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Review

CRISPR/Cas in Grapevine Genome Editing: The Best Is Yet to Come

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Abstract: The advent of Clustered Regularly Interspaced Palindromic Repeat (CRISPR)/CRISPR-associated (Cas) proteins as a revolutionary innovation in genome editing has greatly promoted targeted modification and trait improvement in most plant species. For grapevine (*Vitis vinifera* L.), a perennial woody plant species, CRISPR/Cas genome editing is an extremely promising technique for genetic improvement in a short period. Advances in grapevine genome editing have been achieved by using CRISPR technology in recent years, which promises to accelerate trait improvement in grapevine. In this review, we describe the development and advances in CRISPR/Cas9 and its orthologs and variants. We summarize the applications of genome editing in grapevine and discuss the challenges facing grapevine genome editing as well as the possible strategies that could be used to improve genome editing in grapevine. In addition, we outline future perspectives for grapevine genome editing in a model system, precise genome editing, accelerated trait improvement, and transgene-free genome editing. We believe that CRISPR/Cas will play a more important role in grapevine genome editing, and an exciting and bright future is expected in this economically significant species.

Keywords: CRISPR/Cas; genome editing; grapevine; trait improvement; transgene-free

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1. Introduction

Though the time of the first discovery of Clustered Regularly Interspaced Palindromic Repeats (CRISPRs) dates back to 1987 [1], not until twenty years later had CRISPR been found to provide an antiviral defense in prokaryotes [2–4]. In 2012, two studies revealed that the RNA-guided CRISPR-associated protein 9 (Cas9) system could be used to cut DNA sequences [5,6]. The application of CRISPR/Cas9 gene editing was first achieved in mammalian cells in 2013 [7]. Since then, the CRISPR technology has revolutionized genome editing and has been widely used in various research areas.

Prior to CRISPR/Cas9, zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) had been developed to target genomic sites of interest for gene editing [8–10]. In the two systems, DNA-binding domains are fused with the FokI nuclease domain, which should form a dimer to activate the nuclease activity, thereby cutting the target DNA sequence to create a double-stranded break (DSB) [11]. However, both of the two systems rely on protein–DNA interactions, and new proteins should be engineered for targeting different sites, which is usually labor-intensive, precluding the applications of ZFNs and TALENs. In contrast, Cas9 protein is an RNA-guided site-specific nuclease and

recognizes target DNA sequence by forming Watson–Crick base pairings with its guide RNA [11]. In theory, Cas9 could be programmed to target different sites just by changing the guide RNA. To date, CRISPR/Cas9 has been the primary CRISPR tool for genome editing in both animals and plants.

The applications of CRISPR/Cas9 in plants were reported in 2013 in rice [12,13], tobacco, and Arabidopsis [13]. In horticultural plants, CRISPR/Cas9-mediated genome editing was first documented in tomato and citrus in 2014 [14,15]. Grapevines are economically important perennial fruit crops widely cultivated in the world. Global viticulture is constantly challenged by climate changes and the prevalence of diseases and pests, which result in a reduction in grapevine production and berry quality. Thus, it is imperative to develop elite cultivars of grapevine with superior traits. As is known, conventional breeding, relying on hybridization across species or cultivars, is tedious and time-consuming. The emergence of CRISPR/Cas technology enables precision breeding to genetically improve grapevine traits of interest in a designed fashion, bypassing the labor-intensive and time-consuming process of conventional breeding method. Successful genome editing in grapevine by CRISPR/Cas9 was first reported in 2016 [16,17]; since then, this CRISPR system has been used as an important tool for gene functional research and trait improvement in this species. As in the other plant species, CRISPR/Cas9 is the most commonly used genome editor in grapevine, and gene knockout is still the major editing type. In addition, CRISPR activation systems based on nuclease-dead Cas9 (dCas9) had also been developed for endogenous gene activation in grapevine [18]. Recently, the base editing of grapevine genes had been successfully documented [19]. However, the great potential of CRISPR/Cas technology for grapevine genome editing has not been fully exploited so far. Here, we reviewed the currently used CRISPR/Cas systems and their derived CRISPR tools, applications of CRISPR/Cas technology in grapevine, and the challenges and future prospects in grapevine genome editing, expecting to provide an overview of recent advances and promote the applications of CRISPR/Cas in grapevine genome editing in the future.

