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CRISPR/Cas9-mediated efficient targeted mutagenesis in Chardonnay (*Vitis vinifera* L.)

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The type II clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 system (CRISPR/Cas9) has been successfully applied to edit target genes in multiple plant species. However, it remains unknown whether this system can be used for genome editing in grape. In this study, we described genome editing and targeted gene mutation in 'Chardonnay' suspension cells and plants via the CRISPR/Cas9 system. Two single guide RNAs (sgRNAs) were designed to target distinct sites of the L-idonate dehydrogenase gene (*IdnDH*). CEL I endonuclease assay and sequencing results revealed the expected indel mutations at the target site, and a mutation frequency of 100% was observed in the transgenic cell mass (CM) as well as corresponding regenerated plants with expression of sgRNA1/Cas9. The majority of the detected mutations in transgenic CM were 1-bp insertions, followed by 1- to 3-nucleotide deletions. Off-target activities were also evaluated by sequencing the potential off-target sites, and no obvious off-target events were detected. Our results demonstrated that the CRISPR/Cas9 system is an efficient and specific tool for precise genome editing in grape.

Targeted genome editing (TGE) using site-specific nucleases (SSNs) promises to be critical to basic and applied biology research. These powerful SSN tools, comprising zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system (CRISPR/Cas9), introduce targeted DNA double-strand breaks (DSBs) and subsequently trigger DNA repair pathways involving either non-homologous end-joining (NHEJ) or homologous recombination (HR)¹. Both ZFNs and TALENs, which consist of customizable ZF/TALE DNA-binding domains (DBDs) with *FokI* cleavage domains, have been proven successful in targeted genome modification^{2–4}. However, disadvantages such as the time required for designing the constructs make these two methods difficult and expensive to use^{5,6}.

Recently, the RNA-guided CRISPR/Cas9 system has been widely used in TGE. There are three types of CRISPR/Cas9 systems (I, II and III), and the type II system with a single Cas9 protein has emerged as a simple but highly efficient tool for TGE^{7,8}. The Cas9 nuclease is guided by a duplex formed by CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) which cleaves the complementary sequence of the crRNA accompanied by an NGG protospacer adjacent motif (PAM)^{9,10}. The PAM is indispensable for target recognition as it helps the crRNA-tracrRNA-Cas9 complex combine with the target sequence^{11,12}. In an engineered CRISPR/Cas9 system, the crRNA-tracrRNA duplex has been fused into an artificial single guide RNA (sgRNA) containing a 20-nucleotide (nt) sequence that determines the DNA target site^{7,10,13}. To date, the CRISPR/Cas9 system has been adopted for TGE successfully in many organisms, including mammalian cells^{10,13}, yeast¹⁴, zebrafish embryos¹⁵, mice¹⁶, and numerous herbaceous plants such as *Arabidopsis*^{17–19}, tobacco^{19,20}, rice^{17,21,22}, wheat²³, maize⁶, sorghum²³, soybean²⁴, and tomato^{25,26}. This genome modification is heritable, and the sequence changes in the first generation of stable herbaceous plant transformants persist into the next generation^{27,28}. In addition, the transient expression of Cas9 and sgRNA in rice and wheat protoplasts also successfully directs genome modification²⁹. Nevertheless, the CRISPR/Cas9 system has rarely been reported to effectively work in perennial plants except for poplar and citrus^{30,31}.

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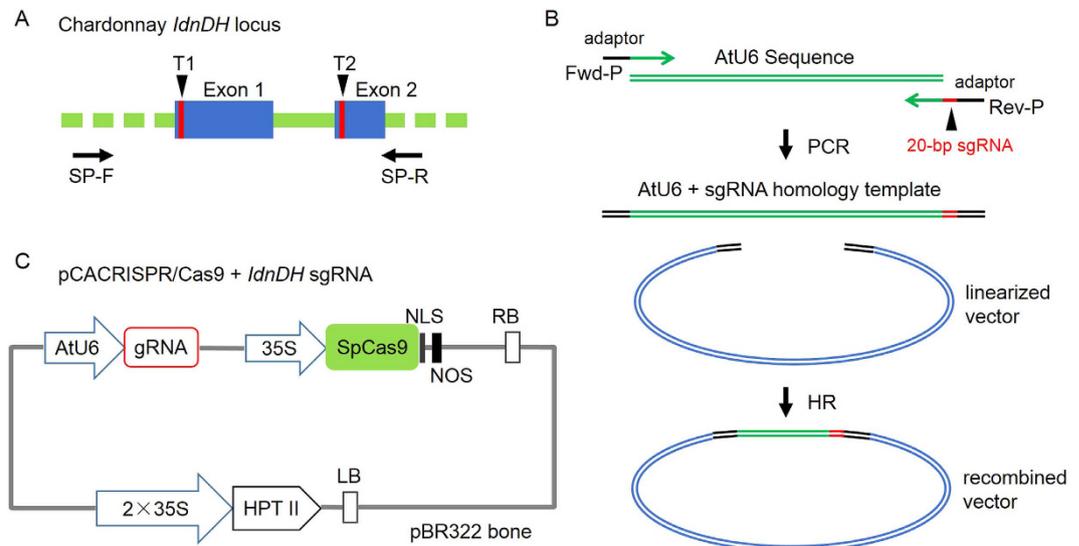


Figure 1. Selection of target sites in the *IdnDH* gene and construction of Cas9/sgRNA expression vector. (A) Schematic illustrating the target sites in the *IdnDH* coding sequence. Blue boxes indicate exons and green lines denote introns. T1 and T2 are selected target sites. SP-F and SP-R are primers used for PCR amplification. (B) Construction process of Cas9/sgRNA expression vector. (C) Schematic diagram of the assembled Cas9/sgRNA expression vector for stable transformation. The vector used in this study was named pCACRISPR/Cas9.

