods, obtaining aromatic foods with pleasant smell and taste, producing decorative products that appeal to visual pleasures, while increasing the yield, especially to meet the increasing food demand of the global population. In recent years, advances in molecular biology and genetic engineering have provided new tools for crop improvement. Genome editing, in particular, has emerged as a promising technique for modifying the genome of crops in a precise and targeted manner [1, 2].

The process of conventional plant breeding is time-consuming despite its significant contribution to the first green revolution in the 1960s. The introduction of molecular marker-assisted selection has helped to speed up the process of plant breeding. Techniques such as mutagenesis and transgenesis have helped to reduce the time required to develop new varieties. However, mutagenesis is a random process that can have undesirable side effects or can result in incomplete and off-target disruption of genes. Genetically modified transgenic plants can also face regulatory challenges. Unlike traditional genetic engineering methods, which involve the insertion of foreign DNA into an organism's genome, genome editing allows scientists to make precise modifications to the existing DNA sequence. Genome editing can be accomplished through different techniques, including CRISPR-Cas9, TALENs, and Zinc Finger Nucleases (ZFNs) [2]. These tools offer an unprecedented level of precision, enabling scientists to make precise, targeted changes to a plant's DNA, resulting in desired traits [3].

Gene families are groups of genes that share a similar DNA sequence and function. They often play critical roles in crop traits such as yield, disease resistance, and environmental adaptation. Although members of a gene family have different specialized functions, they share a common purpose. Therefore, it is important to understand and define the functions of gene family members involved in important plant development or stress resistance in different plant species. Traditional breeding methods are often unable to effectively target and manipulate gene families, as they may contain many members that have overlapping functions or redundancies [1].

The food system faces challenges such as sustainability and food supply stemming from climate change, global conflicts and increasing population [4]. Conventional breeding and agricultural practices come short in terms of meeting the current and future food demand. Therefore, it is necessary to have different approaches to ensure a more resilient and sustainable agrifood system. In this sense, genome-edited crops have the potential to play a role in obtaining elite genotypes with desirable traits in plant breeding. Besides, genome editing has another advantage, as it enables the study of genetic diversity that governs desirable crop characteristics. The knowledge that comes out of such studies could be used in the development of a genome-edited crop as well as in conventional breeding.

In this chapter, we explore the role of genome editing of gene families in crop improvement, its potential benefits, and its challenges.

2 Benefits of Genome Editing in Crop Improvement

Genome editing has several potential benefits for crop improvement. One of the most significant advantages is its precision. Unlike traditional genetic engineering techniques, which often involve the insertion of foreign DNA into an organism's genome, genome editing allows scientists to make precise modifications to the existing DNA sequence. This precision means that genome editing can be used to introduce desirable traits into crops without introducing unwanted traits or disrupting existing genes [1, 3].

Another advantage of genome editing is its efficiency. Traditional breeding methods can take many years to develop new crop varieties with desirable traits. In contrast, genome editing can produce changes in the DNA sequence in a matter of weeks or months. This speed and efficiency mean that genome editing can be used to rapidly develop new crop varieties that are better adapted to changing environmental conditions, such as drought or disease resistance.

According to a survey of experts on the added potential benefits of genome-edited crops compared to those developed through genetic modification and conventional breeding, there is a consensus among experts on the enhanced agronomic performance and product quality of genome-edited crops over alternatives [5]. The majority of experts indicated that genome editing enables faster trait development, lower R&D costs, and trait innovation [6]. High-oleic soybean in the US and tomato with increased φ-aminobutyric acid (GABA) levels in Japan are two current genome edited crops in the market. New ones are expected to follow in the coming years.

In recent studies, the functions of members of gene families have been investigated by many different types of mutation, including knock-out, base editing and allele exchange with multiplex sgRNAs for targeted genes with CRISPR/Cas9 technology. Their role in crop improvement has been summarized in Table 1.1 and discussed below in terms of improved agronomic traits, disease resistance, quality, and nutritional content.