2. CRISPR/Cas Nucleases

As mentioned above, CRISPR/Cas system functions as an adaptive immune system in prokaryotes against invading DNA or RNA molecules [2–4]. Based on the Cas proteins required for the immune response, CRISPR systems have been classified into two major classes: class 1 systems (type I, III, and IV) use multiple effector proteins to form a large complex for target cleavage, while class 2 systems (type II, V, and VI) only require one effector protein for target cleavage [20] (Figure 1). Notably, most of the CRISPR/Cas systems from both class 1 and class 2 systems are found to target DNA molecules, while several CRISPR/Cas systems are directed to target RNA sequences [20]. Therefore, according to the types of target sequences, CRISPR systems can be generally divided into DNA-targeting and RNA-targeting systems.

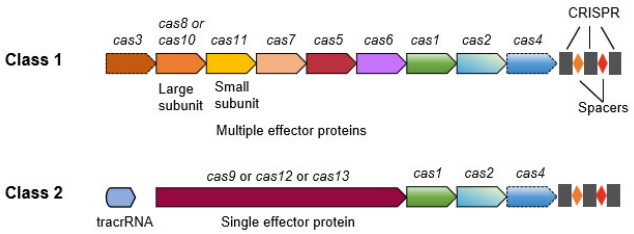

Figure 1. Illustration of the generic organizations of the class 1 and class 2 CRISPR/Cas loci. Class 1 systems have effector modules composed of multiple Cas proteins that function in protein complex during the editing. Class 2 systems have a single, multidomain effector protein that is functionally analogous to the effector protein complex of class 1. Some of the class 2 systems like Cas9 proteins require trans-acting CRISPR RNA (tracrRNA).

2.1. DNA-Targeting Cas Proteins

2.1.1. Cas9 Nucleases

The most widely used *Streptococcus pyogenes* Cas9 (SpCas9) protein is from class 2 type II CRISPR system and requires the CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) duplex to recognize target DNA through a 20 base-pair (bp) sequence called spacer in crRNA [5,7]. In an engineered CRISPR/Cas9 system, the crRNA and tracrRNA were fused into a single guide RNA (sgRNA) [7]. The Cas9 protein is guided by sgRNA to cut the target DNA in the presence of a protospacer-adjacent motif (PAM) located immediately 3′ of the protospacer, resulting in blunt-end DSB [7]. The introduced DSBs are the most deleterious DNA damages and could be repaired by several conserved mechanisms such as non-homologous end-joining (NHEJ), microhomology-mediated end joining (MMEJ, also knowns as alternative EJ), and homology-directed repair (HDR) [21,22]. The predominant NHEJ process, for example, is to re-ligate the broken DNA ends, usually leading to nucleotide deletions or insertions (indels) at the DSB sites (Figure 2). However, NHEJ repair could also be precise, but the restored sequence could be targeted again and again by Cas9 until mutations are produced [22]. MMEJ depends on short microhomology sequences surrounding the broken sites, and increasing evidence shows that MMEJ is an active repair way in human cells [23,24]. HDR is a high-fidelity repair pathway and triggered by the presence of a donor DNA template (Figure 2). However, the efficiency of HDR is less than NHEJ and MMEJ [22].

