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Current achievements in modifying crop genes using CRISPR/Cas system

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With the advent of the new genome editing tool of target-specifically customizable endonucleases, a huge variety of novel opportunities have become feasible. The crop improvement is one of the main applications of genome editing in plant science and plant biotechnology. The amount of publications referring to genome editing and CRISPR/Cas system based molecular tools application in crops is permanently growing. The aim of this study is the systematization and cataloging of these data. Earlier we published the first catalog of targeted crop genome modifications as of February 10, 2017. The current review is an update of the catalog; it covers research papers on crop genome modifications from February 10, 2017 to August 17, 2018, found by searching 47 crop names in the Scopus database. Over one year and a half, 377 articles mentioning CRISPR/Cas and crop names have been published, of which 131 articles describe an experimental application of this tool for editing 193 genes in 19 crops, including rice with the largest number of genes modified (109 genes). Editing 50 of 193 genes was aimed at crop improvement. The catalog presented here includes these 50 genes, specifying the cultivars, each gene and gene product function, modification type and delivery method used. The current full list of genes modified with CRISPR/Cas with the aim of crop improvement is 81 in 16 crops (for 5 years from August 2013 to August 2018). In this paper, we also summarize data on different modifications types in different crops and provide a brief review of some novel methods and approaches that have appeared in crop genome editing research over the reviewed period. Taken together, these data provide a clear view on current progress in crop genome modifications and traits improvement using CRISPR/Cas based genome editing technology.

Key words: biotechnology; CRISPR/Cas; crop plants; genome editing; next-generation breeding; site-directed mutagenesis; target genes.

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Текущие достижения в области модификации генов культурных растений с использованием системы CRISPR/Cas

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С появлением технологии геномного редактирования, основанной на применении сайт-специфических эндонуклеаз, открылось огромное количество новых возможностей. Одной из ключевых задач геномного редактирования в биологии и биотехнологии растений является улучшение культурных растений. Число публикаций, описывающих редактирование генома сельскохозяйственных видов с помощью системы CRISPR/Cas, неуклонно возрастает. Цель работы – систематизация и каталогизация этих данных. Ранее мы проводили анализ индексируемых в базе данных Scopus публикаций, описывающих модификации генома растений, в каталоге, включающем сведения на 10.02.2017. Текущий обзор – это обновление каталога; он охватывает исследовательские работы о модификациях генома сельскохозяйственных культур с 10 февраля 2017 г. по 17 августа 2018 г., найденные путем поиска по 47 названиям культур в базе данных Scopus. В течение полутора лет было опубликовано 377 статей, в которых названия культурных растений упоминались в сочетании с «CRISPR», из них 131 статья описывает экспериментальное применение данного метода для редактирования 193 генов в 19 культурах, включая рис с наибольшим количеством модифицированных генов (109 генов). Редактирование 50 из 193 генов было направлено на улучшение свойств растений. Представлен-

ный каталог описывает эти 50 генов с указанием сортов, функций продуктов генов, типа модификации и используемого метода доставки. Текущее общее количество генов, модифицированных с помощью CRISPR/Cas с целью улучшения признака, составляет 81 в 16 культурах (за пять лет с августа 2013 по август 2018 г.). В этой статье мы также обобщаем данные о различных типах модификаций в культурах растений и даем краткий обзор некоторых новых методов и подходов, которые появились в исследованиях редактирования генома растений за рассматриваемый период. В совокупности эти данные дают четкое представление о текущем прогрессе в области модификации и улучшения генома растений с использованием технологии CRISPR/Cas.
Ключевые слова: CRISPR/Cas; биотехнология; гены-мишени; геномное редактирование; культурные растения; направленный мутагенез; селекция нового поколения.