2.1 Improved Agronomic Traits

Genome editing can be used to enhance the yield of crops by improving their resistance to environmental stressors, such as drought, heat, and cold. By modifying specific genes, scientists can increase the plant's ability to cope with adverse conditions, leading to improved yield.

Allelic variants of the *ARGOS8* gene belonging to the ARGOS gene family were obtained by CRISPR gene editing in maize. These allelic variants were the negative regulator of ethylene responses [7]. The ARGOS8 variants had high levels of *ARGOS8* transcripts relative to the native allele and increased grain yield under flowering stress conditions and had no yield loss in arid conditions.

Table 1.1 Summary of gene families targeted by genome editing for crop improvement in terms of improved agronomic traits, disease resistance, quality, and nutritional content

	Gene family	Observed phenotypes	Methods	Plant	References
Agronomic traits	ARGOS	Improved grain yield	CRISPR-Cas9	*Zea mays*	[7]
	Phospholipase D	Impaired agronomic traits in *PLD1* mutants	CRISPR-Cas9	*Brassica napus*	[8]
	SUMO proteases	Reduced root and shoot biomass in mutant lines	CRISPR-Cas9	*Oryza sativa*	[9]
	The basic helix–loop–helix (bHLH)	Improved salt stress tolerance	CRISPR-Cas9	*Oryza sativa*	[10, 11]
	The cytochrome P450	Higher grain width, grain length, and grain fragrance, increase in 1000 grain weight.	CRISPR/Cas9	*Oryza sativa*	[12]
	Promoter-binding protein-like (SPL) TFs	Improved agronomic traits	CRISPR/Cas9	*Triticum aestivum*	[13]
	Cytokinin oxidase/dehydrogenase	Effected plant development, seed quality, and starch composition.	CRISPR-Cas9 and CRISPR-Cas12a	*Oryza sativa*	[14]
	The Lateral Organ Boundaries Domain (Lbd) proteins	Enhanced drought tolerance	CRISPR/Cas9	*Solanum lycopersicum*	[15]
	Rubisco	Reduced photosynthetic rates and biomass accumulation	CRISPR-Cas9	*Nicotiana tabacum*	[16]
	Pectin methylesterase inhibitors	Reduced plant height, increased growth, improved resistance to Cadmium in mutant lines	CRISPR-Cas9	*Oryza sativa*	[17]
	The transcription factors *bHLH*, *bZIP*, *MYB*, *PHD* and *LBD*	Various effects on male fertility, anther dehiscence, pollen development	CRISPR-Cas9	*Zea mays*	[18]
	Type 2A serine/threonine protein phosphatases (PP2As)	Self-incompatibility in knock-out lines	CRISPR-Cas9	*Brassica napa*	[19]

Disease resistance	miRNA482	Negatively regulated the resistance to tomato wilt disease in mutant lines	CRISPR-Cas9	*Solanum lycopersicum*	[20]
	miR482	Reduced late blight, caused by *Phytophthora infestans*.	CRISPR-Cas9	*Solanum lycopersicum*	[21]
	miR482	A lower disease index for *Verticillium dahliae* infection	CRISPR-Cas9	*Gossypium hirsutum*	[22]
Quality	Starch branching enzymes (SBEs)	A unique starch lacking branching	CRISPR-Cas9 RNP-method	*Solanum tuberosum*	[23]
	Starch branching enzymes (SBEs)	Higher amylose content	CRISPR-Cas9	*Oryza sativa*	[24]
	Lysophosphatidic acid acyltransferase (LPAT)	Decreased oil content, increased size of oil body	CRISPR-Cas9	*Brassica napus*	[25]
	Polyphenol oxidases (PPOs)	Reduced enzymatic browning	CRISPR-Cas9	*Solanum tuberosum*	[26]
	Polyphenol oxidases (PPOs)	Higher polyphenol content in the berries	CRISPR-Cas9	*Solanum melongena*	[27]
Nutritional content	Fatty acid desaturase (FAD2)	Higher oleic acid content	CRISPR-Cas9	*Glycine max*	[28]
	Laccase	Delayed hairy root development, very low lignin content	CRISPR-Cas9	*Salvia miltiorrhiza*	[29]
	Berberine bridge enzyme-like (BBL)	Reduced amount of nicotine	CRISPR-Cas9	*Nicotiana tabacum*	[30]
	α- and γ-gliadin	Decreased gluten immunogenicity	CRISPR-Cas9	*Triticum aestivum*	[31]
	α-amylase /trypsin inhibitors (ATI)	Decreased amount of α-amylase/ trypsin inhibitor proteins for less allergenic wheat variety	CRISPR-Cas9	*Triticum durum*	[32]

