

CRISPR/Cas genome editing in grapevine: recent advances, challenges and future prospects

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Abstract

The development of clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9 (CRISPR/Cas9) system has revolutionized genome editing and plant breeding. Applications of CRISPR/Cas9 technology in fruit crops, including grapevine, enable precise improvement of agronomically important traits. In this review, we first describe genome editing based on the most widely used CRISPR/Cas9 system and recently developed CRISPR technologies. We then focus on applications of CRISPR/Cas9 in improvement of disease resistance, optimization of CRISPR/Cas9 systems, multiplex genome editing and off-target effect analysis in grapevine. We also discuss the challenges facing genome editing that should be overcome to realize the potential of CRISPR technology in grapevine. Finally, we highlight possible future experimental considerations for more precise and efficient genome editing in grapevine.

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Introduction

Fruit crops are an essential part of agriculture and fruits contribute greatly to the world economy. Fruits are also rich sources of food and nutrients that are beneficial to human health. As one of the most important fruit crops, grape is widely cultivated worldwide, and its related foodstuffs, such as grape juice, raisins and wine, are favored globally by consumers. For instance, the most widely consumed fruit wine worldwide is grape wine^[1]. However, cultivars of wine grape (*Vitis vinifera* L.) are peculiarly prone to damage by cold stress^[2]. Conventional breeding is commonly used to develop cold-resistant grapevines by using cold-hardy species such as Amur grape (*V. amurensis*) that can survive low temperature -30 to -40 °C^[3] as breeding material^[4–5]. Though conventional breeding contributes significantly to grapevine improvement, it is accompanied by genetic diversity and loss of fitness, and is also inefficient to obtain multiple desired characteristics simultaneously, as it largely depends on natural allelic variations^[6–7]. Moreover, grapevine has a relatively long juvenile stage and breeding is therefore a time-consuming practice. Hence, novel approaches are required to improve the traits or/and production of grapevine more rapidly and efficiently.

The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) has emerged as a simple but efficient genome editing technology^[8] and shown great promise for crop improvement^[9–11]. In recent years, the CRISPR toolbox has swiftly expanded, and new approaches such as base editing and prime editing have been developed and applied to crop improvement^[10]. CRISPR/Cas9-mediated genome editing in grapevine was first reported in 2016^[12–13]. Since then, genome editing research based on CRISPR/Cas9

technology in this species has been boosted. In this review, we describe CRISPR/Cas platforms designed for plant genome editing and summarize research progress on genome editing based on CRISPR/Cas9 systems in grapevine. The challenges and future prospects of applications of CRISPR/Cas technologies in grapevine are also discussed.

Overview of CRISPR/Cas technologies

CRISPR/Cas systems were initially identified in bacteria and archaea function as adaptive immune systems to protect prokaryotes from invading nucleic acid molecules^[14]. CRISPR/Cas systems can be classified into two different classes based on effector protein organization^[15,16]. The class 1 systems are characterized as multi-protein effectors, whereas the class 2 systems utilize single-protein effectors^[15,16]. The class 2 systems can be divided into three types, with the type II system requiring the Cas9 protein as the effector^[17,18]. Native Cas9 nuclease has a bi-lobed architecture: a recognition (REC) lobe and a nuclease (NUC) lobe. The REC could bind to the guide RNA (gRNA) and therefore determines the specific function of Cas9, while the NUC contains two nuclease domains, namely RuvC and HNH^[19–21]. Once the Cas9-gRNA complex binds to the target sequence through Watson-Crick pairing between gRNA and target DNA, Cas9 nuclease can cleave target DNA strands along with a protospacer adjacent motif (PAM), resulting in a double-strand break (DSB) (Fig. 1). The produced DSBs can be repaired by non-homologous end joining (NHEJ) or the homology-directed repair (HDR) pathway^[22,23]. NHEJ could induce indel (insertion or deletion) mutations, which usually lead to the shift of an open reading frame. By taking advantage of the error-prone NHEJ pathway, CRISPR/Cas9

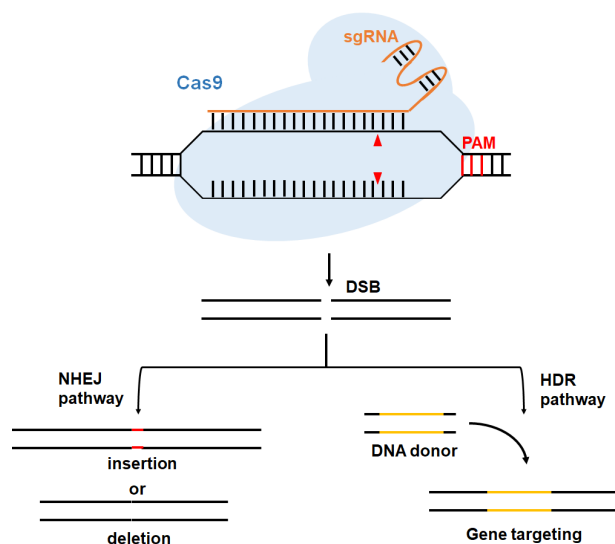


Fig. 1 Schematic diagram of genome editing induced by CRISPR/Cas9 system. Cas9 protein cleaves the target sequence (the cut sites are indicated by red triangles) that is complementary with the single guide RNA (sgRNA) and produces double strand breaks (DSB). The DSB could be repaired through non-homologous end joining (NHEJ) or the homology-directed repair (HDR) pathway, resulting in indel (insertion or deletion) or gene targeting mutation.

system is commonly adopted for gene knockout. In contrast, HDR pathway stimulated by homologous donor templates is an accurate repair mechanism and can be used to accomplish point mutations or fragment knock-in^[24] (Fig. 1). The reports on CRISPR/Cas9-mediated genome editing in plants first appeared in 2013^[25,26], and artificial single gRNA (sgRNA) was used in these studies instead of the naturally occurring gRNA formed by the fusion of bacterial CRISPR RNA (crRNA) and *trans*-activating crRNA (tracrRNA) (Fig. 1). Due to its simplicity and high efficiency, the engineered CRISPR/Cas9 system has since then been widely applied to genome editing in plants.

Modifications of native Cas9 protein result in different Cas9 variants. The Cas9 nickase (Cas9n), with a mutation in RuvC or HNH domain, could cleave a single DNA strand to produce a nick, rather than DSB. The use of Cas9n combined with a pair of sgRNAs could improve the specificity of genome editing and reduce off-target effects^[27,28]. The catalytically inactive Cas9, called dead Cas9 (dCas9), loses the ability of nuclease but is capable of binding to the target site^[29]. The binding of the dCas9-sgRNA complex to the promoter of a gene could repress gene expression^[29]. Moreover, the dCas9 can be fused with activator or repressor domains to develop CRISPR activator (CRISPRa) or CRISPR interference (CRISPRi) systems for gene activation or silencing^[29,30]. In addition, the CRISPR/dCas9 system can also serve as modular platform for epigenetic modification and visualization of targeted genomic locations when fused with epigenetic factors and fluorescent protein, respectively^[31,32].