As one of the most important fruit crops worldwide, grape has tremendous economic value. Since the whole-genome sequence of grape (*Vitis vinifera* L.) was released in 2007³², tremendous gene resources were available for functional studies in *Vitis*. Therefore, a useful and efficient system is crucial for gene functional studies as well as genetic engineering in grape. Ectopic manipulation of gene expression either via overexpression or knock-out is a very useful strategy for gene functional studies³³. Random insertional mutagenesis that proved useful in model plants remains difficult for grape due to its biological characteristics³⁴. As a rapid and efficient tool to generate targeted mutations, the CRISPR/Cas9 system is becoming the primary tool for gene editing in plants. However, there is no report on the efficacy of CRISPR/Cas9 in grape to date, so it is necessary to determine if it can be a useful tool in *Vitis*.

Here we report on targeted gene editing in grape via the CRISPR/Cas9 system by introduction of a single plasmid with a specific sgRNA into grape cells. Though *Agrobacterium*-mediated transformation of grapevine has been reported previously³⁵, the efficiency and reliability of this technology is presently low, and most grape cultivars are recalcitrant for plant regeneration^{34,36,37}. Additionally, somatic mutations frequently occur during the tissue culture-based transformation and many abnormal plantlets may be generated from somatic embryos^{38,39}. Therefore, it is difficult and time-consuming to obtain enough grapevines for our studies, so ‘Chardonnay’ suspension cells were adopted as our main experimental material, and the L-idonate dehydrogenase gene (*IdnDH*, LOC100232980), which controls the biosynthesis of tartaric acid (TA) in grape⁴⁰, was chosen as the target gene to be disrupted site-specifically in suspension cells of ‘Chardonnay’. Knocking out or silencing the gene resulted in failure of TA biosynthesis or accumulation in suspension cells. Mutagenesis in a transgenic cell mass (CM) and resulting grape plants was detected by CEL I endonuclease assays and Sanger sequencing, respectively. The content of TA in the transgenic CM was also measured to determine the effect of mutagenesis. The expression levels of *Cas9* and sgRNAs were measured by quantitative real-time PCR (qRT-PCR). Furthermore, off-target events were evaluated. Taken together, our data demonstrated that the CRISPR/Cas9 system could efficiently induce targeted mutations in ‘Chardonnay’ suspension cells as well as its regenerated plants, indicating the feasibility of this system as a powerful tool for genome modification in grape.

Results

sgRNA design and CRISPR/Cas9 expression vector construction. The L-idonate dehydrogenase gene (*IdnDH*) was selected as the target of Cas9 nuclease. An approximately 1-kb DNA fragment of *IdnDH* was cloned and sequenced, and the result showed that the sequence of *IdnDH* in ‘Chardonnay’ was identical to that of its homologous gene in ‘Pinot Noir’ PN40024 (data not shown). Two 20-bp sequences with tandem guanosine nucleotides (PAM) on their 3’-regions in the *IdnDH* sequence were chosen as sgRNA complementary sites, including one in the first exon and the other in the second exon (Fig. 1A). The construction process of the pCACRISPR/Cas9 binary vectors is shown in Fig. 1B. In brief, the *Arabidopsis* U6 promoter (AtU6) and sgRNA were first combined by PCR, and then the sgRNA expression cassette with adaptors was inserted into the linearized vector by the HR method. Finally, two binary vectors were constructed, where the expression of Cas9 and sgRNA was driven by the CaMV 35S promoter and AtU6, respectively (Fig. 1C).

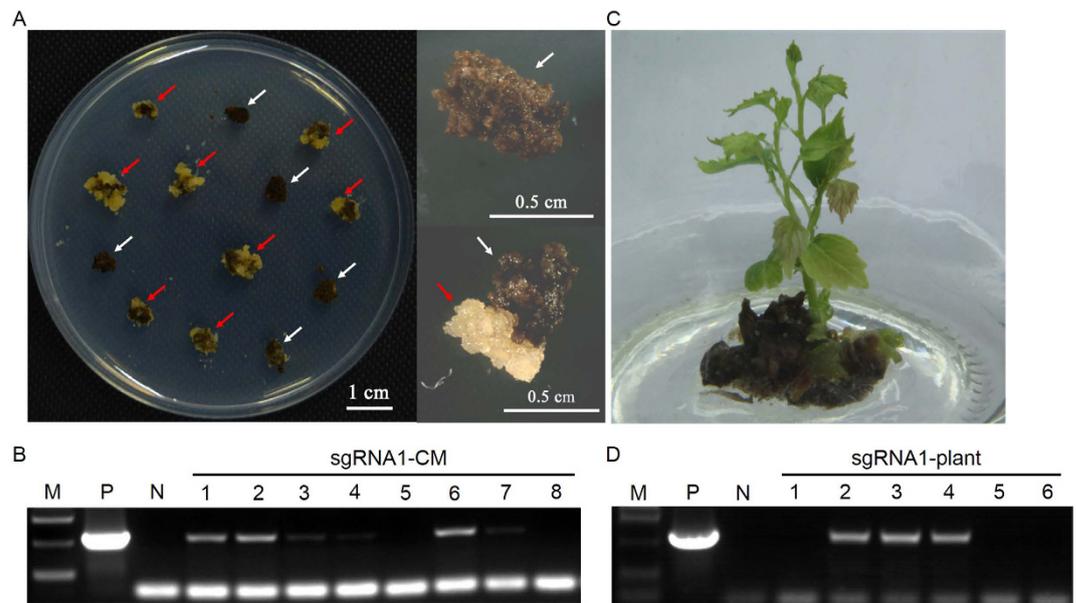


Figure 2. Screening and identification of positive transgenic cell mass (CM) and plantlets. (A) Yellowish resistant CMs generated on selective medium. (B) Identification of exogenous T-DNA insertion in sgRNA1-CMs. Genomic DNA of sgRNA1-CMs was extracted and used as templates for PCR with specific primers for the hygromycin resistant gene. The plasmid of the constructed vector and wild-type DNA were used as a positive control (P) and a negative control (N), respectively. Lanes 1–8 represent different sgRNA1-CMs. (C) Grape plantlets regenerated from transgenic CMs. (D) Identification of exogenous T-DNA insertion in sgRNA1-plants. Genomic DNA was extracted from plants and used as templates for PCR with specific primers for the hygromycin resistant gene. The plasmid of the constructed vector and wild-type DNA were used as a positive control (P) and a negative control (N), respectively. Lanes 1–6 represent different individual sgRNA1-plants.