In order to understand the function of *BnaPLDα1* genes of Phospholipase D Gene Family in *Brassica napus*, *BnaPLDα1* gene was over-expressed and knocked out by CRISPR. Four lines were edited at one sgRNA site and two lines were edited at two sgRNA sites [8]. Compared to the wild type, the PLDα1 protein was found to be more expressed in the over-expressed edited lines while less or not expressed in the knockout lines. When the plants were planted in the field, the plant height was not different between overexpression lines and wild type, but the plant heights of the knocked-out lines were shorter, indicating that the appropriate dose of PLDα1 was required to maintain the normal growth of the plant, indicating that further increase of PLDα1 level had limited effect on it. Many agronomic traits such as plant height, effective branches, silique number in inflorescence, silique seed number were adversely affected in lines with reduced PLDα1 levels [8]. These findings proved that *BnaPLDα1* regulation changes the agronomic traits of *B. napus*.

The loss of function lines of *OsOTS1* gene enhanced sensitivity to salt with reduced root and shoot biomass, suggesting it plays a role in salinity stress. Mutagenesis of *OsOTS1* gene of SUMO proteases gene family to reveal its role against salinity stress was targeted in rice [9]. To investigate the usability of transcription factor (basic helix-loop-helix (bHLH)) sub-U family genes in breeding rice varieties under salinity stress conditions in rice (*Oryza sativa* L.), loss of function lines of *OsbHLH024* and *OsbHLH044* genes was generated by the CRISPR/Cas9 system [10, 11]. The gene editing mutant lines revealed altered morphological and physiological phenotypes. *OsbHLH024* mutant lines had increased shoot weight, the total chlorophyll content, and the chlorophyll fluorescence. They had less reactive oxygen species and stabilized levels of MDA, fewer Na+ but more K+, and a balanced level of Ca^{2+}, Zn^{2+}, and Mg^{2+} in the shoot and root. Therefore, it was concluded that the *OsbHLH024* gene improves salt stress resistance by playing a role as a negative regulator [10]. Considering *OsbHLH044* mutant lines, they showed reduced morphological and physiological parameters, lower antioxidant activities and higher lipid peroxidation and hydrogen peroxide (H_2O_2) accumulation. The loss-of-function of this gene also altered the expression of ion homeostasis-related genes (*OsHKTs*, *OsHAK*, *OsSOSs*, and *OsNHX*) and ABA-responsive gene (*OsLEA3*). The mutant synthesized less stored starch and proteins because of decreased expression of genes coding for starch (*OsAGPL1*, *OsSSIIa*, *OsWx*, and *OsFLO2*) and seed storage proteins (*GluA1* and *Globulin 1*). It was concluded that the *OsbHLH044* gene plays a role as a positive regulator of salt stress and grain quality [11].

In rice, three genes (*Os03g0603100*, *Os03g0568400*, *GL3.2*) of cytochrome P450 family and *OsBADH2 were* multiplex edited with CRISPR/Cas9 and their novel alleles were generated [12]. The high performance of mutant plants homozygous for grain width, grain length, and grain fragrance was recorded. Also, the mutant plants showed high level 2-acetyl-1-pyrroline (2AP). It has been concluded that the edited plant in this study could be used as valuable genetic material for crop breeding to improve rice yield and quality.