A big concern for the use of the CRISPR/Cas9 system is the possible off-target effect. The best studied and most widely used Cas9 protein is isolated from *Streptococcus pyogenes*, and SpCas9 has been shown to generate off-target mutations during genome editing^[33–35]. To minimize off-target effects, several high-fidelity Cas9 variants have been developed^[36–40]. For example, the SpCas9-HF1, a quadruple substitution SpCas9

variant (N497A/R661A/Q695A/Q926A), exhibited nearly no detectable off-target effects without sacrificing the on-target activities^[36]. In addition, PAM sequence is also an important factor that limits the editing scope of SpCas9. The PAMs are essential for recognition of target sites by Cas9-sgRNA complexes^[8]. In fact, SpCas9 protein contains a PAM-interacting domain and recognizes canonical NGG PAMs. However, in rice, the non-canonical NAG PAMs were also reported to be efficiently recognized by SpCas9^[41]. Furthermore, the modified SpCas9 variants VQR-SpCas9 (D1135V/R1335Q/T1337R) and EQR-SpCas9 (D1135E/R1335Q/T1337R), which recognize NGA and NGCG PAMs, respectively, have been used for rice genome editing^[42,43]. Recently, another two newly engineered SpCas9 variants, namely SpCas9-NG and xCas9, have been applied in plants for targeted mutagenesis of the targets with NG PAMs^[44–46]. The use of these SpCas9 variants greatly expands the target scope in genome editing. Alternatively, orthologous Cas9 proteins from *Streptococcus canis* (ScCas9) and *Staphylococcus aureus* (SaCas9) were also reported to cleave targets with altered PAMs in plants^[47–50]. More recently, an engineered SpCas9 variant named SpRY was developed to target almost all PAMs (NRN and NYN), resulting in unconstrained genome editing^[51]. The CRISPR toolbox based on the SpRY has been developed now for plant genome engineering^[52], which is expected to promote plant genome editing in a PAM-less fashion. In contrast to SpCas9, Cas12a (also known as Cpf1) was reported to recognize a T-rich PAM at 5' of the target site and lead to a staggered DSB, which is distant from the PAM, with higher specificity^[53,54]. More importantly, Cas12a only requires a small crRNA to cleave the target DNA, and the RNase III activity of Cas12a allows for pre-crRNA processing^[53], both of which make it powerful for the editing of multiple targets simultaneously.

State-of-the-art technologies for higher plant genome engineering

Base editing

Although HDR-mediated gene targeting could accomplish precise genome modifications, the editing efficiency achieved with HDR is very low, which restricts its applications in plants^[55]. The development of base editors enables more efficient and precise genome editing.

Cytosine base editor (CBE) consists of a Cas9n (D10A), a cytidine deaminase and an uracil DNA glycosylase inhibitor (UGI) (Fig. 2). Cytidine deaminase mediates deamination of cytidines (C) and produces uridines (U), which are protected by UGI from base-excision repair and finally changed into thymines (T) during DNA replication^[56]. CBE was first described to generate C:G>T:A conversions in human cells^[56], and following the development of human CBE, multiple CBE systems have been developed and optimized for plant base editing^[57–59]. In the initial CBE (called BE3) system, rat cytidine deaminase APOBEC1 (rAPOBEC1) was used with an editing window of seven nucleotides from position 3 to 9 within the protospacer^[56]. Additionally, *Petromyzon marinus* cytidine deaminase 1 (PmCDA1), human activation-induced cytidine deaminase (hAID) and human APOBEC3A (hAPOBEC3A) were also employed to develop CBEs for efficient base editing in plants^[57,58,60]. Recently, two CBE variants, A3Bctd-VHM-BE3 and

