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The lowest hanging fruit: Beneficial gene knockouts in past, present, and future crop evolution

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ABSTRACT

It is now well-documented that gene knockout (loss-of-function) alleles have played a prominent role in crop evolution during domestication, diversification, and improvement. This includes dramatic examples from the very origins of agriculture to the monumental yield increases of the Green Revolution. Recent advances in the generation of whole genome sequence data and functional investigations into the molecular genetic basis of crop traits continue to reveal the existence of considerable beneficial loss of function in crop species. These discoveries now inspire contemporary efforts to use targeted gene knockouts powered by modern gene editing tools to accelerate crop breeding. Here we trace the history and future of loss of function as a powerful mechanism of crop evolution and review the reasons gene knockouts might have been particularly important in past crop evolution and why they continue to be a "lowest hanging fruit" for directing crop evolution through molecular breeding in pursuit of more productive, resilient, and nutritious crops.

1. Introduction

Gene knockouts, or loss-of-function (LoF) alleles, are an important source of genetic variation [1,2]. They can be defined conceptually as alleles that disrupt or break a gene's molecular function or more formally as those whose phenotypic consequences are functionally equivalent to total gene loss. Discoveries in the age of functional molecular genomics have led to an increasing appreciation for their importance in crop evolution. It is now known that gene content varies considerably between and within crop species [3–5]. For example, the reference genome of maize was reported to contain only half of the genes from the pan genome [3]. And a large body of evidence has accumulated showing that beneficial gene loss and LoF has contributed to adaptive evolution in diverse crops and the evolution of agriculturally important traits [6–11].

In this review, we synthesize the findings from recent fundamental evolutionary genomic research and applied functional molecular breeding efforts that have jointly revealed LoF mutations to be a powerful source of adaptive genetic variation in crops. We examine the contribution of beneficial LoF mutations to crop evolution of the past during domestication, diversification and improvement. We also see how discoveries of naturally occurring beneficial LoF mutation inspire present efforts to improve crop traits through targeted knockouts using new gene editing approaches. Finally, we look ahead, considering future challenges and opportunities to scale up the discovery and generation of beneficial LoF variants to accelerate molecular breeding in diverse crops.

2. Why knockouts?

To understand how and why gene LoF might play a prominent role in crop evolution it is first important to consider the large mutational target size and phenotypic effects of LoF mutations. The type and number of mutations that can break a gene's molecular function are expected to be much greater than the mutations which lead to specific gain-of-function changes in protein activity or expression [12]. Such mutations include frame-shifts, premature stop codons, large insertions and deletions, splice site disruptions, and amino acid substitutions [9]. Indeed, diverse molecular causes of beneficial LoF have been observed in recently discovered cases involved in crop domestication and diversity (Table 1).

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Table 1
Examples of naturally observed beneficial LoF alleles caused by frameshift, premature stop, and splice site mutations that have contributed to the evolution of different crops and traits. These discoveries inspire present and future efforts to use targeted gene knockouts to accelerate crop improvement. SNP = Single Nucleotide Polymorphism. Indel = insertions or deletions.