Introduction

The rapidly developing complex of genome editing methods provides opportunities for the permanent expansion of technique utilization in different fields. Breeding and crop improvement is a broad field with endless possible applications of targeted genome modifications. However, the progress in its application is unpredictably affected by different factors. There are different limitations. Many methods are still genotype-dependent, and there are crops with only few certain model cultivars amenable for genome editing. The overview of a field and comparative analysis of different types of modifications in different crops is necessary to reveal the "hot points" and "problem zones" of this technology. Such analysis can help predict the success of each modification in each crop and set goals for further research. We published the first catalog of targeted crop genome modifications performed from the date of method emergence until February 10, 2017. Careful registration of all successful events in trait improvement by CRISPR/Cas-based modification after that date (from February 2017 until August 2018) is the aim of the present research.

Genome editing in different crops

The search was carried out using 47 crop names in the Scopus database (www.scopus.com). A total of 377 articles were mined by using the keyword CRISPR with a crop name within article titles, abstracts and keywords. Each article was analyzed, and the data about editing genes with the help of CRISPR/Cas9 were recorded. The number of publications describing experimental applications of CRISPR/Cas-based genome modifications was 131 (≈35 %). These articles describe the editing of the genomes of 19 crops with 193 target genes, including rice with the largest number of genes modified (109 genes). Compared to the previous period (Korotkova et al., 2017), data for the genome editing of the following 9 crops have been added: alfalfa, cassava, cotton, coffee, banana, carrot, switchgrass, oilseed rape and *Brassica carinata* (Ethiopian mustard). Thus, the current total number of edited crops is 24 including cereal and corn crops (barley, maize, rice, switchgrass, wheat), vegetables and melons (cabbage, carrot, cassava, cucumber, potato, rape, tomato, watermelon), fruits (apple, banana, grape, grapefruit, orange), legumes (alfalfa, soybean), technical crops (Ethiopian mustard, cotton, flax) and coffee.

The 131 articles describing the experimental application of CRISPR/Cas-based genome modifications (published from February 10, 2017 until August 17, 2018) were analyzed to distinguish studies aimed at crop improvement from functional genetics investigations or methodological research. A total of 38 papers were identified as describing gene editing aimed at crop improvement. The number of genes related to trait improvement without obvious severe pleiotropic effects on other plant properties was 50 for 11 crops. These genes were included in the catalogue (see the Table). Four crops (cotton, oilseed rape, orange, and switchgrass) were new in comparison with the Catalogue-2017 (Korotkova et al., 2017). Thus, from 24 crops on which CRISPR/Cas was successfully tested, 16 were used in the studies aimed at their improvement (apple, barley, cotton, cucumber, flax, grape, grapefruit, maize, orange, potato, oilseed rape, rice, soybean, switchgrass, tomato, wheat). The previous (Korotkova et al., 2017) and current (see the Table) catalogues include a total of 81 genes (Fig. 1), with the largest gene numbers in rice (34 genes), tomato (14 genes) and wheat (7 genes).

Among the novel genes modified, negative regulators are most frequent, such as negative regulators of rice grain fragrance (Lu et al., 2017) and earliness (Li et al., 2017), negative regulators of powdery mildew resistance in wheat (Zhang et al., 2017) and resistance to fungal pathogens in barley (Kumar et al., 2018), negative regulators of lateral roots growth in cotton (Wang Y. et al., 2017) and parthenocarpy in tomato (Ueta et al., 2017), etc. (see the Table). Knockout of negative regulators by NHEJ (non-homologous ends joining) results in crop improvement or obtaining genotypes with desired properties. For example, knockout of the *Waxy* gene in rice resulted in the development of new transgene-free rice lines with lower amylose content (Zhang et al., 2018). Based on amylose content values, rice is commercially classified into five groups: waxy (0–5 %), very low (5–12 %), low (12–20 %), intermediate (20–25 %), and high (25–33 %) AC. Waxy rice, also called glutinous rice, is especially sticky when cooked. The resulting rice phenotype has special properties that are important for use in the food industry. The further example is the low-gluten transgene-free wheat. It was obtained by multiplex knockout of α-gliadin genes (Sánchez-León et al., 2017). Wheat grain contains gluten proteins. Amongst these, the α-gliadin family is the main protein group associated with the development of coeliac disease and noncoeliac gluten sensitivity. Traditional mutagenesis and plant breeding have failed to obtain low immunogenic wheat varieties for patients with coeliac. It was shown that CRISPR/Cas9 technology can be used to precisely and efficiently reduce the amount of α-gliadins. It could be used to produce low-gluten foodstuff and serve as a source material to introgress this trait into elite wheat varieties.