Screening and identification of positive transgenic CM and plants. After *A. tumefaciens*-mediated transformation and screening on selective medium, yellowish resistant CMs were generated, whereas non-resistant CMs turned brown and were unable to proliferate (Fig. 2A). A total of 62, 58 and 15 independent resistant CMs in empty vector (without 20-bp sgRNA sequence, EV-*IdnDH*), sgRNA1-*IdnDH* and sgRNA2-*IdnDH* vectors, respectively (Table 1). Identification of these CMs by PCR using specific primers for the hygromycin resistant gene (Supplementary Table S2) revealed that 27, 21, and 3 of the examined CMs contained exogenous T-DNA insertions, respectively (Fig. 2B, Table 1). The transgenic CMs identified were subsequently transferred into liquid CSM medium with 10 mg/L hygromycin for rapid propagation. Some of the cells were transferred onto regeneration medium to generate transgenic plants. Finally, 4, 6 and 0 independent normal shoots were generated in EV-*IdnDH*, sgRNA1-*IdnDH* and sgRNA2-*IdnDH*, respectively (Fig. 2C, Table 1). Among these shoots, 25% (1 out of 4) of the EV-*IdnDH* plantlets (EV-plant) and 50% (3 out of 6) of the sgRNA1-*IdnDH* plantlets (sgRNA1-plant) were identified with exogenous T-DNA insertions (Fig. 2D, Table 1).

Detection of CRISPR/Cas9-mediated mutagenesis in the *IdnDH*. Mutations in positive CMs were first detected by using the CEL I endonuclease assay. The DNA fragments containing target sites from transgenic CMs of sgRNA1-*IdnDH* (sgRNA1-CM) were digested with CEL I enzyme and two different fragments were produced, whereas the DNA fragments from wild-type (WT) as well as EV-CMs were not digested with CEL I (Fig. 3A). Similar results were also obtained with CEL I endonuclease assays of DNA fragments from regenerated shoots (Fig. 3A). These results suggested that the DNA fragments from sgRNA1-CM and sgRNA1-plant had mutations, and those from EV-CM and EV-plant might be wild-type. Notably, the presence or absence of WT DNA made no difference in the results, indicating that these CMs may be mosaics (100%, 21/21) and the harvested plants might be heterozygotes or chimeras (100%, 3/3).

To accurately verify the results, the modification of DNA fragments was detected by sequence analysis of 10 EV-CMs, 10 sgRNA1-CMs and 3 sgRNA2-CMs, respectively. As the CM was chimeric, more than 12 clones of PCR amplicons from each sample were sequenced. The results showed that fragments from sgRNA1-CMs contained indel mutations at the target site (Fig. 3B). All the examined sgRNA1-CMs contained deletion or insertion mutations at the target site, with a high mutation rate of 100% (10/10). The high mutation rate was partially due to the fact that the CRISPR/Cas9-induced editing events mainly take place in the transformed cells⁴¹. Most mutations (15.83%, 19 out of 120 clones) were 1-bp (T) insertions, which was consistent with previous reports in *Arabidopsis* and rice^{27,28}, followed by short nucleotide deletions (≤ 3 bp) (Fig. 3B). Only 0.83% (1/120) of the mutations exhibited >20 -bp deletions. In addition, there were mutations of 11-bp and 14-bp deletions (Fig. 3B). Most indel mutations in sgRNA1-CMs led to frame shifts of the open reading frame (ORF) and the majority of encoded amino acid were altered (Fig. 3C). Intriguingly, among the 12 kinds of mutation-containing sequences, 4 (33.33%, 4/12) exhibited stop codons on the 5'-region of their mRNA sequences (Fig. 3C) which would result

Vector	No. of obtained CMs	No. of CMs with T-DNA	No. of examined CMs	No. of CMs with mutation	Mutation rate in CMs (%)	No. of regenerated plants	No. of plants with T-DNA	No. of plants with mutation	Mutation rate in plants (%)
AtU6-(None)-CaMV35S-Cas9	62	27	10	0	0.0	4	1	0	0.0
AtU6-sgRNA1-CaMV35S-Cas9	58	21	10	10	100.0	6	3	3	100.0
AtU6-sgRNA2-CaMV35S-Cas9	15	3	3	1	33.3	0	0	0	0.0

Table 1. Number and percentage of examined transgenic CMs and plants with exogenous T-DNA and mutations.