Gene	Species	Mutation type	Mutation effect (domestication, diversification and improvement)	Reference
Waxy	*Amaranthus caudatus*	Indel - Frameshift	Glutinous grains	[18]
BnTFLC A3b	*Brassica napus*	Indel - Frameshift	Flowering time	[19]
BoAP1	*Brassica oleracea*	SNP - Premature stop	Proliferation of inflorescences	[20]
CsGL3	*Cucumis sativus*	Indel - Frameshift	Glabrous phenotype	[21]
FaNES1	*Fragaria vesca*	SNP - Premature stop	Reduced monoterpene	[22]
FvKSN	*Fragaria vesca*	SNP - Premature stop	Continuous flowering	[23]
Dt1	*Glycine max*	SNP - Premature stop	Determinate shoots	[24]
E2 (GmGIa)	*Glycine max*	SNP - Premature stop	Flowering and maturity	[25]
ELF3	*Glycine max*	SNP - Premature stop	Flowering and maturity	[26]
F3′H	*Glycine max*	SNP - Premature stop	Pubescence color	[27]
GmPdh1	*Glycine max*	SNP - Premature stop	Non-shattering	[28]
LPD1	*Glycine max*	SNP - Premature stop	Oil content	[29]
HaFT1	*Helianthus annuus*	SNP - Frameshift	Flowering time	[30]
AmCEN	*Hordeum vulgare*	SNP - Premature stop	Flowering time	[31]
Hv.Nst1	*Hordeum vulgare*	SNP - Premature stop	Starch Synthesis	[32]
HvFT3	*Hordeum vulgare*	SNP - Premature stop	Flowering time	[33]
INTERMEDIUM-C	*Hordeum vulgare*	SNP - Frameshift	Suppression of tillering and male fertility	[34]
nud	*Hordeum vulgare*	Indel - Frameshift	Reduced hull-caryopsis adhesion	[35]
VRS1	*Hordeum vulgare*	SNP - Premature stop	Row architecture	[34]
GBSSI	*Manihot esculenta*	Indels- Premature stop codon	Amylose synthesis	[36]
Bad2	*Oryza sativa*	Indel - Frameshift	Fragrant grains	[37]
Bh4	*Oryza sativa*	SNP - Premature stop	White hull	[38]
bHLH	*Oryza sativa*	Indel - Frameshift	White pericarp	[39]
DEP1	*Oryza sativa*	InDels- Frameshift	Reduce length of the inflorescence internode, increase number of grains per panicle	[40]
GS3	*Oryza sativa*	SNP - Premature stop	Long grain	[41]
GS3	*Oryza sativa*	InDels - Frameshift	Grain length	[42]
OsGN1A	*Oryza sativa*	InDels- Frameshift	Increase grain number	[43]
OsLG3b	*Oryza sativa*	Indel - Frameshift	Increased seed size	[44]
OsWaxy	*Oryza sativa*	SNP - Splicing defect	Glutinous grains	[45]
Pi21	*Oryza sativa*	InDels- Frameshift	Blast resistance	[46]
PROG1	*Oryza sativa*	SNP - Premature stop	Erect growth	[47]
qSH1	*Oryza sativa*	Indel - Frameshift	Non-shattering	[48]
SLR1	*Oryza sativa*	SNP - Premature stop	Dwarf plant	[49]
GBSSI	*Panicum miliaceum*	Indel - Frameshift	Glutinous grains	[50]
PvTFL1	*Phaseolus vulgaris*	Indel - Frameshift	Determinate shoots	[51]
PsMLO1	*Pisum sativum*	InDels- Frameshift SNP - Premature Stop	Powdery mildew resistance	[52]
FAS	*Solanum lycopersicum*	SNP - Premature stop	Larger fruit	[53]
mlo	*Solanum lycopersicum*	InDels- Frameshift	Powdery mildew resistance	[54]
OVATE	*Solanum lycopersicum*	SNP - Premature stop	Expansion of fruit neck region	[55]
LGS1	*Sorghum bicolor*	InDels- Frameshift	*Striga hermonthica* resistance	[56]
SbGBSS	*Sorghum bicolor*	SNP - Premature stop	Low-amylose grains	[57]
SbSh1	*Sorghum bicolor*	Indel - Splicing defect	Non-shattering	[58]
QAP2	*Triticum aestivum*	SNP - Premature stop	Reduction in shattering	[59]
TaHRC	*Triticum aestivum*	InDels- Frameshift	Blight resistance	[60]
Tsn1	*Triticum aestivum*	InDels- Frameshift SNP - Premature Stop	Disease resistance	[61]
VvMYBA2	*Vitis vinifera*	SNP - Frameshift	White berry color	[62]
DET2	*Zea mays*	SNP - Premature stop	Dwarf plant	[63]
Kn1	*Zea mays*	SNP - Frameshift	Branche and spikelet number	[64]
RAMOSA1	*Zea mays*	Indel - Frameshift	Kernel organization	[65]
Tga1	*Zea mays*	SNP - Premature stop	Softer glume	[66]
ZmSh1	*Zea mays*	Indel - Frameshift	Non-shattering	[58]

(continued on next page)

Table 1 (*continued*)

Gene	Species	Mutation type	Mutation effect (domestication, diversification and improvement)	Reference
ZmSUGARY1	*Zea mays*	SNP - Premature stop	Starch biosynthesis	[67]

The number of different mutations that can cause LoF is also large because many positions along the length of a gene are vulnerable to disruptive mutations - frameshift mutations at most positions of the coding sequence of a gene are likely to produce a severely disrupted protein product.