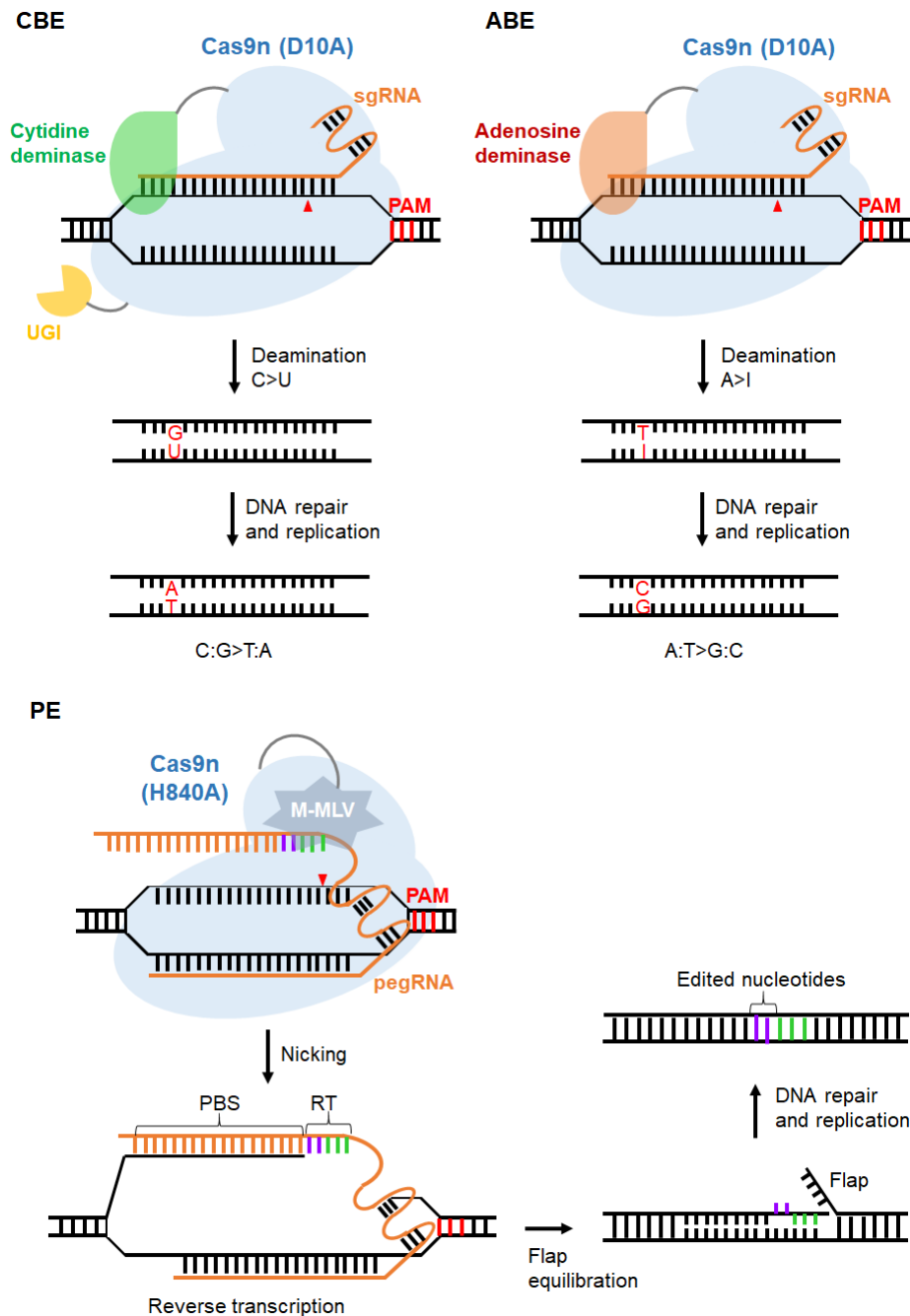


Fig. 2 Schematic overview of base editing and prime editing. In the cytosine base editor (CBE) system, a Cas9 nickase (Cas9n) is fused with a cytidine deaminase and a uracil DNA glycosylase inhibitor (UGI). The CBE could mediate C-to-T substitutions. In the adenine base editor (ABE) system, a Cas9n protein is fused with an engineered *Escherichia coli* adenosine deaminase (ecTadA), which catalyzes the conversion of adenine (A) to inosine (I). The I is recognized as guanine (G) by DNA polymerase during replication, resulting in A-to-G substitutions. Prime editing (PE) is accomplished by M-MLV-Cas9n-pegRNA complex, in which pegRNA functions as a guide RNA and also provides primer binding site (PBS) and reverse transcriptase (RT) template. The nicked target DNA sequence hybridizes to the PBS, priming reverse transcription of the template into the DNA sequence. The desired mutations within the pegRNA are indicated in purple. PAM, protospacer adjacent motif.

A3Bctd-KKR-BE3, were developed by using rationally designed hAPOBEC3B and showed no DNA off-target edits in rice^[61].

In contrast to CBE, adenine base editor (ABE), composed of a Cas9n and an adenosine deaminase effector, induces A:T>G:C conversions^[62] (Fig. 2). Adenosine deaminase deaminates adenines (A) to inosines (I), which are recognized as guanines by DNA polymerase during DNA replication^[10]. Due to the lack of natural adenosine deaminase, the *Escherichia coli* tRNA adenosine deaminase (ecTadA) was used for extensive directed

evolution, and evolved ecTadA variants (ecTadA*) were used to develop ABEs^[62]. Plant-compatible ABEs have been successfully applied in *Arabidopsis*, rapeseed, rice, wheat, tomato, and soybean^[63–65]. Intriguingly, the promoters used for the expression of ABEs in plants were reported to have an impact on editing efficiency^[63,65]. For example, the *RPS5A* promoter is more efficient than CaMV35S or YAO promoter in driving the expression of ABEs in *Arabidopsis* and rapeseed^[63]. Similarly, in tomato, seven different promoters were tested with ABEs and

the *SIEF1α* promoter outperformed the others and resulted in the highest editing efficiency^[65].

CBE and ABE could be combined to create dual base editor (DuBE), in which a cytidine deaminase, an adenosine deaminase, a Cas9n (D10A) and an UGI are fused together, to conduct C:G > T:A and A:T > G:C editing simultaneously^[66–68]. Plant DuBEs such as saturated targeted endogenous mutagenesis editors (STEMEs) and pDuBE1 have been developed to mediate robust dual editing^[67,68], which is very useful for crop trait improvement and plant direct evolution.

Prime editing

Although base editors could perform precise genome modifications, only certain base substitutions can be achieved. However, many plant traits are associated with different heterogeneous mutations. To overcome the limitation problem, a 'search-and-replace' genome editing technology named prime editing was recently developed to perform multiple types of modifications as designed^[69]. Prime editors (PEs) consist of a Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) and Cas9n (H840A) (Fig. 2). A prime editing guide RNA (pegRNA) that contains a RT template and primer binding site (PBS) region was used instead of sgRNA (Fig. 2), and the information carried by the RT template could be copied directly and introduced into the target site after prime editing^[69]. Compared with previous base editor systems, PEs exhibited higher specificity and the editing efficiency was comparable with that obtained using base editors. As a game-changing technology, PEs have been adapted rapidly for plant genome editing^[70–73]. However, low editing efficiency is the major factor that limits the applications of PEs in plants. Lin et al.^[72] found that optimizing the melting temperature (T_m) of the PBS could improve prime editing efficiency. By comparing multiple pegRNAs with different PBS T_m , the researchers found that pegRNAs with the PBS T_m approaching 30 °C exhibited higher activities. Moreover, adoption of a dual-pegRNA strategy further improved prime editing efficiency^[72]. A user-friendly online tool named PlantPegDesigner was developed accordingly^[72], which is expected to facilitate the applications of PEs in plants.

Chromosome engineering

Due to the advances in CRISPR technologies, CRISPR/Cas-mediated chromosome engineering has been possible in plants. Chromosomal inversions of up to 18 kb have been accomplished using SaCas9 protein combined with an egg cell-specific promoter in *Arabidopsis*^[74]. The method designed for generating heritable chromosomal translocations was recently established in *Arabidopsis*, and these CRISPR/Cas9-induced reciprocal translocations were achieved between chromosome 1 and 2, and chromosome 1 and 5, respectively^[75]. Both inversions and translocations play an important role in adaptation, speciation and genome evolution^[76]. Furthermore, these chromosomal rearrangements generally alter genetic linkages between plant traits. For clonally propagated crops with high genome heterozygosity, deleterious mutations linked together cannot be removed through conventional breeding. In grapevine, for instance, deleterious alleles are usually hidden at the heterozygous state in the genome^[77]. Moreover, the color polymorphism of grape berry was reported to be involved in chromosomal rearrangements^[78,79]. CRISPR/Cas-induced chromosome engineering provides a way to break the linkage of

deleterious genes or to fix genetic linkage of target genes contributing to desirable traits.

Genome editing in grapevine

Grape is an economically important fruit crop, and the CRISPR/Cas9 system has been used for precise genome editing in this species (Table 1). In recent years, several grape characteristics such as quality and disease resistance, have been improved by using CRISPR/Cas9 technology. Moreover, optimization of the currently used CRISPR/Cas9 system was also reported in grapevine. In general, embryogenic callus (or cells) induced from anthers and filaments are used for stable transformation, which is usually mediated by *Agrobacterium* cells that contain CRISPR/Cas9 constructs (Fig. 3; Table 1). In addition, protoplasts isolated from grape callus or leaves are also suitable for CRISPR experiments (Fig. 3; Table 1).

Targeted editing of genes involved in quality and growth

In 2016, CRISPR/Cas9-mediated genome editing was first reported in 'Chardonnay' by targeting the L-idonate dehydrogenase (*IdnDH*) gene, which encodes the enzyme that functions in biosynthesis of tartaric acid (TA) in grape^[12]. Disruption of the *IdnDH* gene resulted in the decrease of TA accumulation in grape cells^[12]. The trans-acting small-interfering locus4 (*TAS4*) and *MYBA7* genes are thought to be associated with pathogen-related anthocyanin accumulation in grape, and targeted mutagenesis of the two genes were conducted using the CRISPR/Cas9 system in grapevine rootstock '101-14' to address this relationship^[80]. Two *TAS4b* edited lines and five bi-allelic lines of *MYBA7* were obtained, however no visible anthocyanin accumulated in these transgenic plants, possibly due to the functional redundancy of *TAS4c* and *MYBA5/6* loci^[80]. Furthermore, targeted knockout of *V. vinifera* phytoene desaturase (*VvPDS*) gene was achieved in table grape 'Neo Muscat', and *VvPDS* mutation in grapevine plants led to the development of albino leaves, with the editing efficiencies ranging from 2% to 86%^[81]. Carotenoid cleavage dioxygenase 7 (*CCD7*) and *CCD8* are two enzymes that are required for biosynthesis of strigolactones, which control axillary bud outgrowth in plants^[82]. To investigate the role for *CCD7* and *CCD8* in the control of shoot branching in grapevine, the *VvCCD7* and *VvCCD8* genes were mutated in grapevine rootstock '41B' by using CRISPR/Cas9, and the obtained *ccd8* mutants showed increased shoot branches^[82].