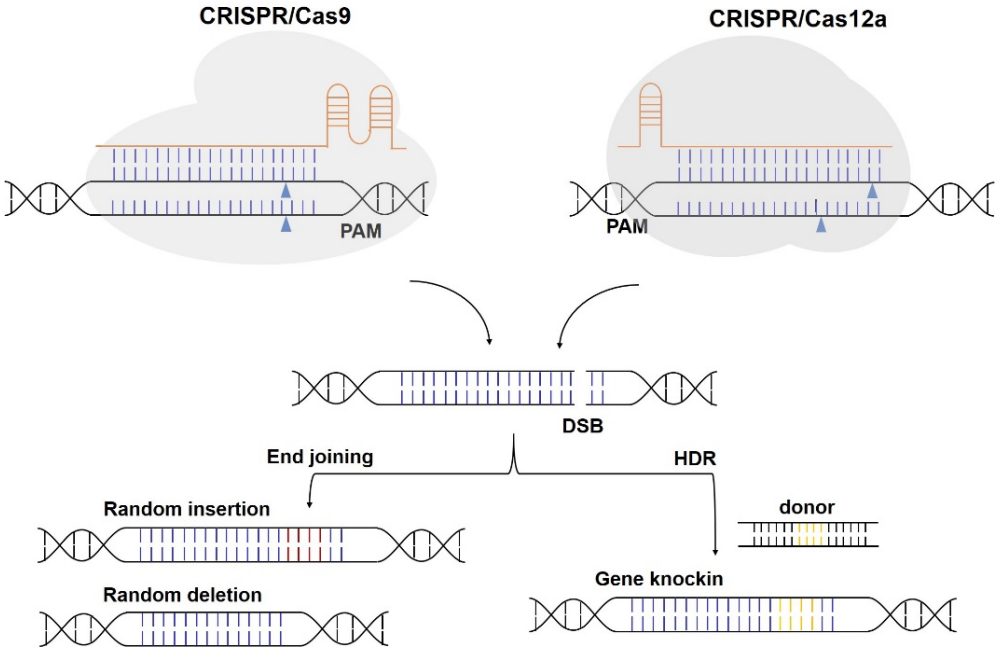

Figure 2. Genome editing induced by CRISPR/Cas9 and CRISPR/Cas12a. Both CRISPR/Cas9 and CRISPR/Cas12a generate double-stranded break (DSB), which can be repaired via end joining or the homology-directed repair (HDR) pathway, resulting in indel (random insertion or deletion) or knock-in mutations. The inserted nucleotides via end joining and desired mutations carried by donor template are indicated in red and yellow, respectively.

The native Cas9 protein contains two distinct nuclease domains, namely, a HNH nuclease domain and RuvC-like nuclease domain. The mutation of either of the two nuclease domains generates a Cas9 nickase (Cas9n), which could be used to improve editing specificity when combined with a pair of sgRNAs [25,26]. The catalytically inactivation of both nuclease domains produces dCas9 that can be repurposed for different applications, such as transcriptional regulation and epigenetic modifications [27–29].

The widely used SpCas9, consisting of 1368 amino acids, recognizes a common NGG (where N is an A, T, G, or C) or a weaker NAG PAM [30]. PAM availability limits the

applications of CRISPR/Cas9 during genome editing. To expand the targeting scope of SpCas9, researchers have developed a number of Cas9 variants harboring mutations of different amino acids. For instance, the SpCas9 mutants, SpCas9-EQR, SpCas9-VQR, and SpCas9-VRER, recognize NGAG, NGA, and NGCG PAMs, respectively [31]. xCas9-3.7 exhibits higher activities on NGT and NGA PAMs than that of SpCas9 [32,33]. In addition, SpCas9-NG (targets NG PAMs), SpG (targets NGN PAMs), and SpRY (targets NRN/NYN PAMs, where Y is C or T) have successively been developed with less restrictive PAM compatibilities [34,35]. In addition to SpCas9 variants, various Cas9 orthologs have been isolated and characterized from *Staphylococcus aureus* [36], *Streptococcus thermophiles* [37], and many other organisms [38–41]. These Cas9 proteins have different overall size, guide RNA structure, and PAM requirement. For example, the Cas9 protein isolated from *Staphylococcus aureus* (SaCas9) contains 1053 aa and recognizes NNGRRT (where R is an A or G) PAMs [36]. All these Cas9 orthologs have been studied and developed as tools for genome editing in bacteria or mammalian cells. Some of them have been tested and used in plant genome editing.