The large mutational target size of LoF has important implications for their role in crop evolution because it can influence the types of variants that contribute to the “arrival of the fittest” [13]. That is, LoF may be common during rapid adaptation simply because they are the first to emerge via *de novo* mutation. Indeed, these dynamics have been observed in experimental evolutionary studies showing that LoF can contribute disproportionately to rapid adaptation, presumably in part because of its high mutation rate [14,15]. For crops, this phenomenon - an enriched contribution of LoF alleles - might be particularly true for traits that are likely maladaptive under ancestral conditions. This is because for such conditions evolution is mutation limited due to the expectation that standing genetic variation is unlikely to be present [16]. A well studied example is the loss of seed shattering, which is generally believed to be maladaptive in natural environments but an important trait in the evolution of many crops [16].

The molecular consequences of LoF are also by definition large. A resulting hypothesis therefore is that the consequences on agriculturally important traits could also be greater than other broad functional classes of alleles such as more minor change-of-function alleles. Because evolutionary theory also predicts that adaptation will involve alleles of large effect when populations are far from fitness optima [17], LoF may be particularly important during evolutionary responses to strong selection such as during rapid adaptation to agricultural environments and intensive selective breeding.

3. Loss-of-function mutations during crop domestication, diversification and improvement

In recent decades, the molecular genetic basis of domestication has been uncovered by genetic mapping key traits. When this body of literature is viewed in aggregate it allows hypotheses about the relative importance of LoF in past evolution to be evaluated. Previous reviews have provided extensive catalogs of the genes and mutations involved in domestication [6–8]. As predicted based on the aforementioned characteristics of LoF mutations, these surveys have revealed that the evolution of domestication traits often involved mutations disrupting gene function. In some cases, convergent LoF has occurred in distantly related species during domestications. For example, putatively disruptive mutations of *sh1* orthologs are implicated in the independent loss of seed shattering in domesticated rice, sorghum, and maize [58]. The potential for LoF mutations to facilitate domestication are further exemplified in recent efforts to domesticate wild species using gene editing. Targeted knockouts of just six genes in wild tomato (*Solanum pimpinellifolium)*, resulted in dramatic improvements in fruit traits characteristic of domesticated tomatoes [11].

It is important to note that the tendency for investigations of the molecular genetic basis of domestication traits to reveal putative LoF variants may be in part explained by ascertainment bias. That is, LoF are simply easy to detect when examining molecular sequence data. For example, frameshift and premature stop mutations are relatively straightforward to identify because they have very obvious impacts on gene function. Yet such variants underlying major quantitative trait loci (QTL), despite being of large effect, can sometimes still only explain a fraction of genetic variance for domestication traits [68]. It is possible that the unaccounted for fraction of variance is therefore explained by cryptic gain- or change-of-function mutations, such as cis-regulatory variants altering patterns of expression which can also contribute significantly to domestication traits but are more difficult to identify from raw sequence data [6]. Nevertheless, a recent genome wide survey identified 116 genes in which LoF was fixed in domesticated soybean [69] suggesting that the contribution of LoF could be greater than the handful of closely studied genes identified by prior investigations. An interesting direction for future work will be distinguishing LoF variants that contribute to domestication traits from those which are fixed (and potentially deleterious) in crop domesticates because of historical population bottlenecks [70,71].