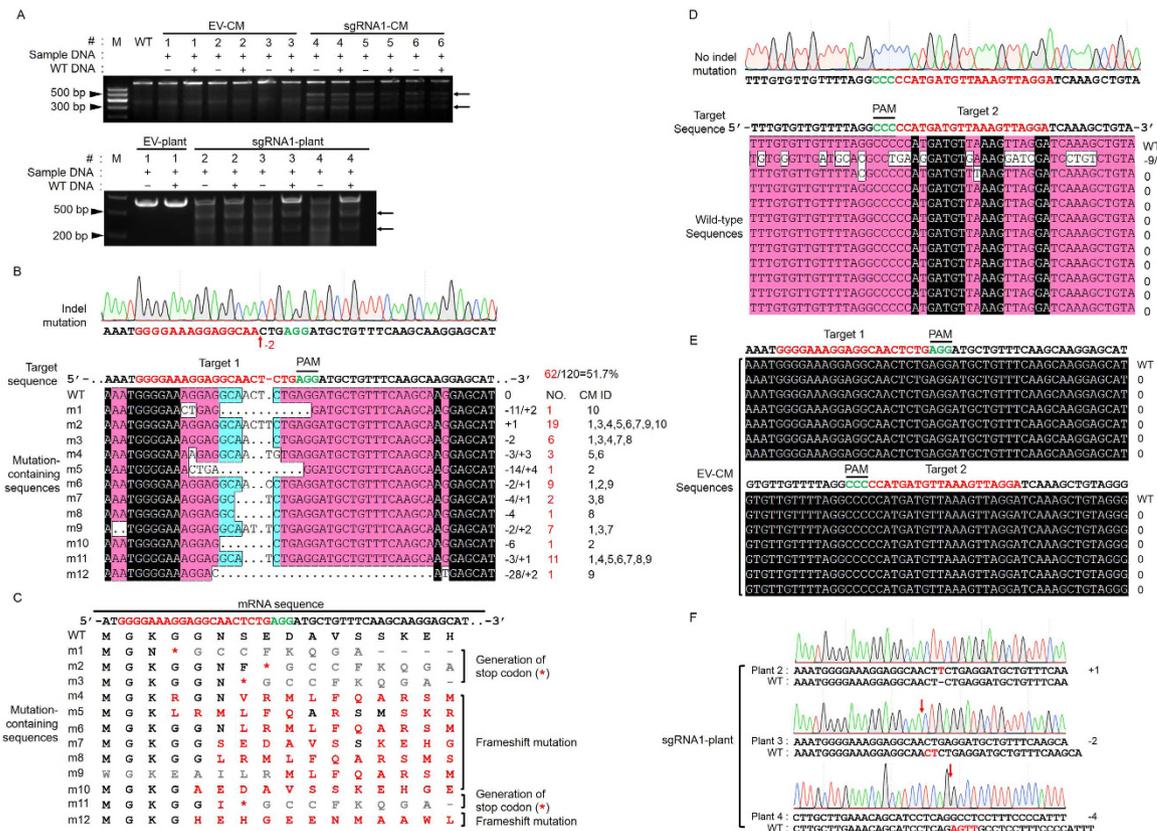


Figure 3. Analysis of induced mutations in target sequence. (A) Detection of target mutations by CEL I endonuclease assay. The target fragments were amplified by PCR from genomic DNA of wild-type (WT), EV-*IdnDH* and sgRNA1-*IdnDH*, and then digested with CEL I endonuclease. Arrows indicate the digested fragments by CEL I. +: PCR products were added. -: no PCR products were added. Lanes 1–6 represent different samples. (B) An example chromatogram showing a microdeletion, as well as representative sequences with indel mutations identified from 120 clonal amplicons of sgRNA1-CMs. The homologous nucleotides are shaded and different colors indicate different homology levels. The nucleotides with a homology level of 100% are shaded in black and those with a homology level of $\geq 75\%$ and $\geq 50\%$ are shaded in red and blue, respectively. Red numbers on the right indicate the number of detected clones with the same mutation type. (C) Mutations of amino acids in corresponding mutated sequences in (B). (D) An example chromatogram and representative sequences identified from 120 clonal amplicons of sgRNA2-CMs. (E) The sequencing results of 36 clonal amplicons of EV-CMs. (F) The chromatograms showing the indel mutations in sgRNA1-plants.

in premature translation termination. However, most DNA fragments from sgRNA2-CMs showed no changes at the target site (Fig. 3D), except that one fragment from sgRNA2-CM 3 contained multiple nucleotide mutations within the 20-bp target and adjoining sequence, which was different from typical mutations induced by CRISPR/Cas9^{26–28}. As expected, no mutations were detected in DNA fragments from EV-CMs (Fig. 3E), suggesting the indispensable role of 20-bp sgRNA sequence in TGE mediated by Cas9/sgrNA. The modification of the targeted sequence in sgRNA1-plants was investigated as well. The DNA fragments from three independent plantlets (plants 2, 3 and 4), which were regenerated from sgRNA1-CM 1, sgRNA1-CM 4 and sgRNA1-CM 8, respectively, contained a 1-bp insertion, or 2-bp and 4-bp deletions at the target site, respectively (Fig. 3F). The results were consistent with that in sgRNA1-CMs (Fig. 3B).

Samples	CM ID	Content of TA (mg/g)	Range of values	P-value
EC-1	1	91.73 ± 3.98	87.89–97.21	ND
EC-2	3	90.35 ± 5.78	88.36–95.95	0.70
sgRNA1-1	1	83.53 ± 2.08*	81.52–85.67	0.02
sgRNA1-2	2	87.96 ± 2.97	84.60–90.25	0.23
sgRNA1-3	4	82.73 ± 1.96*	80.82–84.73	0.02
sgRNA1-4	9	58.67 ± 1.17**	57.35–59.75	0.00

Table 2. Determination of TA in EV- and sgRNA1-CM. The content of TA was analyzed by HPLC and the data obtained from at least three replicates are presented as mean values ± SD. A P-value was determined by Student's *t*-test. Asterisks indicate statistically significant differences between EC-1 and sgRNA1-CM. *P < 0.05 and **P < 0.01. ND, not determined.

sgRNA	Putative off-target site	Putative off-target sequence	Putative off-target locus	Putative off-target region	No. of examined CMs	No. of CMs with mutations
sgRNA1	1	GGAAAAATGAGGGAAGCTCTGTGG	chr3_random: +1026707	Intergenic	10	0
	2	GGGGAAACTCTGCAACTCTGAAG	chr13_random: -2503776	Intergenic	10	0
sgRNA2	1	TTAAAAATTTTAACATCATGGAGG	chr16: -2516563	Intergenic	3	0
	2	TCAGGACTCTAACATCATGGAGG	chr1: -20476929	Intergenic	3	0

Table 3. Analysis of mutation in potential off-target sites. PAM sequence (NGG and the analogue NAG) is indicated in green. Mismatched nucleotides are marked in red.