In some cases, NHEJ was used not for knockout of the target gene but for a finer change in the gene. For example, in tomato, the *GAD2* and *GAD3* genes encoding a key enzyme in γ-aminobutyric acid biosynthesis, deletion of the autoinhibitory domain by introducing a stop codon was

achieved, resulting in an increased level of γ-aminobutyric acid (non-proteinogenic amino acid that has hypotensive effects) in tomato fruits (Nonaka et al., 2017). Tomato fruits with an increased level of γ-aminobutyric acid, GABA (a non-proteinogenic amino acid that has hypotensive effects), were obtained. Such tomatoes can be useful for patients' treatment with mild high blood pressure or high-normal blood pressure.

In 5 of the 50 genes modified (see the Table), amino acid substitutions or allelic replacements were made using the homologous recombination (HR) approach or chimeric single-guide RNA (cgRNA) repair method. The exact replacement of the existing *NRT1.1B* allele in commercial varieties with the elite allele in rice was produced (Li et al., 2018). Without additional selection pressure, the Japonica *NRT1.1B* allele was successfully replaced with an elite allele in just one generation at a frequency of 6.72 %. This work demonstrates the feasibility of replacing any genes with elite alleles within one generation, greatly expanding our ability to improve agriculturally important traits.

For the *ALS* gene (related to resistance to herbicides), besides the HR approach, the target-AID (target-activation induced cytidine deaminase) method was used to make amino acid substitution (Shimatani et al., 2017, 2018a, b). Adenine and cytidine deaminases convert their respective nucleotides into other DNA bases, thereby offering many possibilities for DNA editing.

In addition to target-AID, some other novel methods and approaches appeared in crop genome editing research during the reviewed period. Besides Cas9 nuclease, Cpf1 nuclease has been used. Cpf1 enzymes are a family of type V CRISPR nucleases that includes both endoribonuclease and endodeoxyribonuclease activities. AsCpf1 recognizes the 5′-TTTN-3′ protospacer adjacent motif, thus it can be used for targeting AT-rich genomes (Yamano et al., 2016). In addition, Cpf1 nucleases were shown to have lower rates of off-target edits relative to Cas9 nucleases (Begemann et al., 2017). Further qualification and broad introduction of the Cpf1 genome editing technology have the potential to make a vast impact on plant biotechnology.

DNA-free genome editing in crops develops further. Previously, efficient delivery of both Cas9 (Svitashev et al., 2016) and Cpf1 (Kim et al., 2017) as ribonucleoprotein complexes (RNPs) with guide RNAs was reported for maize embryos and soybean protoplasts, respectively. The detailed protocol is now available for wheat (Liang et al., 2018); RNP delivery to protoplast with subsequent regeneration of plants is reported for potato (Andersson et al., 2018).

The combination of double-haploid technology and genome editing enables one to efficiently produce homozygous plants with modified genomes. The utilization of double haploid plants regeneration from pollen of primary transformants was demonstrated in barley (Hensel et al., 2018). Targeted mutagenesis in pollen grains with subsequent regeneration of double haploid plants in one generation was proposed in wheat (Bhowmik et al., 2018).