Doebley et al. [6] observed that LoF mutations were even more important for the generation of varietal diversification and trait improvement than for domestication. Later surveys of the genetic basis of crop evolution have largely supported this view [7,8]. One explanation is that “unconscious” selection for domestication traits was relatively weak [72], in contrast to stronger natural or artificial selection on beneficial crop traits related to variety improvement. Thus, the increased contribution of LoF alleles to crop improvement that has been described by others is at least consistent with the classical prediction that responses to very strong selection tend to involve fewer mutations of larger effect [17]. Indeed, it is now known that responses to intensive breeding that ultimately led to monumental yield increases of the Green Revolution in the mid 20th century were largely attributed to convergent LoF alleles in *GA20-OX* genes which produced semi-dwarf varieties in both rice and barley [73–75]. Such dramatic examples of the importance of LoF alleles as in the Green Revolution captures their ability to play a key role in the rapid improvement of crop traits and inspires current efforts to use targeted gene knockouts as a breeding tool toward accelerated crop improvement.

4. Targeted knockout mutations to improve crops

There are multiple reasons that targeted knockout mutations are becoming a particularly compelling direction for modern breeding efforts. First, as reviewed above, studies of the genetic basis of past crop evolution have revealed that LoF mutations can provide a significant contribution to crop improvement. Second, because they are often easy to identity from sequence data (ie. frameshift mutations, premature stop), they are currently the easiest to immediately annotate from whole genome sequence data in search of candidates for molecular breeding. Third, current genome editing technologies, especially CRISPR/Cas based gene editing are particularly good at generating targeted knockout mutations [76] – indeed, the CRISPR system originally evolved as a mechanism of breaking foreign viral DNA [77]. During the error-prone non-homologous end joining (NHEJ) repair of double strand breaks at target sites of CRISPR/Cas, small insertions and deletions are often introduced (Fig. 1). If such mutations occur inside of the coding region of a target gene and are not a multiple of 3 base pairs, the effect will be a frameshift mutation that is likely to severely disrupt the function of the target gene and introduce a premature stop codon. In contrast, the editing efficiency of creating specific gain-of-function alleles (e.g. a particular base pair substitution in promotor or important amino acid position) is generally much lower, and thus faces additional levels of complexity and challenges. Therefore, from a practical perspective, targeted knockouts remain the “lowest-hanging fruit” in terms of using CRISPR to improve crops. Below we highlight recent examples of where knockouts were used to progress common breeding goals: increased yield, greater stress tolerance, and improved nutritional quality.

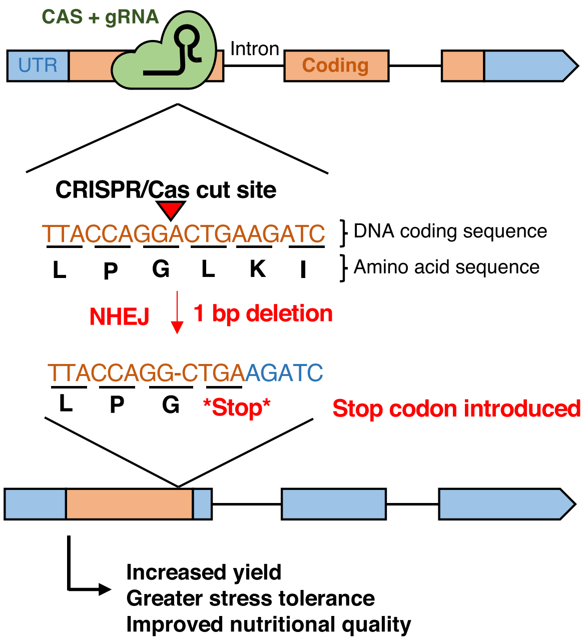

Fig. 1. Schematic of CRISPR/Cas as a tool for generating a targeted knockout mutation. During error prone NHEJ repair of the double stranded break induced by the CRISPR/Cas editing system, small insertions and deletions are common. These can cause frameshift mutations which disrupt the downstream amino acid sequence and will usually eventually lead to a premature stop codon. Shown here is a cartoon example of the effect of a 1 bp deletion in the coding region of a target gene.