Measurement of TA contents in transgenic CM. The TA content of transgenic CMs was measured to evaluate the effect of mutagenesis in *IdnDH*. The TA contents in EV-CM (EC) exhibited no significant difference. Among the four sgRNA1-CMs, three exhibited significant differences in TA content compared to that of EC-1 (Table 2). Actually, indels of -3/+3 bp or -4/+1 bp may not lead to frameshifts of the ORF, consequently resulting in no defect in phenotype. More significantly, the presence of wild-type cells or cells without mutations in the mosaic CM may affect the determination of TA content. In short, the results suggested that the N' terminus of *IdnDH* was an effective target site and frameshift mutations of *IdnDH* were likely to disrupt its expression.

Expression analysis of Cas9 and sgRNAs. qRT-PCR was conducted to investigate the expression profiles of *Cas9* and sgRNA. There was not much difference in expression levels of *Cas9* driven by the 35S promoter between EV-CMs and sgRNA1-CMs. However, the expression levels of sgRNA1 driven by the AtU6 promoter in sgRNA1-CMs were highly increased (Supplementary Figure S1). Both *Cas9* and sgRNA2 in sgRNA2-CMs exhibited lower expression abundance compared to that of EC-1, with the exception being sgRNA2-1, where the transcript level of sgRNA2 was about 4.7-fold that of EC-1 (Supplementary Figure S1). However, the three sgRNA1 plantlets had higher expression levels of *Cas9* and sgRNA1 compared to that of EV-plant (Supplementary Figure S1). These results suggested that the abundance of sgRNA as well as *Cas9* was significant in triggering genome editing.

Off-target analysis. In two sgRNA-*IdnDH* constructs, only the sgRNA1-*IdnDH* construct successfully generated mutations at the target site (Fig. 3). To detect off-target events in transgenic CMs, potential off-target sites predicted with the online tool CRISPR-P were selected for further study (Table 3). Genomic DNA extracted from those CMs (10 sgRNA1-CMs and 3 sgRNA2-CMs) adopted for sequence analysis was used as a template to amplify DNA fragments containing the putative off-target sites by PCR with specific primers (Supplementary Table S2). At least 10 clones of PCR amplicons from each CM were sequenced. Apart from variation of some nucleotides, no mutation was found at the putative off-target sites in the tested transgenic CMs (Table 3 and Supplementary Figure S2). The results indicated that the mutagenesis induced by CRISPR/Cas9 system in grape was highly specific.

Discussion

As a simple but efficient site-directed genome editing technology, CRISPR/Cas9 system has been widely used for genome editing in multiple organism^{4,6,8,15}. However, the efficiency and application of this system in grape (grape cells or grapevines) has not been reported to date. Like most woody plants, grape is classically improved through vegetative methods or conventional breeding. Application of any gene technology to grape requires an efficient transformation system that can be applied to a wide range of cultivars⁴². Despite significant progress in genetic engineering based on *Agrobacterium*-mediated transformation, some cultivars of *Vitis vinifera* are still recalcitrant to transformation³⁷. Furthermore, efficiency and reliability of this technology has been low, and many factors such as *Agrobacterium* strain, culture medium and grape genotype can play an important role in transformation efficiency^{34,37}. Moreover, it takes a relatively long time (over a 32 week period) to obtain transgenic

embryos from a small amount of embryogenic callus, during which somatic mutations frequently occur and abnormal plantlets are usually generated^{36,38,39,42}. Hence, a stable and efficient transformation and regeneration method is needed for application of the CRISPR/Cas9 system in grape.

In this study, the gene *IdnDH*, which regulates the stable accumulation of TA in grape cells, was selected as the target gene to verify the efficacy of the CRISPR/Cas9 system in 'Chardonnay'. Two different sgRNA expression cassettes were inserted into the binary pCACRISPR/Cas9 vector via HR, and were then used to transform 'Chardonnay' suspension cells by infection with *A. tumefaciens*. Identification of exogenous T-DNA insertion by PCR in the CMs obtained revealed an average transformation rate of 37.78% (Table 1). CEL I endonuclease assays and Sanger sequencing showed that the sgRNA1 had successfully directed the target-editing in transgenic CMs and plants (Fig. 3A,B,F), whereas the sgRNA2 did not result in indel mutation at the target site (Fig. 3D). However, there was one piece of sequence from sgRNA2-CM 3 containing several nucleotide mutations (Fig. 3D). Interestingly, most nucleotides of the 12-nucleotide 'seed sequence' located at the target site and adjoining the PAM were conserved, and there was no deletion occurred at the 4th base of the PAM, which have been regarded as one of the typical mutations induced by CRISPR/Cas9 system^{27,28}. Importantly, it cannot be ruled out that these nucleotide mutations might be introduced by the DNA polymerase during PCR amplification. Actually, the greatest likelihood is that sgRNA2 was inactive for on-target editing⁴³. Thus, the sgRNA2 failed to induce site-directed cleavage in grape cells. In contrast, a high mutation rate of 100% was detected in the sgRNA1-CMs tested (Fig. 3B, Table 1). In three sgRNA1 plantlets, insertion and deletion mutations were detected at the target site (Fig. 3B). Previous reports have shown that the GC content of target sequences may influence editing efficiency in plants, and a higher GC content (>50%) could generate high editing efficiency while a lower GC content (≤40%) results in low editing efficiency^{26,38,44}. In our study, the GC content of sgRNA1 and sgRNA2 was 60.0% and 35.0%, respectively (Supplementary Table S1). Thus, the high GC content of sgRNA1 may contribute to the high mutation rate, and the failure of sgRNA2 to edit might be due to the low GC content. In addition, CRISPR/Cas9-induced editing efficiency in plants may also be affected by expression levels of *Cas9* and sgRNA^{26,38,45}. The results of expression analyses showed that the expression level of *Cas9* increased in sgRNA1-CMs and sgRNA1-plants, but was down-regulated in sgRNA2-CMs (Supplementary Figure S1). In rice and *Arabidopsis*, transgenic plants with no targeted mutations generally have very low expression levels of sgRNA³⁸. The transcript level of sgRNA1 was extremely high, and the signal was over 2000-fold that of EC-1 (Supplementary Figure S1). However, the transcript abundance of sgRNA2 was lower than that of EC-1, except that sgRNA2-1 had a higher level of sgRNA but a lower expression level of *Cas9* (Supplementary Figure S1). These results suggested that the GC content and the abundance of sgRNA and *Cas9* were significant limiting factors for genome editing.