Targeted editing of genes involved in disease resistance

Wang et al.^[83] used the CRISPR/Cas9 system to edit the transcription factor gene *VvWRKY52* in 'Thompson Seedless' and obtained 22 independent mutants, among which 15 plants were identified as bi-allelic mutants. Four out of the 22 edited plants were tested with enhanced resistance to *Botrytis cinerea*^[83]. Similarly, in two recent studies, the grapevine cultivar 'Thompson Seedless' was engineered to improve its resistance to powdery mildew^[84,85]. In the first study, Wan et al.^[84] employed the CRISPR/Cas9 system to edit the mildew resistance Locus O3 (*VvMLO3*) and *VvMLO4* genes, with the editing efficiency varying from 0 to 38.5%. Four *VvMLO3*-edited plants showed enhanced powdery mildew resistance^[84]. In the other study, pathogenesis-related protein 4b (*VvPR4b*) gene

Table 1. Applications of the CRISPR/Cas9 system in grape.

Target genes	sgRNA/Cas9 promoter	Explant	Delivery method	Modification	Target traits	Editing efficiency	Off-target effect	Reference
<i>IdnDH</i>	AtU6/35S	Embryogenic cells of 'Chardonnay'	<i>Agrobacterium</i> -mediated transformation	KO	Tartaric acid synthesis	100%	No	Ren et al. ^[12]
<i>MLO-7</i>	Not mentioned	Embryogenic calli of 'Chardonnay'	PEG-mediated transformation	KO	Powdery mildew resistance	0.1%	ND	Malnoy et al. ^[13]
<i>VvPDS</i>	AtU6/PcUbi4-2	Embryonic calli of 'Neo Muscat'	<i>Agrobacterium</i> -mediated transformation	KO	Albino phenotype	2%–86%	No	Nakajima et al. ^[81]
<i>VvPDS</i>	AtU6/2×35S	Embryogenic cells of 'Chardonnay' and '41B'	<i>Agrobacterium</i> -mediated transformation	KO	Albino phenotype	22.2%–59.9% ('Chardonnay') 30.3%–86.6% ('41B')	ND	Ren et al. ^[89]
<i>VvWRKY52</i>	AtU3, AtU6/2×35S	Proembryonal masses (PEM) of 'Thompson Seedless'	<i>Agrobacterium</i> -mediated transformation	KO	<i>Botrytis cinerea</i> resistance	31%	No	Wang et al. ^[83]
<i>CCD8</i>	AtU6/35S	Embryogenic cells of '41B'	<i>Agrobacterium</i> -mediated transformation	KO	Strigolactones biosynthesis and shoot branching	66.7%	No	Ren et al. ^[82]
<i>VvPR4b</i>	AtU6/35S	PEM of 'Thompson Seedless'	<i>Agrobacterium</i> -mediated transformation	KO	Powdery mildew resistance	68.8%	No	Li et al. ^[85]
<i>VvMLO3</i> , <i>VvMLO4</i>	AtU3, AtU6/2×35S	PEM and somatic embryos of 'Thompson Seedless'	<i>Agrobacterium</i> -mediated transformation	KO	Powdery mildew resistance	12.8%–38.5%	ND	Wan et al. ^[84]
<i>TAS4</i> , <i>MYBA7</i>	MtU6.6/ ZmUbi	Embryogenic callus of rootstock '101-14'	<i>Agrobacterium</i> -mediated transformation	KO	Anthocyanin accumulation related to Pierce disease (PD) and <i>Grapevine Red Blotch Virus</i> (GRBV)	Not mentioned	Yes	Sunitha & Rock ^[80]
<i>PDS</i> , <i>TMT1</i> , <i>TMT2</i>	AtU6, VvU3, VvU6/35S, VvUbi	Embryogenic cells of '41B'	<i>Agrobacterium</i> -mediated transformation	KO	Albino phenotype Sugar accumulation	23.5%–43.2% (<i>PDS</i>) 10.4%–20.9% (<i>TMTs</i>)	ND	Ren et al. ^[90]

Abbreviations: AtU6, *Arabidopsis thaliana* U6 promoter; MtU6.6, *Medicago truncatula* U6.6 promoter; VvU6, *Vitis vinifera* U6 promoter; PcUbi4-2, *Petroselinum crispum* ubiquitin4-2 promoter; ZmUbi, *Zea mays* ubiquitin promoter; VvUbi, *V. vinifera* ubiquitin promoter; 35S, CaMV 35S promoter; KO, Knock out; ND, Not determined.

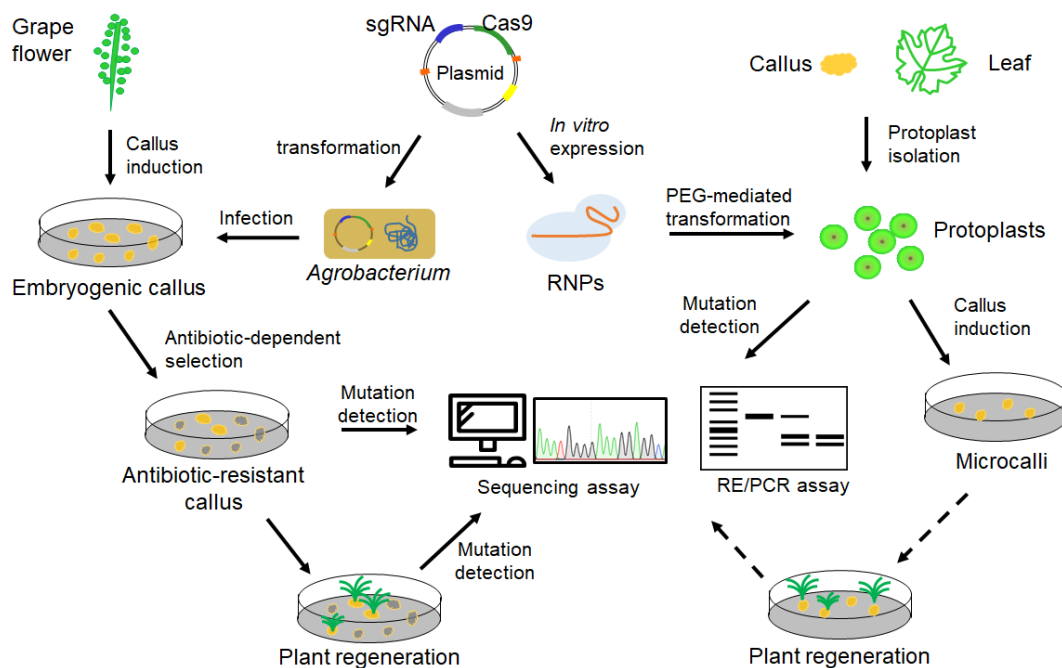


Fig. 3 Pipeline of CRISPR/Cas9-mediated genome editing in grape. Both embryogenic callus and protoplasts could be used as materials for grape genome editing. CRISPR/Cas9 reagents such as plasmid constructs and CRISPR/Cas9 ribonucleoproteins (RNPs) can be delivered into the embryogenic callus and protoplasts, respectively. Edited grapevine plants could regenerate from embryogenic callus. Microcalli could be induced from grape protoplasts, but plant regeneration from the protoplasts-induced calli (indicated by broken lines) has not been achieved.

was successfully edited, and mutation of the *VvPR4b* gene increased the susceptibility of grapevine mutants to pathogen

Plasmopara viticola, which suggested that the *VvPR4b* gene may contribute to powdery mildew resistance in grapevine^[85].