Another major research focus has been the development of Cas9 variants with higher DNA specificity. As described above, paired Cas9n combined with two sgRNAs targeting opposing strands of the DNA target can reduce off-target effects [25,26]. Engineered SpCas9 variants, eSpCas9(1.1) and SpCas9-HF1, were developed with reduced off-target editing by decreasing the binding affinity between Cas9/sgRNA complex and DNA targets [42,43]. The combination of mutations used in eSpCas9(1.1) and SpCas9-HF1 makes the HeFSpCas9 variant [44]. An enhanced fidelity variant named HypaCas9 was later developed using rational design approaches [45]. The selection of a library of SpCas9 variants with different mutations in yeast and *Escherichia coli* identified the evoCas9 and Sniper-Cas9 with greater fidelity, respectively [46,47].

2.1.2. Cas12 Nucleases

Unlike Cas9, Cas12 nucleases contain a single RuvC-like nuclease domain for the DNA cleavage of both strands. Moreover, many Cas12 effectors only require a crRNA for efficient DNA cleavage. Cas12a (also named Cpf1) is the first characterized and widely used Cas12 protein for genome editing [48]. Cas12a has RNase activity and could process its pre-mature crRNAs to generate mature crRNAs. Cas12a recognizes T-rich PAMs and cuts the target regions distal to the PAM sequences [48,49] (Figure 2). Cas12a orthologs from *Acidaminococcus* sp. *BV3L6* (AsCas12a), *Francisella novicida* (FnCas12a), *Lachnospiraceae bacterium ND2006* (LbCas12a), *Eubacterium rectale* (ErCas12a), and many other organisms have been studied and applied for genome editing in plants [50–55].

Notably, Cas12a is more sensitive to temperatures than Cas9, and much effort had been made to develop Cas12a variants with lower temperature sensitivity [56–59]. Temperature tolerant LbCas12a (ttLbCas12a and LbCas12a-D156R) was first developed to display enhanced editing efficiency at a low temperature [58]. LbCas12a-RRV and LbCas12a-RVQ were also constructed by conducting saturation mutagenesis in *E. coli* [56]. Based on structure-guided protein engineering, AsCas12a-Plus was developed with increased activity and specificity [57]. Additionally, to expand the targeting scope of Cas12a, nine Cas12a orthologs were tested in plants, and an engineered Mb2Cas12a-RVRR variant was finally identified with more relaxed PAM requirements [55]. Moreover, Cas12a has also been repurposed for transcriptional regulation and base editing [51,60,61].

Recently, phylogenetic analysis revealed that TnpB and Fanzor of the OMEGA (obligate mobile element guided activity) system might be the evolutionary ancestor of Cas12, and experimental evidence demonstrated that TnpB of Deinococcus radiodurans ISDra2 and Fanzor are RNA-guided nucleases; both of them could be reprogrammed for human genome engineering applications [62,63], suggesting that TnpB and Fanzor could be used as novel systems for genome editing.