Off-target mutations are the main concern in the application of the CRISPR/Cas9 system. The probability of off-target events induced by the CRISPR/Cas9 system varies among different organisms^{46,47}. Generally, the probability of off-target mutations is low in plants^{28,48,49}. In our study, no off-target events were observed in the tested putative off-target sites by sequence assays (Supplementary Figure S2), suggesting the high specificity of the CRISPR/Cas9 system in grape. There are several factors affecting the specificity of RNA-guided genome editing. The selection of the target sequence is vital for the specificity and efficacy of the CRISPR/Cas9 system. As mentioned above, target sequences with a high GC content (50–70%) has relatively high editing efficiency³⁸, yet a high GC content may potentially lead to the risk of off-targeting⁴⁴. Furthermore, the secondary structure of sgRNAs and local chromatin structure may affect the specificity of CRISPR/Cas9 system^{8,38,50}. The specificity of Cas9 also depends upon the relative abundance of effective Cas9-sgRNA complexes with respect to the effective target concentration⁴⁴. As the PAM and 'seed sequence' are necessary and significant for the specific binding of Cas9, it is the most effective strategy to determine a highly specific target sequence with the help of online tools⁵¹. In addition, the use of Cas9 nickase, which consists of an inactivating mutation in the endonuclease cleavage domains and produces single-strand breaks, could reduce off-target mutations^{7,52,53}. The newly found CRISPR/Cpf1 system can recognize the T- and T/C-dependent PAMs and produce a staggered DSB to activate the DNA repair process, through which the efficiency and specificity of the system could be improved^{54,55}.

In this study, the CRISPR/Cas9 system was used to successfully generate TGE for *IdnDH* in 'Chardonnay' suspension cells and regenerated plantlets. The contents of TA, as well as the expression levels of *Cas9* and sgRNAs, were measured to verify the results of TGE. No off-target mutations were detected in the tested putative off-target sites, suggesting a specificity of the CRISPR/Cas9 system in grape. In short, our study proves that the CRISPR/Cas9 system is a promising and useful tool for investigating the function of candidate genes and conducting genetic improvement in grape.

Methods

Cloning of *IdnDH* gene. The primers (Supplementary Table S2) used for amplification of genomic DNA fragments of *IdnDH* in 'Chardonnay' were designed based on its homologous gene (LOC100232980) sequence in 'Pinot Noir'. The PCR reaction was carried out with High-Fidelity DNA polymerase KOD-plus Neo (TOYOBO, Japan) in a total volume of 50 μ L at 95 °C for 5 min; 34 cycles of 95 °C for 15 s, 60 °C for 25 s and 68 °C for 30 s; followed by a final extension at 68 °C for 10 min. The PCR product was cloned into pLB-Simple vector (TIANGEN, China) and around 5 clones were sequenced.

Design of sgRNA and assembly of CRISPR/Cas9 construct. The verified sequence of *IdnDH* was input into the online tool Grape-CRISPR⁵⁶ (<http://biodb.sdau.edu.cn/gc/>) and CRISPR-P⁵⁷ (<http://cbl.hzau.edu.cn/cgi-bin/CRISPR#>) to find potential Cas9 target sites, and the output target sites were selected for designing target sgRNAs based on their location in the gene and off-target possibilities. The backbone of the pCACRISPR/Cas9 vector carrying plant-optimized *Streptococcus pyogenes* Cas9 protein coding gene was derived from the pPIC4 vector (Genloci, China). For constructing the AtU6-sgRNA cassette, the 20-bp sgRNA sequence as well

as an adaptor were synthesized as reverse primers for amplification of AtU6 promoter fragments. The amplified AtU6-sgRNA fragments containing adaptors could be inserted into the homologous sites of the linearized pCAC-RISPR/Cas9 binary vector via HR.

Growth, transformation of Chardonnay suspension cells and regeneration of plants. The anthers of ‘Chardonnay’ at the mid-late uninucleate stage were placed on induction medium (MS basal medium supplemented with 2 mg/L 6-benzyladenine [6-BA], 1 mg/L 2,4-dichlorophenoxyacetic acid [2,4-D], 60 g/L sucrose, 3 g/L phytigel, pH 5.8) to induce embryogenic callus. The suspension cells, derived from induced embryogenic callus, were cultured in 250 mL flasks fitted with 50 mL of liquid CSM medium (MS basal medium supplemented with 0.5 g/L glutamic acid, 1 mg/L 2-naphthoxyacetic acid [NOA], 5.0 mL/L glycerol, 20 g/L maltose, pH 5.8), and shaken at 117 rpm at $26 \pm 1^\circ\text{C}$ in the dark. All the suspension cells were sub-cultured every 7 days. The final binary vectors were introduced into the *Agrobacterium* strain EHA105 by the freeze-thaw method. For *A. tumefaciens*-mediated transformation, the cultures of suspension cells were collected by short centrifugation, and then inoculated with a prepared *A. tumefaciens* suspension. The explants were transferred onto selective medium (MS basal medium supplemented with 1 mg/L 2,4-D, 2 mg/L 6-BA, 1 mg/L NOA, 3 g/L phytigel, pH 5.8) with 10 mg/L hygromycin until resistant CMs were generated. These resistant CMs were subsequently transferred into liquid CSM medium with 10 mg/L hygromycin for rapid propagation. Some of sub-cultured cells were transferred onto regeneration medium (MS basal medium supplemented with 0.2 mg/L kinetin, 0.1 mg/L NOA, 30 g/L sucrose, 3 g/L phytigel, pH 5.8) to generate plants.