Although the efficacy of the CRISPR/Cas12a system in grape genome editing has not yet been demonstrated, a method known as plasmonic CRISPR Cas12a assay has been recently developed for colorimetric detection of grapevine redblotch virus infection. In this detection system, the presence of viral marker induced the degradation of the single stranded substrate (S) that serves as a linker between two DNA functionalized plasmonic gold nanoparticles, resulting in a visible blue-to-red color transition^[86]. This CRISPR/Cas12a-based detection method could be used to detect virus infections in the vineyard.

Optimization of the CRISPR/Cas9 system in grapevine

Following the successful applications of the CRISPR/Cas9 system in grape genome editing^[12,13], in 2018 the protocols for CRISPR experiments in grape based on plasmid-mediated and CRISPR/Cas9 ribonucleoproteins (RNPs)-mediated procedures were established^[87]. In addition, the CRISPR database, designed for easy selection of sgRNAs for grape genome editing, is also available^[88]. Successful genome editing is expected to be accomplished using these tools. The editing efficiency, however, could be further improved. To optimize editing efficiency, Ren et al.^[89] surveyed the effect of three key parameters, i.e., GC content of sgRNA, expression level of *Cas9*, and genotypes of grape, on grape genome editing. According to the results, CRISPR/Cas9 editing efficiency increased with sgRNA GC content. In addition, the expression level of *Cas9* and grape genotypes also had an influence on editing efficiency^[89]. Very recently, grape U3/U6 and ubiquitin (UBQ) promoters were isolated and used to drive the expression of sgRNA and *Cas9* instead of the *Arabidopsis* U6 (AtU6) and CaMV35 promoters, respectively, during genome editing in grape^[90]. The use of grape promoters significantly improved the editing efficiency by increasing the expression of sgRNA and *Cas9*^[90].

Multiplex genome editing

CRISPR/Cas9 toolkits for multiplex genome editing were first designed and applied in *Arabidopsis* and crops, such as rice and maize^[91,92]. Two strategies are commonly used to generate multiple sgRNAs targeting different gene loci: (i) constructing different sgRNA expression cassettes into all-in-one vector^[91,92], and (ii) producing multiple sgRNAs together with *Cas9* from a single transcription unit (STU) by using endoribonuclease *Csy4*, tRNA, or ribozymes (RZs)^[93,94]. In the study reported by Wang et al.^[83], four sgRNAs designed for targeting the *VvWRKY52* gene were assembled into the pYLCRISPR/Cas9P35S-N binary vector for genome editing. However, multiplex genome editing involving different genes was not reported until recently. Simultaneous editing of the grape tonoplast monosaccharide transporter (*TMT*) gene *TMT1* and *TMT2* was conducted by using conventional multiple sgRNA expression cassettes (Multi-sgRNA/Cas9) and polycistronic tRNA-sgRNA cassette (PTG/Cas9), respectively, with the overall editing efficiencies being higher than 10%^[90]. Simultaneous mutations of *TMT1* and *TMT2* resulted in low sugar accumulation in grape cells^[90]. The development of Multi-sgRNA/Cas9 and PTG/Cas9 systems paves the way for multiplex genome editing involving different traits in grapevine.

Off-target effect in grape genome editing

In previous studies, most of the editing events in grape were specific and no off-target effect was detected^[12,81–83,85]. In contrast, during the editing of *TAS4* and *MYBA7* in rootstock

'101-14', an off-target effect was detected in *TAS4b*^[80]. In general, the possible off-target sites are predicted using online tools, such as CRISPR-P (<http://crispr.hzau.edu.cn/CRISPR2>) and Plant-CRISPR (www.grapeworld.cn/pc/design.html), and then the predicted off-target sequences were amplified by PCR followed by Sanger sequencing assays^[12,81–83,85]. Most recently, whole-genome sequencing (WGS) was employed to assess off-target effect in *Cas9*-edited grapevine plants^[95]. A number of three wild-type plants and seven CRISPR/Cas9-edited plants were analyzed in greater depth by using WGS analysis, and among the 3,272 tested off-target sites, only one off-target site was identified with indel mutation^[95]. These results suggest that the CRISPR/Cas9 system shows high specificity in grapevine genome editing.

Delivery of CRISPR/Cas9 components

CRISPR/Cas9 constructs are commonly delivered into grape callus or cells via *Agrobacterium*-mediated transformation^[81–85]. The transformation method based on *Agrobacterium* is mostly efficient and low-cost, but some grapevine varieties, however, are found to be refractory to *Agrobacterium* infection^[96]. Biolistic delivery could be used to solve the problem, given that delivery of CRISPR/Cas9 reagents could be accomplished by particle bombardment with physical force. Alternatively, polyethyleneglycol (PEG)-mediated transformation was also employed to deliver CRISPR/Cas9 RNPs into grape protoplasts^[13]. Intriguingly, microcalli could be induced from edited grapevine protoplasts^[87], which provides a possible way to obtain edited grapevine plants. Novel approaches, such as nanoparticle-assisted transformation for plant genetic engineering, have been recently reviewed^[97]. Although nanoparticles could serve as cargo carriers for plant transformation, there are many challenges when using this technology. The biggest problem of nanoparticles-based genetic transformation is the regeneration of transgenic plants (except magnetic transfection of pollen)^[97]. Whether this approach could be used for grapevine transformation needs to be further investigated in the future.

Detection of targeted mutagenesis in grape genome

A critical step of genome editing is the detection of targeted mutagenesis. In grape, targeted mutagenesis of genes of interest is generally detected using typical approaches. Firstly, to detect mutations in grape callus or cells after transformation, the target sequences were amplified from the grape genome and the resulting PCR products were analyzed by direct sequencing. Targeted mutations could be judged by the presence of overlapping peaks in sequencing chromatograms^[82]. This method could be used to quickly confirm the existence of targeted mutagenesis. However, desired mutations might not be detected using this method in the presence of large numbers of unedited cells. Secondly, the PCR fragments could be treated with T7EI or CEL I endonuclease. The wild-type-mutated DNA hybrids could be recognized and digested by T7EI and CEL I endonucleases, resulting in the generation of two smaller DNA fragments^[12,89,90]. In addition to the mutations, editing efficiencies can also be calculated according to the intensities of digested bands^[89,90]. However, homologous or bi-allelic mutations could not be detected using this method. Additionally, the restriction enzyme (RE)/PCR approach is also adopted for mutagenesis detection^[90]. Nevertheless, this approach requires the presence of proper endonuclease recognition sites within the target sequence. Thirdly, to explore

Genome editing in grape

mutation types accurately, the amplified target sequences are usually cloned into a cloning vector (like pLB vector) and then analyzed by Sanger sequencing. This detection method has been applied in both grape cells and plants^[12,81–85,90]. The editing efficiencies could be obtained by calculating the mutated amplicons relative to the total analyzed^[12,81]. Other approaches like high resolution melting (HRM) and high-throughput illumina sequencing are also adopted for detection of mutations in plants^[98]. However, compared with the typical methods described above, these approaches are still more expensive and require specific instruments.