5. Increased yield

As others have noted, it might be counterintuitive that disrupting gene function could improve a trait [78], but the evolutionary history of crops (Table 1) demonstrates that breaking the right genes can have a positive impact on numerous traits including yield. Knowledge of the potential for LoF alleles to increase crop yields motivates current efforts to knockout target genes as a breeding tool. In rice for example, CRISPR/Cas was used to create knockout alleles in elite landraces mimicking natural LoF alleles in *sd1* (GA20-ox) already known to be responsible for the semi-dwarf rice varieties of the Green Revolution [79]. Likewise, *DEP1* and *GN1A* are two genes associated with rice yield. Loss of these genes results in an increased number of grains per panicle and an increase in grain yield. Plants edited for LoF in *GN1A* showed a 21 % increase in yield per plant over the functional allele while in the case of *DEP1* the yield increased by 40.9 % compared to a functional control [80]. Recently, knockout lines of *RSPR1* were produced that increased rice grain width, length, and weight by 11 %, 16 %, and 23 %, respectively [81]. And grain production of rice was increased by as much as 31 % in knockout lines of *pyl1/4/6* [82].

In addition to being powerful test cases highlighting the efficacy of accelerating yield gains through targeted knockouts, the experiments above illustrate an important feature of the use of targeted gene knockouts for crop improvement. Because they are arguably the easiest class of mutation to generate using gene editing [83], researchers can introduce a desirable allele into a new genetic background much faster than via traditional introgression of a beneficial QTL (especially complex or cryptic gain-of-function allele where gene editing is intractable) which requires generations of crossing and backcrossing [84]. Such has been the challenge for introgressing the dominant *CMD2* allele for resistance to Cassava Mosaic Disease (CMD) through traditional methods into Latin-American germplasm to render it amenable for cassava breeding programs in Africa [85]. It took at least ten years to test the stability of Latin-American germplasm carrying *CMD2* in Nigerian fields [86,87].

The discovery and subsequent application of targeted knockout mutations can even extend beyond species boundaries [11], unlike traditional breeding which is generally limited to crosses between species that can produce fertile offspring. This is important, because it means discoveries made in crop relatives - a beneficial LoF polymorphism - can be more readily translated into downstream molecular breeding. For example, based on knowledge of genes in tomato, the orphan crop *Physalis pruinosa* (groundcherry; Solanaceae) was improved by CRISPR-mediated targeting of three orthologous genes influencing plant architecture, flower production and fruit size [88]. The average number of fruits per five nodes raised from 10 to almost 16 in CRISPR-mutated plants, maintaining fruit mass and diameter about the same, which has important implications in already established post-harvest processing methods.

6. Greater stress tolerance

There are compelling biological reasons for why LoF might be particularly effective at conferring resistance to certain kinds of biotic stress. Because many plant pests and pathogens interact directly with plant molecular pathways [89], crops can benefit from knockouts of these genes. This expectation is consistent with discoveries of natural mechanisms of disease resistance (Table 1) and broader surveys of natural genetic diversity that have found LoF to be common in genes annotated as involved in antagonistic biotic interactions. For example, the parasitic plant *Striga hermonthica* uses the product of the gene *LGS1*, which is a molecule secreted by sorghum roots into the soil, to find host plants. And it was recently discovered that sorghum varieties lacking functional alleles of this gene have evolved greater resistance [56]. Such discoveries are interesting both for what they reveal about the functional genomic basis of arms races between crops and pests, but also for providing information directly useful for molecular breeding.

Knowledge about crop-pest interactions and the molecular genetic basis of resistance are already being translated into gene editing applications to knockout specific genes. In rice for example, CRISPR-mediated frameshift mutations of *OsERF922*, a gene whose expression is induced by the pathogenic fungus *Magnaporthe oryzae* [90], were used to engineer genotypes that exhibit an approximate 60–70 % reduction susceptibility to *M. oryzae* [91]. More recently, CRISPR-mediated indels disrupting the target sites of pathogen effector proteins produced rice varieties resistant to several *Xanthomonas* strains [92]. In cassava, resistance (43–45 % reduction in viral load) to *cassava brown streak viruses* was achieved through induced knockout mutations in *nCBP-1* and *nCBP-2*, genes which interact directly with viral genome encoded proteins [76]. And in wheat the effects of a natural LoF in *TaHRC* which is responsible for resistance to fusarium head blight resistance was replicated using a CRISPR-mediated frameshift in the coding region leading to a >40 % reduction in disease susceptibility [60]. Similarly, CRISPR-mediated deletions of *TcNPR3*, a repressor of pathogen defense response, led to a >60 % reduction in leaf lesions in cacao caused by *Phytophthora tropicalis* [93,94].