New PAMs (for example, NAG PAM) for CRISPR/Cas9 gene editing were tested and showed good activity (Meng et al., 2018). It was revealed that the most widely used wild type SpCas9 is robust in recognizing both NAG and NGG PAMs in rice. The NAG PAM could be chosen alone or together with

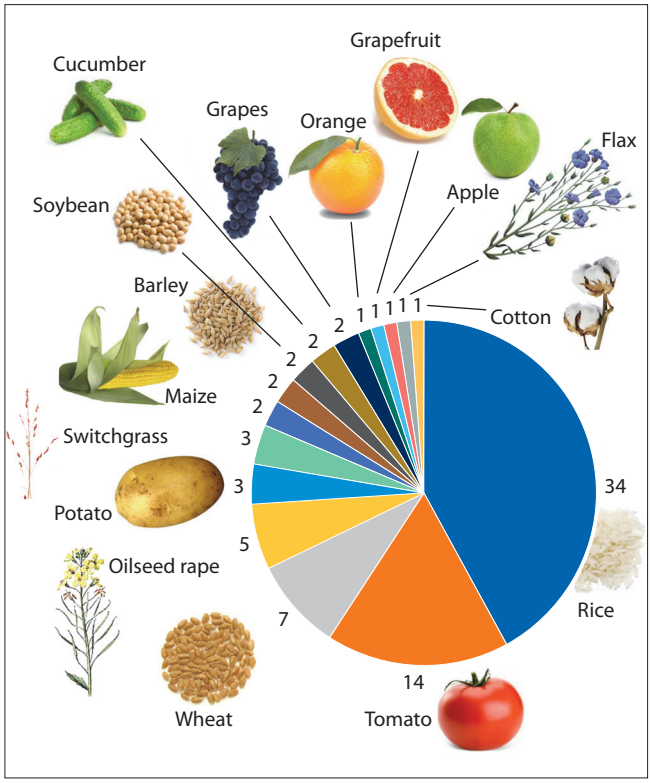

Fig. 1. Number of genes modified using CRISPR/Cas system with the aim of crops improvement, summarized from (Korotkova et al., 2017) and the Table for the period from August 2013 till August 2018.

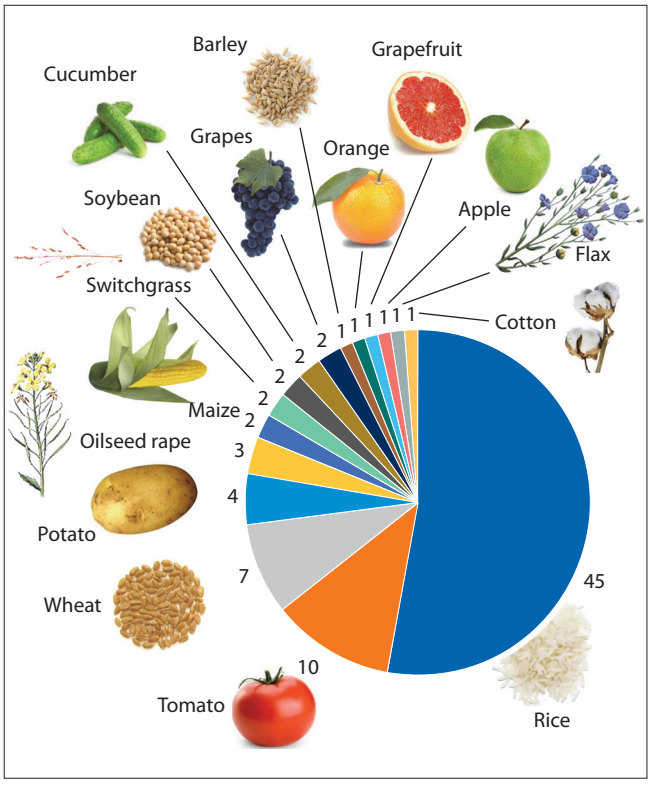

Fig. 2. Number of genotypes modified using CRISPR/Cas system with the aim of crops improvement, summarized from (Korotkova et al., 2017) and the Table for the period from August 2013 till August 2018.