Extraction of genomic DNA. Genomic DNA was extracted from WT and resistant CM as well as plants using the typical CTAB method as previously described³⁰.

Identification of exogenous T-DNA insertion by PCR. The specific primers for the hygromycin resistant gene (Supplementary Table S2) were used for PCR. The PCR product was separated on an ethidium bromide-stained agarose gel (1.0%) and then recovered and cloned into the pLB-Simple vector for Sanger sequencing.

CEL I endonuclease assay and sequencing analysis. The prepared genomic DNA was used as a template to amplify genomic fragments containing the target sites. The sequences of specific primers (SP) used for PCR were identical to that of cloning primers. For CEL I assay, the PCR products were digested with the Surveyor Mutation Detection Kit (Transgenomic, USA) according to the manufacturer’s instruction. The PCR products were further verified by Sanger sequencing.

Off-target analysis. The potential off-target sites of the target sequence were predicted simultaneously during the process of designing sgRNAs with the online tool of CRISPR-P. Two top-ranking potential off-target sites of each sgRNA were selected for off-target analysis, respectively. The genomic DNA fragments containing the potential off-target sites were amplified using specific primers (Supplementary Table 2) and then analyzed by Sanger sequencing.

Quantitative RT-PCR analysis. Total RNA was extracted from ‘Chardonnay’ CM and regenerated plants using TRIzol reagent (Invitrogen, USA). The first cDNA was synthesized with 1 μg of RNA using the HiScript Q RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme, China) according to the manufacturer’s instruction. qRT-PCR was performed in a volume of 20 μL on a CFX96 Real-Time System (Bio-Rad, USA) with *Cas9* and sgRNAs specific primers (Table S2). The grape *Actin1* (accession number AY680701) was used as internal control. The relative expression level was calculated using the $2^{-\Delta\Delta\text{CT}}$ method⁵⁸.

Determination of TA by HPLC. To measure the content of TA, 1 g fresh weight of CM powder was homogenized with 6 mL ultrapure water and incubated for 2 h, then centrifuged at $20,000 \times g$ for 10 min. A 2 mL volume of supernatant was removed and passed through a C18 extraction column (Waters, Milford, MA) and 0.22 μm filter, and organic acids were separated by HPLC. The mobile phase was 20 mM KH_2PO_4 adjusted to pH 2.4 with phosphoric acid at a flow rate of 0.8 mL/min.

References

1. Symington, L. S. & Gautier, J. Double-strand break end resection and repair pathway choice. *Annu. Rev. Genet.* **45**, 247–271 (2011).
2. Townsend, J. A. *et al.* High-frequency modification of plant genes using engineered zinc-finger nucleases. *Nature* **459**, 442–445 (2009).
3. Shan, Q. *et al.* Rapid and efficient gene modification in rice and Brachypodium using TALENs. *Mol. Plant* **6**, 1365–1368 (2013).
4. Zhang, Y. *et al.* Transcription activator-like effector nucleases enable efficient plant genome engineering. *Plant Physiol.* **161**, 20–27 (2013).
5. DeFrancesco, L. Erratum: Move over ZFNs. *Nat. Biotechnol.* **30**, 112 (2012).
6. Chen, K. L. & Gao, C. Targeted genome modification technologies and their applications in crop improvements. *Plant Cell Rep.* **33**, 575–583 (2014).
7. Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823 (2013).
8. Wu, X. *et al.* Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. *Nat. Biotechnol.* **32**, 670–676 (2014).
9. Gaj, T., Gersbach, C. A. & Barbas, C. R. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol.* **31**, 397–405 (2013).
10. Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 (2012).
11. Nishimasu, H. *et al.* Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* **156**, 935–949 (2014).
12. Sternberg, S. H., Redding, S., Jinek, M., Greene, E. C. & Doudna, J. A. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* **507**, 62–67 (2014).
13. Mali, P. *et al.* RNA-guided human genome engineering via Cas9. *Science* **339**, 823–826 (2013).