It is important to note that not all beneficial knockout mutations will be universally beneficial. For example, there may be a knockout mutation that increases resistance to one pathogen but decreases resistance to others. Indeed, inactivation of BIK1 can increase resistance to *Pseudomonas syringae* but increase susceptibility to necrotrophic fungal pathogens [95]. Whether such tradeoffs are more common for LOF than other types of mutations is uncertain, but such pleiotropic effects might limit the breeding value of some conditionally beneficial knockout mutations.

Targeted knockouts have proven effective for increasing abiotic stress tolerance as well [96,97]. In rice, a T-DNA induced knockout mutation of *OsGSK1*, a negative regulator of brassinosteroid signaling, led to a >30 % reduction in wilting responses to extreme salt, heat, and cold treatments [98] and a knockout mutation of *OsLDC-like* 1 performed 70 % better under experimental oxidative stress [99].

Impressively, RNA interference based knockdown of *OsERF109* increased survivorship after drought treatments by as much as 250 % [100]. More recently, CRISPR-mediated knockouts have been used to increase salinity tolerance in rice by as much as 96 % through knockouts of the transcription factor *OsRR22* [101]. Together, these results highlight a quality of LoF alleles that may have contributed to their important role in crop evolution and value for modern breeding: their large molecular effect size which could in turn lead to large phenotypic effects.

7. Improved nutritional quality

One of the most fruitful uses of targeted knockout to accelerate crop breeding is for improved nutritional traits such as the increased bioavailability of macro- and micro-nutrients and decrease of undesirable toxins. The logic for the particular value of LoF in the evolution of such traits is that desirable phenotypes may be achieved by knocking out genes directly responsible for the production, uptake or tissue specific accumulation of toxic, anti-nutritive, or undesirable elements or molecules. Toxic heavy metals such as cadmium can accumulate in many crops, posing a major threat to communities that rely on food grown in contaminated soils. Groups investigating molecular breeding approaches to reduce cadmium accumulation found that targeted knockouts of *NRAMP5*, a gene directly involved in cadmium uptake [102], can lower cadmium accumulation in rice by over 97 % without compromising yield [103,104]. Because a breeding program based on knockouts can readily translate discoveries between crop species, *NRAMP5* and other members of this gene family are emerging as a promising target for molecular engineering low-cadmium varieties of a number of other crops such as cacao [105].

The capacity to impact nutritional composition of plants with LoF alleles may be a reflection of the pool and flux nature of biosynthetic pathways - concentrations of a target molecule are directly influenced by removing upstream or downstream fluxes (i.e. enzymes). In oilseed crops, this principle has been applied to engineer fatty acid profiles that are healthier and have longer shelf lives - CRISPR-mediated knockouts of *FAD2* genes (Δ12 oleic acid desaturases) reduced both linolenic and linoleic acid by over 70 % in Camelina [106]. And in cassava, knocking out the genes *CYP79D1* and *CYP79D2* (enzymes that catalyze the synthesis of main cyanogens) in bitter varieties has been used to reduce cyanide by 92 % [107]. For many crops, it is well-known that naturally occurring LoF alleles in *GBSSI (waxy)*, an amylose synthase gene, are responsible for crop products with low amylose content, a desirable culinary trait for many people (Table 1). Armed with this knowledge, CRISPR-mediated knockouts of GBSSI have been generated in Cassava that completely eliminate amylose content in roots [36,108]. Conversely, high-amylose starch can be desirable because it benefits individuals with diabetes [109,110]. Gene silencing of *BE1* and *BE2* - both genes involved in amylopectin biosynthesis - in cassava produced plants with amylose contents 130 % greater than the wild type. Hence, knocking out these genes with CRISPR/Cas could lead to high-amylose cassava starch with an impact in combating diabetes [111].