Challenges for genome editing in grapevine

Applications of CRISPR/Cas9 rely on efficient transformation systems. The lack of efficient transformation methods is a bottleneck that restricts the application of CRISPR technologies in grapevine. The grape flowers, or rather, the anthers and filaments have been demonstrated to be suitable explants for induction of embryonic callus^[81,87], which could be used for *Agrobacterium*-mediated transformation and plant regeneration. However, whether this method could be used in other grape varieties or species is still unknown. In addition, some cultivars are found to be recalcitrant to *Agrobacterium*-mediated transformation^[96], hindering the delivery of CRISPR/Cas9 reagents. Furthermore, it is laborious and inefficient to discriminate transgenic or edited calli from a pool of grape materials using current screening methods, which relies on antibiotic-dependent selection, followed by PCR identification and Sanger sequencing assay^[82,83,85]. Considering the low transformation rate of grape calli, more efficient screening methods need to be developed. It also usually takes a long time (> 12 months) to obtain the regenerated plants from embryonic calli^[81,83,85], and as a perennial fruit crop, grapevine has a long juvenile stage. All these factors make it difficult to evaluate and optimize CRISPR/Cas systems in grapevine plants, and the effect of targeted editing of candidate genes on grape berry development or quality can be difficult to identify in a short period of time. More importantly, grapevine plants are vegetatively propagated, and it is hard to obtain transgene-free editing plants through next generation segregation.

Conclusions and future perspectives

As a robust and versatile technology, applications of CRISPR/Cas9 greatly facilitate the basic and applied research of grapevine. However, most of the up-to-date CRISPR technologies have not yet been applied in grapevine. The current genome editing in grapevine is mainly focused on gene knockout, and it remains a long and arduous task to enrich the CRISPR toolbox for grape genome editing. Precise genome editing technologies, i.e., base editing and prime editing, will facilitate the improvement of desirable traits. Due to the difficulties in grape transformation, alternative transformation systems based on protoplasts and hairy roots could be used to evaluate efficacy and efficiency of new CRISPR platforms in grape. A recent study reported a method of *de novo* meristem induction to generate transgenic plants, which promises to overcome the bottleneck of plant tissue culture^[99]. Although this strategy has been proven to be effective in grapevine^[99], the generation of chimeric plants is an unavoidable problem

when using this method. In addition, rapid and robust detection methods are also needed to screen edited grape calli or plants more efficiently. In a previous study, recovery of non-functional-enhanced green fluorescence protein (nEGFP) was used as an indicator of successful editing in grape cells^[100]. Visible markers that could be detected by the naked eye are preferred and can be used without the need for specific instruments. Considering the public concerns on genetically modified organisms, in the future, genome editing methods should be developed to produce transgene-free edited grapevine plants. Site-specific recombinases and newly developed PEs promise to remove T-DNA sequences from the plant genome. In conclusion, the use of CRISPR/Cas technologies has already revolutionized, and will continue to revolutionize, basic and applied research of grapevine.

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Conflict of interest

The authors declare that they have no conflict of interest.

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REFERENCES

1. Yang H, Cai G, Lu J, Gómez Plaza E. 2021. The production and application of enzymes related to the quality of fruit wine. *Critical Reviews in Food Science and Nutrition* 61:1605–15
2. Wang Z, Wong DCJ, Wang Y, Xu G, Ren C, et al. 2021. GRAS-domain transcription factor PAT1 regulates jasmonic acid biosynthesis in grape cold stress response. *Plant Physiology* 186:1660–78
3. Fennell A. 2004. Freezing tolerance and injury in grapevines. *Journal of Crop Improvement* 10:201–35
4. Wang J, Wang S, Liu G, Edwards EJ, Duan W, et al. 2016. The synthesis and accumulation of resveratrol are associated with veraison and abscisic acid concentration in Beihong (*Vitis vinifera* × *Vitis amurensis*) berry skin. *Frontiers in Plant Science* 7:1605
5. Wang J, Ma L, Xi H, Wang L, Li S. 2015. Resveratrol synthesis under natural conditions and after UV-C irradiation in berry skin is associated with berry development stages in 'Beihong' (*V. vinifera* × *V. amurensis*). *Food Chemistry* 168:430–38
6. Karkute SG, Singh AK, Gupta OP, Singh PM, Singh B. 2017. CRISPR/Cas9 mediated genome engineering for improvement of horticultural crops. *Frontiers in Plant Science* 8:1635
7. Meyer RS, Purugganan MD. 2013. Evolution of crop species: genetics of domestication and diversification. *Nature Reviews Genetics* 14:840–52
8. Cong L, Ran FA, Cox D, Lin S, Barretto R, et al. 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339:819–23
9. Manghwar H, Lindsey K, Zhang X, Jin S. 2019. CRISPR/Cas system: recent advances and future prospects for genome editing. *Trends in Plant Science* 24:1102–25
10. Zhu H, Li C, Gao C. 2020. Applications of CRISPR-Cas in agriculture and plant biotechnology. *Nature Reviews Molecular Cell Biology* 21:661–77