14. Wei, C. *et al.* TALEN or Cas9-rapid, efficient and specific choice for genome modifications. *J. Genet. Genomics* **40**, 281–289 (2013).
15. Hwang, W. Y. *et al.* Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotech.* **31**, 227–229 (2013).
16. Yang, H. *et al.* One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas mediated genome engineering. *Cell* **154**, 1370–1379 (2013).
17. Feng, Z. *et al.* Efficient genome editing in plants using a CRISPR/Cas system. *Cell Res.* **23**, 1229–1232 (2013).
18. Mao, Y. *et al.* Application of the CRISPR-Cas system for efficient genome engineering in plants. *Mol. Plant* **6**, 2008–2011 (2013).
19. Li, J. F. *et al.* Multiplex and homologous recombination mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. *Nat. Biotechnol.* **31**, 688–691 (2013).
20. Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J. D. G. & Kamoun, S. Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* **31**, 691–693 (2013).
21. Jiang, W. Z. *et al.* Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. *Nucleic Acids Res.* **41**, e188 (2013).
22. Miao, J. *et al.* Targeted mutagenesis in rice using CRISPR-Cas system. *Cell Res.* **23**, 1233–1236 (2013).
23. Shan, Q. *et al.* Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat. Biotechnol.* **31**, 686–688 (2013).
24. Jacobs, T. B., LaFayette, P. R., Schmitz, R. J. & Parrott, W. A. Targeted genome modifications in soybean with CRISPR/Cas9. *BMC Biotechnol.* **15**, 16 (2015).
25. Brooks, C., Nekrasov, V., Lippman, Z. B. & VanEck, J. Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 system. *Plant Physiol.* **166**, 1292–1297 (2014).
26. Pan, C. *et al.* CRISPR/Cas9-mediated efficient and heritable targeted mutagenesis in tomato plants in the first and later generations. *Sci. Rep.* **6**, 24765 (2016).
27. Feng, Z. *et al.* Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **111**, 4632–4637 (2014).
28. Zhang, H. *et al.* The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. *Plant Biotechnol. J* **12**, 797–807 (2014).
29. Shan, Q. W., Wang, Y. P., Li, J. & Gao, C. Genome editing in rice and wheat using the CRISPR/Cas system. *Nat. Protoc.* **9**, 2395–2410 (2014).
30. Fan, D. *et al.* Efficient CRISPR/Cas9-mediated targeted mutagenesis in Populus in the first generation. *Sci. Rep.* **5**, 12217 (2015).
31. Jia, H. & Wang, N. Targeted genome editing of sweet orange using Cas9/sgRNA. *PLoS One* **9**, e93806 (2014).
32. Jaillon, O. *et al.* The grapevine genome sequence suggest ancestral hexaploidization in major angiosperm phyla. *Nature* **449**, 463–465 (2007).
33. Ferreira, A. A transgenic perspective on plant functional genomics. *Transgenic Res.* **9**, 245–260 (2000).
34. Vidal, J. R. *et al.* Use of gene transfer technology for functional studies in grapevine. *Aust. J. Grape Wine Res.* **16**, 138–151 (2010).
35. Mullins, M. G., Archie Tang, F. C. & Facciotti, D. Agrobacterium-mediated genetic transformation of grapevine: transgenic plants of *Vitis rupestris* Scheele and buds of *Vitis vinifera* L. *Biotechnology* **8**, 1041–1045 (1990).
36. Harst, M., Bornhoff, B.-A., Zyprian, E. & Topfer, R. Influence of culture technique and genotype on efficiency of Agrobacterium-mediated transformation of somatic embryos (*Vitis vinifera*) and their conversion to transgenic plants. *Vitis* **29**, 99–102 (2000).
37. Torregrosa, L., Iocco, P. & Thomas, M. R. Influence of Agrobacterium strain, culture medium, and cultivars on the transformation efficiency of *Vitis vinifera* L. *Am. J. Enol. Vitic.* **53**, 183–190 (2002).
38. Ma, X. *et al.* A robust CRISPR/Cas9 system for convenient, high-efficient multiplex genome editing in monocot and dicot plants. *Mol. Plant* **8**, 1274–1284 (2015).
39. Dai, L. *et al.* Establishment of a picloram-induced somatic embryogenesis system in *Vitis vinifera* cv. chardonnay and genetic transformation of a stilbene synthase gene from wild-growing *Vitis* species. *Plant Cell Tiss. Organ Cult.* **121**, 397–412 (2015).
40. Debolt, S., Cook, D. R. & Ford, C. M. L-tartaric acid synthesis from vitamin C in higher plants. *Proc. Natl. Acad. Sci. USA* **103**, 5608–5613 (2006).
41. Ma, X., Chen, L., Zhu, Q. & Liu, Y. Rapid decoding of sequence-specific nuclease-induced heterozygous and biallelic mutations by direct sequencing of PCR products. *Mol. Plant* **8**, 1285–1287 (2015).
42. Iocco, P., Franks, T. & Thomas, M. R. Genetic transformation of major wine grape cultivars of *Vitis vinifera* L. *Transgenic Res.* **10**, 105–112 (2001).
43. Doench, J. G. *et al.* Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nat. Biotechnol.* **32**, 1262–1267 (2014).
44. Tsai, S. Q. *et al.* GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat. Biotechnol.* **33**, 187–197 (2015).
45. Osakabe, Y. *et al.* Optimization of CRISPR/Cas9 genome editing to modify abiotic stress responses in plants. *Sci. Rep.* **6**, 26685 (2016).
46. Pattanayak, V. *et al.* High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat. Biotechnol.* **31**, 839–843 (2013).
47. Fu, Y. *et al.* High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* **31**, 822–826 (2013).
48. Sun, X. *et al.* Targeted mutagenesis in soybean using the CRISPR-Cas9 system. *Sci. Rep.* **5**, 10342 (2015).
49. Xu, R. *et al.* Generation of inheritable and “transgene clean” targeted genome-modified rice in later generations using the CRISPR/Cas9 System. *Sci. Rep.* **5**, 11491 (2015).
50. Kescu, C., Arslan, S., Singh, R., Thorpe, J. & Adli, M. Genome-wide analysis reveals characteristics of off-target sites bound by the Cas9 endonuclease. *Nat. Biotechnol.* **32**, 677–683 (2014).
51. Xie, K., Zhang, J. & Yang, Y. Genome-wide prediction of highly specific guide RNA spacers for CRISPR-Cas9-mediated genome editing in model plants and major crops. *Mol. Plant* **7**, 923–926 (2014).
52. Fauser, F., Schiml, S. & Puchta, H. Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in Arabidopsis thaliana. *Plant J* **79**, 348–359 (2014).
53. Mei, Y., Wang, Y., Chen, H., Sun, Z. S. & Ju X. D. Recent Progress in CRISPR/Cas9 Technology. *J. Genet. Genomics* **43**, 63–75 (2016).
54. Zetsche, B. *et al.* Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* **163**, 759–771 (2015).
55. Ran, F. A. *et al.* Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* **154**, 1380–1389 (2013).
56. Wang, Y. *et al.* Identification of genomic sites for CRISPR/Cas9-based genome editing in the *Vitis vinifera* genome. *BMC Plant Biol.* **16**, 96 (2016).
57. Lei, Y. *et al.* CRISPR-P: A web tool for synthetic single-guide RNA design of CRISPR-system in plants. *Mol. Plant* **7**, 1494–1496 (2014).
58. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402–408 (2001).

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Author Contributions

Z.L., C.R. and S.L. designed the research. C.R., X.L. and Z.Z. performed the experiments. C.R., Y.W. and W.D. analyzed data. C.R., Z.L. and S.L. wrote the manuscript. All authors read and approved the manuscript.

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