Some molecules are essential for crop physiology, so knocking out genes directly responsible for their production is untenable. Yet targeted knockout can still be effective at reducing their accumulation in consumed parts of the plant. Phytic acid acts as an antinutrient, blocking the bioavailability of micronutrients [112] however, many alleles that directly reduce phytic acid are lethal in a homozygous state [113]. This challenge has been overcome by targeting genes involved in phytic acid transport. Knockout of *SPDT*, a rice gene that controls the allocation of phosphorus to the grain, has proven effective at reducing accumulation specifically in rice grains by 30 % without negative impacts on other agronomic traits [114]. Similarly, low phytic acid genotypes have been generated with knockout mutations in transporters *PvMRP1* in common bean (90 % reduction, [115]) and *BnMRP5* in oilseed rape (15 % reduction, [116]).

8. Future discoveries of beneficial knockouts

To date, many of the examples of using targeted gene knockouts to improve crops have involved genes already known to improve traits or with well studied functions previously elucidated from model systems. While serving as valuable cases studies and proofs of concept demonstrating the efficacy of knockouts as a tool for breeding, the next generation of breeding by knockouts calls for much larger scale discovery of new target genes. While molecular breeders now have access to an unprecedented capacity to use tools such as CRISPR to knockout target genes, the critical question that remains is, which genes should be targeted?

Recent advances in nucleic acid sequencing have made functional annotation of genomic diversity in crops possible at an unprecedented scale (Fig. 2). The ability to generate whole genome sequence data for large populations represents a quantum leap in the ability to uncover genes where LoF has contributed to trait evolution and identify candidate genes for targeted knockout. Previous genotyping approaches involved in identifying genomic regions associated with traits of interest in crop populations, such as genotyping by sequencing, reduced representation sequencing, and SNP arrays all shared the common characteristic that while they provide a thorough summary of genetic diversity at genomic scales, they are poorly suited to characterize genomic diversity at functional molecular resolutions (i.e. identification of specific causal genetic variants). The assumption behind the use of these data to find genomic regions associated with agriculturally important traits with genome wide association (GWA) scans has been that variants such as single nucleotide polymorphisms are in physical linkage with functionally important alleles [117]. Unfortunately, this may not be the case, especially for beneficial LoF variants which suffer from the phenomenon of allelic heterogeneity (multiple LoF alleles at same locus) due to their high effective mutation rate [118]. Indeed, traditional GWAS is mainly based on common variants while LoF variants are often present at low frequency. Of ~18,000 LoF variants identified in soybean, 80 % have an allele frequency of below 10 % [119].

In contrast to earlier forms of genetic data, whole genome sequencing data provides details of functional molecular diversity in the context of genomic elements such as protein coding genes. The potential effect of every variant in the genome can be predicted with an array of tools [120–122]. From whole genome sequence data, predicted LoF alleles have been readily identified in numerous crop species in recent years [70,119,123–132]. Indeed, one of the interesting discoveries that has emerged from genome resequencing projects is the scale of LoF variation segregating in crops. For example, in rice 198,609 premature stop codon inducing single nucleotide variants were found when 3010 genotypes were sequenced (Fig. 2c, [133]). However, the phenotypic consequences of this vast amount of potential LoF variation is largely unknown. But given the prominent role that LoF alleles have played throughout crop evolutionary history, deeper investigations of these variants are likely to yield new discoveries of beneficial genetic knockouts. For example, these and other LoF variants such as frameshift mutations can be used in function-based GWA where associations of functional allele states (non-functional vs functional) are compared to identify candidate loci [134,135].

Application of these genomic analyses in crops presents an opportunity to sort out the deleterious LoF variation from those which have contributed to beneficial crop evolution and are thus valuable additional targets for improvement through knockout mutations. In addition to potentially yielding candidates for further investigation, surveys of functional genomic diversity could be valuable for filtering out candidates that are poor targets - genes in which natural LoF are never observed might be too constrained to be useful for breeding through knockout mutations.