Indel mutations in the *TaSPL13* gene of SQUAMOSA promoter-binding protein-like (SPL) transcription factors gene family were created to increase grain yield in bread wheat (*Triticum aestivum*) [13]. The microRNA 156 recognition element

indel mutations in 3′ UTR of *TaSPL13* brought about the abundance of TaSPL13 transcripts and a decrease in flowering time, tiller number, and plant height and the consequent grain size and number increased in the mutant plants.

Eleven members of the Cytokinin oxidase/dehydrogenase gene family (*OsCKX1-OsCKX11*) were knocked out using CRISPR/Cas9 and CRISPR-Cas12a to reveal their functions in rice [14]. CRISPR-Cas12a performed better than CRISPR-Cas9 for generating multigene mutants. The *OsCKX* members showed functional redundancy and affected plant development, seed quality, and starch composition by regulating endogenous cytokinins. *OsCKX1/2* and *OsCKX3/8/10* genes had roles in the control of panicle architecture and grain number. *OsCKX4/5/9* genes regulated the development of roots and plant height and tillers.

Knock-out and over-expression of *SlLBD40* (*Solyc02g085910*) gene of The Lateral Organ Boundaries Domain (Lbd) Protein Family were created to understand its role against drought stress in tomato [15]. The results showed that *SlLBD40* knock-out lines had improved water-holding ability and relieved the physical harm of the photosynthetic system of tomato seedlings, while *SlLBD40* overexpressing plants showed vigorous wilting symptoms under drought stress, suggesting that *SlLBD40* is a negative regulator of drought tolerance.

In order to increase photosynthetic efficiency in tetraploid tobacco (*Nicotiana tabacum*), it was aimed to obtain novel alleles of genes (*rbcS*) coding the small subunit of CO_2-fixing enzyme Rubisco family so that the efficiency of carbon assimilation through better Rubisco enzymes could be increased [16]. The phenotyping analysis of mutant plants showed reduced Rubisco content, photosynthetic rates, and biomass accumulation. In this study, it has been shown that it is possible to produce superior non-native Rubisco enzymes by multiplex gene editing with CRISPR/Cas9 in polyploid species.

The function of the *OsPMEI12* gene, which belongs to the pectin methylesterase inhibitors (*OsPMEI*) gene family for growth, cell wall development, and response to phytohormone and heavy metal stress, was elucidated by mutating it in rice (*Oryza sativa* L.) [17]. It has been revealed that the *OsPMEI12* gene plays a role in the regulation of methyl esterification during growth, thus pectin status is controlled by hormones and cadmium stress.

Discovery of genetic male sterility (GMS) genes will allow to unravel the molecular mechanism of anther development and to develop male sterility systems for crop breeding and hybrid seed production. Therefore, the researchers focused on elucidating the roles of the transcription factors genes (*bHLH*, *bZIP*, *MYB*, *PHD* and *LBD* gene families) responsible for the GMS agronomic trait in highly heterosis maize [18]. Mutation of transcription factor gene families with CRISPR yielded the following results; that *ZmbHLH51* and *ZmbHLH122* are necessary for male fertility, *ZmTGA10* has affected anther dehiscence. *ZmMYB84* is essential for maize pollen development. *ZmPHD11* is responsible for the formation of anther cuticle and Ubisch bodies. Two MYB33 paralogs are important for GMS. *ZmLBD27* has an impact on the reduced ratio of normal pollen grains. ZmPHD11/27 influenced viable pollen formation.

Two homologous genes (*Bra018924* and *Bra014296*) of PP2A, a 55 kDa B regulatory subunit (PR55/B), were edited through the CRISPR/Cas9 system to develop self-compatible lines controlled by type 2A serine/threonine protein phosphatases (PP2As) in Chinese cabbage [19]. The *PR55/B* gene knock-out line did not show self-incompatibility and produced seeds, suggesting that the *PR55/B* gene was responsible for the self-incompatibility.