Crop genes modified with the CRISPR/Cas system to improve agronomically important traits (according to publications indexed in Scopus; from February 10, 2017 to August 17, 2018)

Target gene	Function of the encoded product	Modification	Desired trait	Cultivar/line name	Transformation method	Modified plants frequency, %	Transgene-free plants	Reference
			RICE					
AAP3	Amino acid transporter	Knockout	Increased tiller number and panicle number (increased grain yield)	Zhonghua 11 Kongyu 131	Agrobacterium-mediated transformation	No data	–	Lu et al., 2018
ALS	A key enzyme for the biosynthesis of branched-chain amino acids (major targets for herbicides)	Substitutions via the use of chimeric single-guide RNA (cgRNA) repair method	Resistance to herbicides	Nipponbare	Protoplast transformation Agrobacterium-mediated transformation	16.88	–	Butt et al., 2017
ALS	A key enzyme for the biosynthesis of branched-chain amino acids (major targets for herbicides)	Substitutions via Target-AID*	Resistance to herbicides	Nipponbare	Agrobacterium-mediated transformation	Up to 18.3	+	Shimatani et al., 2017, 2018a, b
ARM1	Arsenite-responsive R2R3 MYB transcription factor	Knockout	Increased tolerance to arsenic stress	Nipponbare Dongjing SSBM	Agrobacterium-mediated transformation	No data	+	Wang F. et al., 2017
BADH2	Negative regulator of grain fragrance	Knockout	Fragrant grain	Xidao	Agrobacterium-mediated transformation	70	+	Lu et al., 2017
DEP1	Regulator of inflorescence architecture and plant height	Multiplex knockout	Dense erect panicle, semi-dwarf phenotype	Nipponbare	Agrobacterium-mediated transformation	83	–	Shen L. et al., 2017
EP3	Regulator of inflorescence		Erect panicle			50		
GS3	Negative grain size regulator		Larger grain size			100		
GW2	Negative grain weight regulator		Increased grain weight			67		
Gn1a	Negative regulator of grain number		Enhanced grain number per panicle			97		
BADH2	Negative regulator of grain fragrance		Fragrant grain			81		
Hd1	Delayed flowering time under log day conditions		Early flowering			78		
FAD2-1	Fatty acid desaturase 2	Knockout	Increased oleic acid content; decrease of linoleic acid content to undetectable levels	Nipponbare	Agrobacterium-mediated transformation	No data	–	Abe et al., 2018
HAK1	K^+ transporter	Knockout	Dramatically reduced Cs+ uptake by rice plants	Nipponbare	Agrobacterium-mediated transformation	83	+	Nieves-Cordones et al., 2017

Hd2, *Hd4*, *Hd5*	Negative regulators of earliness (suppressors of the *Early heading date 1* – *Ehd1* gene expression)	Knockout	Extremely early flowering	Longdao16 Longdao18 Daohuaxiang2 Songjing19 Dongnong430 Dongnong429 Longqingdao2 Heinuomi Banpohe Bijing45	Agrobacterium-mediated transformation	77.8	–	Li et al., 2017
LCT1	Cd transporter	Knockout	Reduction of Cd transportation into rice grain	Xidao	Agrobacterium-mediated transformation	70	+	Lu et al., 2017
Nramp5	Metal transporter gene	Knockout	Reduction of Cd transportation into rice grain	Huazhan Longke 638S	Agrobacterium-mediated transformation	Up to 82.4	+	Tang et al., 2017
NRT1.1B	Nitrogen transporter	Allelic replacement	Increase in nitrogen use efficiency	Zhonghua 11	Particle bombardment	6.72	–	Li et al., 2018
PYL1, *PYL4*, *PYL6*	PYR1-like regulatory components of the ABA receptor	Multiplex knockout	Faster growth and higher yield in natural paddy field conditions without negative effect on dormancy	Nipponbare	Agrobacterium-mediated transformation	No data	–	Miao et al., 2018
SaF. SaM	Hybrid male sterility regulators	Knockout	Overcoming the hybrid male sterility in rice breeding	T65	Agrobacterium-mediated transformation	No data	+	Xie et al., 2017
SBEIIb	Starch branching enzyme	Knockout	Increased amylose content and resistant starch in grain	Kitaake	Agrobacterium-mediated transformation	Up to 40	+	Sun et al., 2017
Sc-j	Regulator of hybrid incompatibility	Knockout	Overcoming the reproductive barrier for hybrid breeding	E5	Agrobacterium-mediated transformation	No data	–	Shen R. et al., 2017
Waxy (*GBSS*)	Granule-bound starch synthase (GBSS)	Knockout	Increase in the amylopectin/amylose ratio (Change of starch properties)	Xiushui134 Wuyunjing 7	Agrobacterium-mediated transformation	Up to 86	+	Zhang et al., 2018
			WHEAT					
EDR1	Negative regulator of powdery mildew resistance	Knockout of three copies	Increased resistance to powdery mildew	Bread wheat KN199	Biolistic transformation	No data	+	Zhang et al., 2017
Gli	Grain storage proteins α-gliadin gene family	Multiplex knockout of more than 40 copies	Low-gluten wheat with immunoreactivity reduced by 85 %	Bread wheat BW208 and THA53 durum wheat Don Pedro	Biolistic transformation	Up to 75	+	Sánchez-León et al., 2017