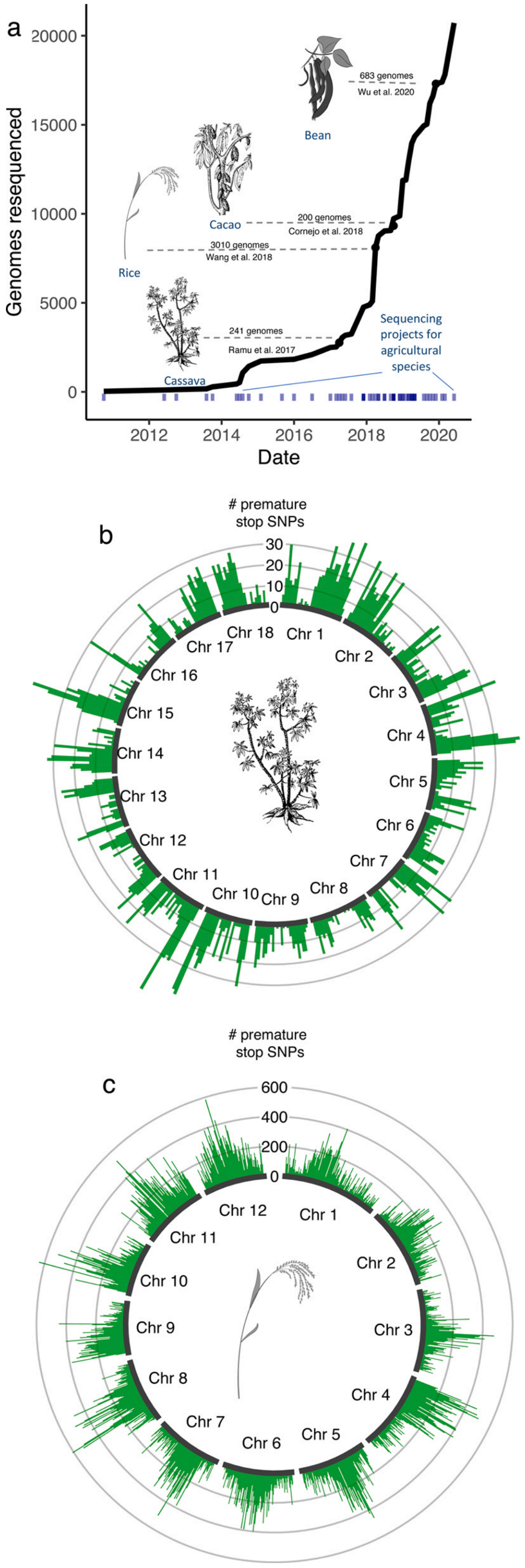

Fig. 2. Scaling up the discovery of natural knockout alleles. (a) Survey of population whole genome resequencing projects in agricultural species. Ticks above x-axis indicate individual projects. Highlighted are sequencing projects in cassava [70], rice [133]. cacao [136]. and common bean [137]. Examples of natural knockout alleles discovered through resequencing: (b) 4,399 premature stop inducing single nucleotide polymorphisms found among 200 cassava genomes (data from Ramu et al. [70]) and (c) 198,609 premature stop polymorphisms found in 3010 rice genotypes (data from Wang et al. [133]).

9. Concluding remarks

The study of LoF in crop evolution reminds us of the value of synthesizing fundamental research of evolutionary biology to inform decisions of applied value in agriculture. For evolutionary biologists interested in understanding rapid evolution and adaptation, domestication and improvement of crop species has always been of interest. Investigations into the functional genomic basis of this evolution has revealed an important role for gene LoF. These discoveries in turn inspire current and future efforts to use gene editing to accelerate crop improvement through targeted knockouts. We now know that monumental yield increases of the Green Revolution were largely caused by natural LoF mutations. Today agriculture is faced with the challenge of growing greater quantities of more nutritious food with less input - all in the face of rapidly changing environments. Some have described the change required to achieve these goals as no less than a Second Green Revolution. The use of targeted knockouts to reach this Second Green Revolution can therefore largely be viewed as inspired by the First. Armed with a deeper understanding of how and why LoF mutations shaped past crop evolution and with technological advances empowering the future discovery and application of targeted knockouts, this goal is becoming increasingly within reach.

Author statement

All authors contributed to the conceptualization, writing, and editing of this manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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