2.2 Improved Disease Resistance

Plant diseases are a significant threat to crop productivity, with losses estimated to cost billions of dollars annually. Genome editing can be used to enhance the resistance of crops to diseases by editing genes that encode for disease resistance.

MicroRNAs act as regulators of the plant immune system by silencing the genes involved in pathogen virulence or by regulating the expression of target genes. MicroRNAs belonging to the miRNA482/2118 superfamily targeted R-genes of the class NBS-LRR (nucleotide-binding site-leucine rich repeat). In tomato, knockout mutants for *SlymiR482e-3p* gene, a member of the miR482/2118 family, were generated to detect its role against tomato wilt disease resistance [*Fusarium oxysporum* f. sp. *lycopersici* (race 2) (Fol)] [20]. The results showed that *SlymiR482e-3p* gene negatively regulated the resistance to wilt disease. In another study with miR482 gene family in tomato, mutations in the two genes (*miR482b* and *miR482c*) were generated [21]. The expression levels of their target NBS-LRR genes were increased, followed by reduced late blight, caused by *Phytophthora infestans,* disease symptoms in mutant plants. Interestingly, it was concluded that knocking out these two genes could lead to expression perturbation of other miRNAs, suggesting cross-regulation between miRNAs. In cotton (*Gossypium hirsutum* L.), the MIR482 mutant collection was obtained by knocking out *MIR482* genes in miR482 gene family with dozens of members using CRISPR/Cas9. This mutant collection allow us to examine the role of individual *MIR482* genes against pathogen response and to identify miR482-NLR module(s) that respond to, particularly fungal pathogen *Verticillium dahliae* agent for Verticillium wilt and other pathogens. It has also been reported that this collection may be a useful genetic resource for the development of new disease-resistant cotton varieties [22].

2.3 Improved Quality

Quantitative trait loci (QTLs) are genomic regions that are associated with complex traits such as crop quality. QTLs can be used to improve crop quality by identifying genomic regions associated with desirable traits. Many genes in QTLs have been targeted with genome editing.

Starch branching enzymes (SBEs) are involved in the biosynthesis of starch in plants and there have been several studies on the use of genome editing *SBE* genes in crops to modify starch content and properties. For example, one study used CRISPR-Cas9 RNP-method to induce mutations in *SBE* genes to develop a unique potato starch lacking branching [23]. Another study used CRISPR/Cas9 to generate high-amylose rice by targeting *SBEI* and *SBEIIb* genes [24].

In *Brassica napus*, an oilseed plant that is the raw material for both cooking oil and biodiesel production, loss-of function of the *BnLPAT2* and *BnLPAT5* genes belonging to lysophosphatidic acid acyltransferase (LPAT) family allowed to lay out their precise roles in oil biosynthesis. That is, the oil content decreased, the size of oil body increased in mutant lines [25]. The results of this study illustrated that these genes could be used for oil production improvement.

In order to prevent enzymatic browning, negatively affecting tubers during harvest and post-harvest procedures, researchers targeted the Polyphenol Oxidases (PPOs) gene family and mutated the *StPPO2* gene in *Solanum tuberosum* L. [26]. A lower PPO causing oxidation of polyphenols (natural antioxidants) in tuber was observed in mutant lines and enzymatic browning was reduced. Another study with the polyphenol oxidases (PPOs) gene family was carried out in eggplant (*Solanum melongena* L.) [27]. In this study, the researchers stated that it would be possible to develop eggplant varieties that retain a high polyphenol content beneficial to human health in the berries during harvest and post-harvest processes by knocking out the three genes (*SmelPPO4*, *SmelPPO5*, *SmelPPO6*).

2.4 Improved Nutritional Content

Genome editing can also be used to enhance the nutritional content of crops by increasing the levels of essential nutrients, such as oil, phenolic acid, vitamins and minerals [4]. This is particularly important in developing countries, where nutrient deficiencies are prevalent and access to a diverse diet is limited.