Table (end)

Target gene	Function of the encoded product	Modification	Desired trait	Cultivar/line name	Transformation method	Modified plants frequency, %	Transgene-free plants	Reference
			BARLEY					
MORC1	Negative regulator of resistance to fungal pathogens	Knockout	Increased resistance to fungal pathogens	Golden Promise	Agrobacterium-mediated transformation	77	–	Kumar et al., 2018
			SWITCHGRASS					
Pv4CL	A key enzyme of phenylpropanoid biosynthesis	Knockout	Lignin reduction and increased sugar release for improved bioethanol production	NFCX1	Agrobacterium-mediated transformation	10 % tetra-allelic mutations	+	Park et al., 2017
tb1a, *tb1b*	Negative regulator of outgrowth of lateral branches	Knockout	Increased tiller numbers	Alamo	Agrobacterium-mediated transformation	95.5 and 11	+	Liu et al., 2018
			POTATO					
St16DOX	A key enzyme of steroidal glycoalkaloids (SGA) biosynthesis	Knockout	Significant decrease in the SGA content	Mayqueen	Agrobacterium-mediated transformation (in a potato hairy root culture)	No data	–	Nakayasu et al., 2018
			TOMATO					
ALC (*alcobaca*)	Fruit shelf life related gene	Amino acid substitution	Long-shelf life of tomato fruits	M82	Agrobacterium-mediated stable transformation	72.73	+	Yu et al., 2017
ETR1	Ethylene receptor	Amino acid substitutions via Target-AID*	Control of plant of parthenocarpy and shelf life period	Micro-Tom	Agrobacterium-mediated transformation	Up to 53.8	+	Shimatani et al., 2017
GAD2, *GAD3*	A key enzyme in γ-aminobutyric acid biosynthesis (a target is C-terminal extension region involved in auto-inhibition)	Deletion of the auto-inhibitory domain by introducing a stop codon	Increased level of γ-aminobutyric acid (non-proteinogenic amino acid that has hypotensive effects) in tomato fruits	Micro-Tom	Agrobacterium-mediated transformation	No data	+	Nonaka et al., 2017
IAA9	Negative regulator of parthenocarpy	Knockout	Parthenocarpy	Micro-Tom Ailsa Craig	Agrobacterium-mediated transformation	Almost 100	+	Ueta et al., 2017
Mlo1	Negative regulator of powdery mildew resistance	Knockout	Increased resistance to powdery mildew	Moneymaker	Agrobacterium-mediated transformation	No data	+	Nekrasov et al., 2017
MYB12	R2R3-MYB transcription factor for flavonoid biosynthesis	Knockout	Changing fruit color from red to pink	TB0249 TB0240 TB0216 TB0115	Agrobacterium-mediated transformation	Up to 100	+	Deng et al., 2017