11. Zhan X, Lu Y, Zhu J, Botella JR. 2021. Genome editing for plant research and crop improvement. *Journal of Integrative Plant Biology* 63:3–33
12. Ren C, Liu X, Zhang Z, Wang Y, Duan W, et al. 2016. CRISPR/Cas9-mediated efficient targeted mutagenesis in Chardonnay (*Vitis vinifera* L.). *Scientific Reports* 6:32289
13. Malnoy M, Viola R, Jung MH, Koo OJ, Kim S, et al. 2016. DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. *Frontiers in Plant Science* 7:1904
14. Horvath P, Barrangou R. 2010. CRISPR/Cas, the immune system of bacteria and archaea. *Science* 327:167–70
15. Makarova KS, Zhang F, Koonin EV. 2017. Snapshot: Class 1 CRISPR-Cas systems. *Cell* 168:946–946.e1
16. Makarova KS, Zhang F, Koonin EV. 2017. Snapshot: Class 2 CRISPR-Cas systems. *Cell* 168:328–328.e1
17. Chylinski K, Makarova KS, Charpentier E, Koonin EV. 2014. Classification and evolution of type II CRISPR-Cas systems. *Nucleic Acids Research* 42:6091–105
18. Mohanraju P, Makarova KS, Zetsche B, Zhang F, Koonin EV, et al. 2016. Diverse evolutionary roots and mechanistic variations of the CRISPR-Cas systems. *Science* 353:aad5147
19. Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, et al. 2014. Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* 156:935–49
20. Jinek M, Jiang F, Taylor DW, Sternberg SH, Kaya E, et al. 2014. Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science* 343:1247997
21. Anders C, Niewoehner O, Duerst A, Jinek M. 2014. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature* 513:569–73
22. Chang HHY, Pannunzio NR, Adachi N, Lieber MR. 2017. Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nature Reviews Molecular Cell Biology* 18:495–506
23. Scully R, Panday A, Elango R, Willis NA. 2019. DNA double-strand break repair-pathway choice in somatic mammalian cells. *Nature Reviews Molecular Cell Biology* 20:698–714
24. Tsai SQ, Joung JK. 2016. Defining and improving the genome-wide specificities of CRISPR-Cas9 nucleases. *Nature Reviews Genetics* 17:300–12
25. Feng Z, Zhang B, Ding W, Liu X, Yang D, et al. 2013. Efficient genome editing in plants using a CRISPR/Cas system. *Cell Research* 23:1229–1232
26. Mao Y, Zhang H, Xu N, Zhang B, Gou F, et al. 2013. Application of the CRISPR-Cas system for efficient genome engineering in plants. *Molecular Plant* 6:2008–11
27. Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, et al. 2013. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 154:1380–89
28. Schiml S, Fauser F, Puchta H. 2014. The CRISPR/Cas9 system can be used as nuclease for *in planta* gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny. *The Plant Journal* 80:1139–50
29. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, et al. 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152:1173–83
30. Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, et al. 2013. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 154:442–51
31. Rusk N. 2014. CRISPRs and epigenome editing. *Nature Methods* 11:28
32. Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, et al. 2013. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* 155:1479–91
33. Cho SW, Kim S, Kim Y, Kweon J, Kim HS, et al. 2014. Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Research* 24:132–41
34. Pattanayak V, Lin S, Guillinger JP, Ma E, Doudna JA, et al. 2013. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nature Biotechnology* 31:839–43
35. Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, et al. 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature Biotechnology* 31:822–26
36. Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, et al. 2016. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* 529:490–95
37. Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, et al. 2016. Rationally engineered Cas9 nucleases with improved specificity. *Science* 351:84–88
38. Chen JS, Dagdas YS, Kleinstiver BP, Welch MM, Sousa AA, et al. 2017. Enhanced proofreading governs CRISPR-Cas9 targeting accuracy. *Nature* 550:407–10
39. Lee JK, Jeong E, Lee J, Jung M, Shin E, et al. 2018. Directed evolution of CRISPR-Cas9 to increase its specificity. *Nature Communications* 9:3048
40. Vakulskas CA, Dever DP, Rettig GR, Turk R, Jacobi AM, et al. 2018. A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells. *Nature Medicine* 24:1216–24
41. Meng X, Hu X, Liu Q, Song X, Gao C, et al. 2018. Robust genome editing of CRISPR-Cas9 at NAG PAMs in rice. *Science China Life Sciences* 61:122–25
42. Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, et al. 2015. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature* 523:481–85
43. Hu X, Wang C, Fu Y, Liu Q, Jiao X, et al. 2016. Expanding the range of CRISPR/Cas9 genome editing in rice. *Molecular Plant* 9:943–45
44. Hua K, Tao X, Han P, Wang R, Zhu JK. 2019. Genome engineering in rice using Cas9 variants that recognize NG PAM sequences. *Molecular Plant* 12:1003–14
45. Ren B, Liu L, Li S, Kuang Y, Wang J, et al. 2019. Cas9-NG greatly expands the targeting scope of the genome-editing toolkit by recognizing NG and other atypical PAMs in rice. *Molecular Plant* 12:1015–26
46. Zeng D, Li X, Huang J, Li Y, Cai S, et al. 2020. Engineered Cas9 variant tools expand targeting scope of genome and base editing in rice. *Plant Biotechnology Journal* 18:1348–50
47. Wang M, Xu Z, Gosavi G, Ren B, Cao Y, et al. 2020. Targeted base editing in rice with CRISPR/ScCas9 system. *Plant Biotechnology Journal* 18:1645–47
48. Xu Y, Meng X, Wang J, Qin B, Wang K, et al. 2020. ScCas9 recognizes NNG protospacer adjacent motif in genome editing of rice. *Science China Life Sciences* 63:450–52
49. Qin R, Li J, Li H, Zhang Y, Liu X, et al. 2019. Developing a highly efficient and widely adaptive CRISPR-SaCas9 toolset for plant genome editing. *Plant Biotechnology Journal* 17:706–8
50. Jia H, Xu J, Orbović V, Zhang Y, Wang N. 2017. Editing citrus genome via SaCas9/sgRNA system. *Frontiers in Plant Science* 8:2135
51. Walton RT, Christie KA, Whittaker MN, Kleinstiver BP. 2020. Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants. *Science* 368:290–96
52. Ren Q, Sretenovic S, Liu S, Tang X, Huang L, et al. 2021. PAM-less plant genome editing using a CRISPR-SpRY toolbox. *Nature Plants* 7:25–33
53. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, et al. 2015. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163:759–71
54. Xu R, Qin R, Li H, Li D, Li L, et al. 2017. Generation of targeted mutant rice using a CRISPR-Cpf1 system. *Plant Biotechnology Journal* 15:713–17
55. Huang TK, Puchta H. 2019. CRISPR/Cas-mediated gene targeting in plants: finally a turn for the better for homologous recombination. *Plant Cell Reports* 38:443–53
56. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. 2016. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 533:420–24
57. Shimatani Z, Kashojiya S, Takayama M, Terada R, Arazoe T, et al. 2017. Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. *Nature Biotechnology* 35:441–43
58. Zong Y, Wang Y, Li C, Zhang R, Chen K, et al. 2017. Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nature Biotechnology* 35:438–40