The oleic acid content of soybean seeds was modulated by knocking out GmFAD2-1A and GmFAD2 genes belonging to the fatty acid desaturase (FAD2) family [28]. The loss of function of these genes has paved the way for cultivating soybean genotypes with high oleic acid content.

In *Salvia miltiorrhiza*, the *SmLAC*s genes of the laccase family, which has 29 family members with high homology, were multiplex silenced by targeting their conserved sequences to reveal their role in the production of medicinal phenolic acid compounds [29]. It has been concluded that *SmLAC*s play a role in lignin formation and phenolic acid biosynthesis in the roots due to decreased expression of target genes and delayed hairy root development, larger and looser xylem cells, low RA and SAB accumulation, and very low lignin content in edited lines compared to wild types.

By knocking out six genes (*BBLa*, *BBLc*, *BBLd.2*, *BBLb*, *BBLd.1*, *BBLe*) belonging to the berberine bridge enzyme-like (BBL) family using a single sgRNA, a nicotine-free tobacco variety was developed and the amount of nicotine was reduced [30]. In this way, the use of nicotine-free tobacco by smokers may protect their health.

The genes in α- and γ-gliadin gene families were silenced using six sgRNAs, and a less allergenic bread wheat variety containing gluten with fewer immunogenic epitopes, which would be beneficial in the diet of coeliac patients, was obtained [31]. A less allergenic durum wheat variety was produced [32]. The multiplex editing of *CM3* and *CM16* genes belonging to α-amylase/trypsin inhibitors (ATI) family subunit U form was carried out using seven gRNAs targeting the two genes of interest. The editing of these genes decreased the amount of ATI transcripts. It was reported that this might have happened due to nonsense-mediated mRNA decay. Interestingly, it was noted that ATI 0.28 pseudogene were activated after knocking out *CM3* and *CM6* genes.

3 Challenges of Genome Editing in Crop Improvement

Despite its potential benefits, genome editing in crops also faces several challenges. One of the biggest challenges is the regulatory landscape. Many countries have regulations that govern the use of genetically modified organisms (GMOs), and it is not yet clear how gene-edited crops will be regulated. In some cases, gene-edited crops may be subject to the same regulations as GMOs, even though the changes made to the DNA sequence are much smaller and more precise.

Another challenge is the potential for unintended consequences. Although genome editing is a precise technique, there is still a risk of off-target changes in the host genome [4]. These unintended changes could have unforeseen consequences for the crop's performance or safety. To mitigate this risk, scientists must employ rigorous testing and validation processes to ensure that the desired changes are made without causing any unintended effects. Therefore, it is important to carefully assess the safety and efficacy of gene-edited crops before they are released into the environment or consumed by humans.

4 Summary

Genome editing is a promising technique for crop improvement that has the potential to produce crops that are better adapted to changing environmental conditions and have improved nutritional content. However, there are also challenges that need to be addressed, including regulatory issues and potential unintended consequences. With careful consideration and regulation, genome editing has the potential to revolutionize crop improvement.

The use of genome editing in crop improvement also raises ethical considerations. For instance, there are concerns around the ownership and control of the technology, as well as the potential for gene-edited crops to create new monopolies in the seed industry. Inadequate stewardship, enhanced inequity between rich and poor, lack of transparency, an unclear intellectual property landscape, and inadequate public sector institutional infrastructures to support the use of genome-editing technologies are also challenges that need to be addressed [33].

Regulatory policy is another challenge. The global regulatory policy for genome-edited crops is still emerging and will shape the path of genome editing innovation. Genome editing is a relatively new technology, and its regulatory framework is still evolving. The regulation of gene-edited crops varies between countries, with some countries adopting a more stringent approach than others. As genome editing techniques become more widespread, there is a need for a coordinated global approach to regulating their use in agriculture to ensure that the technology is used responsibly and safely.

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