PROCERA	DELLA growth regulator	Deletion of 3 nt	Control of plant height and parthenocarpy	Moneymaker	Agrobacterium-mediated transformation	No data	+	Tomlinson et al., 2019
PROCERA	DELLA growth regulator	Amino acid substitutions via Target-AID*, deletion of 12 nt	Control of plant height and parthenocarpy	Micro-Tom	Agrobacterium-mediated transformation	Up to 53.8	+	Shimatani et al., 2017
			CUCUMBER					
WIP1	An inhibitor of carpel development	Knockout	Gynoecious phenotype, with the upper nodes bearing only female flowers	CU2	Agrobacterium infection	64.3	+	Hu et al., 2017
			GRAPE					
WRKY52	Negative regulator of resistance to *Botrytis cinerea*	Knockout	Increased the resistance to *Botrytis cinerea*	Thompson Seedless	Agrobacterium-mediated transformation	Up to 27	–	Wang X. et al., 2017
			ORANGE					
CsLOB1	Negative regulator of resistance to citrus canker promoter contains effector	Indels in effector binding element of the CsLOB1 promoter	Increased resistance to citrus canker	Wanjincheng	Agrobacterium-mediated transformation	Up to 64.7	–	Peng et al., 2017
			COTTON					
ARG	Negative regulator of lateral roots growth	Knockout of 2 copies	Significantly improved lateral root system development	R18	Agrobacterium-mediated transformation	Up to 98	–	Wang Y. et al., 2017
			OILSEED RAPE					
ALC (*ALCATRAZ*)	Negative regulator of shutter resistance	Knockout of 2 copies	Increased shatter resistance (decreased seed loss during mechanical harvest)	Haydn	Agrobacterium-mediated transformation	No data	+	Braatz et al., 2017
CLV3	CLAVATA3 – a peptide ligand that interacts with the CLV1/CLV2 receptor complex to limit the number of stem cells in the shoot apical meristem	Knockout of 2 copies	Increased seed production	J9707	Agrobacterium-mediated transformation	Up to 48.65	+	Yang et al., 2018
DA1, *DA2*	Negative regulators of organ size	Knockout	Increases in organ size	Westar	Agrobacterium-mediated transformation	Up to 100	+	Yang et al., 2017
FAD2	Fatty acid desaturase 2	Knockout	Increased oleic acid content	Westar	Agrobacterium-mediated transformation	Up to 50	+	Okuzaki et al., 2018

* Target-AID (target-activation induced cytidine deaminase).

NGG PAM for efficient genome editing, and have a relatively low off-target effect.

To assess possibility for a widespread practical application of genome editing in breeding programs, we summarized data on a number of improved genotypes for each crop (Fig. 2). The biggest progress was achieved on rice (45 genotypes), tomato (10 genotypes) and wheat (7 genotypes). In most crops, only one (apple, barley, cotton, flax, grapefruit, orange) or two (cucumber, grapes, maize, soybean, switchgrass) genotypes were modified (see Fig. 2). The genotype-dependent efficacy of *in vitro* cultivation is a frequent problem for most species. Furthermore, in some species, like barley, finding transformation-efficient genotypes is a rare case. Hisano et al. (2017) performed a high-resolution mapping of the TFA (transformation amenability) loci and proposed a marker-assisted approach for selection of transformation-efficient barley genotypes. The use of this approach in barley marker-assisted breeding may increase the possibility for wider application of genome editing for improvement of future barley cultivars. Thus, considering genome editing in tight association with classical and marker-assisted breeding tools can provide the most efficient and most realistic way for next-generation breeding.

Conclusion

Targeted knockout via indels introduction and subsequent frameshift mutation induction has been successfully applied in many crops and is now being routinely used for agriculturally valuable traits improvement. Base editing shows rapid development and seems to be a promising tool for many applications in crops. Precise genome editing via HR remains challenging. The majority of genome modifications have been performed for rice and tomato, involving many different cultivars and all types of modifications. The technology is currently applied for many important crops including main cereal crops, oilseed, cotton, vegetables and fruits.

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