59. Zong Y, Song Q, Li C, Jin S, Zhang D, et al. 2018. Efficient C-to-T base editing in plants using a fusion of nCas9 and human APOBEC3A. *Nature Biotechnology* 36:950–53
60. Ren B, Yan F, Kuang Y, Li N, Zhang D, et al. 2018. Improved base editor for efficiently inducing genetic variations in rice with CRISPR/Cas9-guided hyperactive hAID mutant. *Molecular Plant* 11:623–26
61. Jin S, Fei H, Zhu Z, Luo Y, Liu J, et al. 2020. Rationally designed APOBEC3B cytosine base editors with improved specificity. *Molecular Cell* 79:728–740.E6
62. Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, et al. 2017. Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. *Nature* 551:464–71
63. Kang BC, Yun JY, Kim ST, Shin Y, Ryu J, et al. 2018. Precision genome engineering through adenine base editing in plants. *Nature Plants* 4:427–31
64. Li C, Zong Y, Wang Y, Jin S, Zhang D, et al. 2018. Expanded base editing in rice and wheat using a Cas9-adenosine deaminase fusion. *Genome Biology* 19:59
65. Niu Q, Wu S, Xie H, Wu Q, Liu P, et al. 2021. Efficient A-T to G-C base conversions in dicots using adenine base editors expressed under the tomato EF1 α promoter. *Plant Biotechnology Journal* In Press
66. Grünwald J, Zhou R, Lareau CA, Garcia SP, Iyer S, et al. 2020. A dual-deaminase CRISPR base editor enables concurrent adenine and cytosine editing. *Nature Biotechnology* 38:861–64
67. Li C, Zhang R, Meng X, Chen S, Zong Y, et al. 2020. Targeted, random mutagenesis of plant genes with dual cytosine and adenine base editors. *Nature Biotechnology* 38:875–82
68. Xu R, Kong F, Qin R, Li J, Liu X, et al. 2021. Development of an efficient plant dual cytosine and adenine editor. *Journal of Integrative Plant Biology* 63:1600–5
69. Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, et al. 2019. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 576:149–57
70. Lin Q, Zong Y, Xue C, Wang S, Jin S, et al. 2020. Prime genome editing in rice and wheat. *Nature Biotechnology* 38:582–85
71. Li H, Li J, Chen J, Yan L, Xia L. 2020. Precise modifications of both exogenous and endogenous genes in rice by prime editing. *Molecular Plant* 13:671–74
72. Lin Q, Jin S, Zong Y, Yu H, Zhu Z, et al. 2021. High-efficiency prime editing with optimized, paired pegRNAs in plants. *Nature Biotechnology* 39:923–27
73. Tang X, Sretenovic S, Ren Q, Jia X, Li M, et al. 2020. Plant prime editors enable precise gene editing in rice cells. *Molecular Plant* 13:667–70
74. Schmidt C, Pacher M, Puchta H. 2019. Efficient induction of heritable inversions in plant genomes using the CRISPR/Cas system. *The Plant Journal* 98:577–89
75. Beying N, Schmidt C, Pacher M, Houben A, Puchta H. 2020. CRISPR–Cas9-mediated induction of heritable chromosomal translocations in *Arabidopsis*. *Nature Plants* 6:638–45
76. Rönspies M, Schindele P, Puchta H. 2021. CRISPR/Cas-mediated chromosome engineering: opening up a new avenue for plant breeding. *Journal of Experimental Botany* 72:177–83
77. Zhou Y, Minio A, Massonnet M, Solares E, Lv Y, et al. 2019. The population genetics of structural variants in grapevine domestication. *Nature Plants* 5:965–79
78. Pelsy F, Dumas V, Bévilaqua L, Hocquigny S, Merdinoglu D. 2015. Chromosome replacement and deletion lead to clonal polymorphism of berry color in grapevine. *PLoS Genetics* 11:e1005081
79. Carbonell-Bejerano P, Royo C, Torres-Pérez R, Grimplet J, Fernandez L, et al. 2017. Catastrophic unbalanced genome rearrangements cause somatic loss of berry color in grapevine. *Plant Physiology* 175:786–801
80. Sunitha S, Rock CD. 2020. CRISPR/Cas9-mediated targeted mutagenesis of *TAS4* and *MYBA7* loci in grapevine rootstock 101-14. *Transgenic Research* 29:355–67
81. Nakajima I, Ban Y, Azuma A, Onoue N, Moriguchi T, et al. 2017. CRISPR/Cas9-mediated targeted mutagenesis in grape. *PLoS One* 12:e0177966
82. Ren C, Guo Y, Kong J, Lecourieux F, Dai Z, et al. 2020. Knockout of *VvCCD8* gene in grapevine affects shoot branching. *BMC Plant Biology* 20:47
83. Wang X, Tu M, Wang D, Liu J, Li Y, et al. 2018. CRISPR/Cas9-mediated efficient targeted mutagenesis in grape in the first generation. *Plant Biotechnology Journal* 16:844–55
84. Wan D, Guo Y, Cheng Y, Hu Y, Xiao S, et al. 2020. CRISPR/Cas9-mediated mutagenesis of *VvMLO3* results in enhanced resistance to powdery mildew in grapevine (*Vitis vinifera*). *Horticulture Research* 7:116
85. Li M, Jiao Y, Wang Y, Zhang N, Wang B, et al. 2020. CRISPR/Cas9-mediated *VvPR4b* editing decreases downy mildew resistance in grapevine (*Vitis vinifera* L.). *Horticulture Research* 7:149
86. Li Y, Mansour H, Wang T, Poojari S, Li F. 2019. Naked-eye detection of grapevine red-blotch viral infection using a plasmonic CRISPR Cas12a assay. *Analytical Chemistry* 91:11510–13
87. Osakabe Y, Liang Z, Ren C, Nishitani C, Osakabe K, et al. 2018. CRISPR-Cas9-mediated genome editing in apple and grapevine. *Nature Protocols* 13:2844–63
88. Wang Y, Liu X, Ren C, Zhong G, Yang L, et al. 2016. Identification of genomic sites for CRISPR/Cas9-based genome editing in the *Vitis vinifera* genome. *BMC Plant Biology* 16:96
89. Ren F, Ren C, Zhang Z, Duan W, Lecourieux D, et al. 2019. Efficiency optimization of CRISPR/Cas9-mediated targeted mutagenesis in grape. *Frontiers in Plant Science* 10:612
90. Ren C, Liu Y, Guo Y, Duan W, Fan P, et al. 2021. Optimizing the CRISPR/Cas9 system for genome editing in grape by using grape promoters. *Horticulture Research* 8:52
91. Xing H, Dong L, Wang Z, Zhang H, Han C, et al. 2014. A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biology* 14:327
92. Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, et al. 2015. A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. *Molecular Plant* 8:1274–84
93. Tang X, Ren Q, Yang L, Bao Y, Zhong Z, et al. 2019. Single transcript unit CRISPR 2.0 systems for robust Cas9 and Cas12a mediated plant genome editing. *Plant Biotechnology Journal* 17:1431–45
94. Tang X, Zheng X, Qi Y, Zhang D, Cheng Y, et al. 2016. A single transcript CRISPR-Cas9 system for efficient genome editing in plants. *Molecular Plant* 9:1088–91
95. Wang X, Tu M, Wang Y, Yin W, Zhang Y, et al. 2021. Whole-genome sequencing reveals rare off-target mutations in CRISPR/Cas9-edited grapevine. *Horticulture Research* 8:114
96. Iocco P, Franks T, Thomas MR. 2001. Genetic transformation of major wine grape cultivars of *Vitis vinifera* L. *Transgenic Research* 10:105–12
97. Lv Z, Jiang R, Chen J, Chen W. 2020. Nanoparticle-mediated gene transformation strategies for plant genetic engineering. *The Plant Journal* 104:880–91
98. Chen L, Li W, Katin-Grazzini L, Ding J, Gu X, et al. 2018. A method for the production and expedient screening of CRISPR/Cas9-mediated non-transgenic mutant plants. *Horticulture Research* 5:13
99. Maher MF, Nasti RA, Vollbrecht M, Starker CG, Clark MD, et al. 2020. Plant gene editing through de novo induction of meristems. *Nature Biotechnology* 38:84–89
100. Ren C, Guo Y, Gathunga EK, Duan W, Li S, et al. 2019. Recovery of the non-functional EGFP-assisted identification of mutants generated by CRISPR/Cas9. *Plant Cell Reports* 38:1541–49



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