2.1.3. Base Editors

Base editors could introduce targeted point mutations without generating DSBs. There are three main types of base editors that have been developed and are currently in use: cytosine base editors (CBEs), adenine base editors (ABEs), and glycosylase base editors (GBEs) [64–66]. CBE was initially developed by fusing a rat cytidine deaminase rAPOBEC1 to the N-terminus of dCas9 [64]. The rAPOBEC1 can deaminate C into U (uracil) in the non-target DNA strand, and C-to-T substitution could be achieved through subsequent DNA repair and replication [64]. However, the G:U base pair could be detected as a mismatch by cellular base excision repair mechanism, and the resulting U is likely to be removed by uracil *N*-glycosylase (UNG), which results in a low editing efficiency of the CBE1 system [64]. Thus, an uracil DNA glycosylase inhibitor (UGI) was fused to the C-terminus of dCas9 in the CBE1 to develop the CBE2, which was tested with improved editing efficiency [64]. In the CBE3, a Cas9n (D10A) was used instead of dCas9 to generate a nick in the target strand to increase editing efficiency by promoting the cellular repair process [64]. The fourth-generation CBE, termed CBE4, was constructed by fusing two UGIs to the C-terminus of Cas9n in the CBE3, and the new CBE4 could enhance base editing efficiency and decrease the frequency of undesired C-to-A or C-to-G transversions [67].

The applications of CBEs are limited by its narrow editing window and strict PAM requirement for SpCas9. To expand the editing window, different deaminases were tested for base editing. The human AID mutant (hAID*Δ), Petromyzon marinus cytidine deaminase (PmCDA1), and human deaminase APOBEC3A (hA3A) were all successfully used to develop CBEs [68–70]. The phage-assisted continuous evolution of current deaminases is a promising way to obtain superior deaminase variants. For example, the test of a series of evolved TadA8e mutants led to the development of several TdCBEs, which enable higher editing accuracy and efficiency [71,72]. Moreover, the early versions of CBEs were developed with SpCas9, which generally recognizes NGG PAMs. To expand the targeting scope of base editors, Cas9 orthologs and variants, such as ScCas9, SpCas9-NG, xCas9, and SpRY, were used instead of SpCas9 to develop CBE variants recognizing alternative PAMs [73–75].

Similar to CBEs, ABEs consist of a Cas9n and an artificially evolved adenosine deaminase, which catalyzes the conversion of A to I (inosine). A:T-to-G:C substitutions are created through subsequent DNA repair and replication [65]. The first developed ABE7.10 was further improved by codon optimization and the addition of nuclear localization sequence (NLS) [76]. Additional NLS was added to both ends of ABE7.10 to develop the ABEmax, which could install A:T-to-G:C conversions in rice with the efficiencies ranging from 17.6% to 62.3% [77,78]. To achieve high editing efficiency, a more efficient adenine deaminase mutant named TadA8e had been evolved from TadA7.10 [79,80]. The developed ABE8e with TadA8e displayed significantly enhanced efficiency of A-to-G conversions [80]. When applied in rice, ABE8e was optimized by combining codon-optimized TadA8e with bis-bpNLS [81]. The resulting rice ABE8e (rABE8e) displayed nearly 100% editing efficiency at most tested targets [81]. Single-stranded DNA binding domain (DBD) was fused with TadA8e to develop high-efficiency ABE (PhieABE) toolbox based on the SpCas9 nickase variants SpCas9n, SpGn, and SpRYn [82]. Among these PhieABEs, hyper ABE8e-DBD-SpRYn (hyABE8e-SpRY) was tested with extremely high editing efficiency, and a high proportion of homozygous base substitutions were also detected [82]. Two mutations (V82S and Q154R) introduced into TadA8e resulted in the generation of TadA9, which expands the editing window in rice when compared to the previous version [83].

Both CBEs and ABEs catalyze only base transitions (C-to-T and A-to-G). GBEs were developed to produce base transversions. GBEs are composed of a Cas9n, a cytidine deaminase, and a UNG [66,84]. As mentioned before, the U base created by cytidine deaminase could be excised by UNG, resulting in the formation of an apurinic/apyrimidinic (AP) site that induces the DNA repair process [84]. GBEs containing AID-Cas9n-UNG and rAPOBEC1-Cas9n-UNG had been successfully used in *E. coli* and mammalian cells, respectively [84]. An optimized GBE was also generated to achieve C-to-G editing in

rice [85]. The combination of an ABE with hypoxanthine excision protein N-methylpurine DNA glycosylase makes the adenine transversion base editor, AYBE, for A-to-Y base editing in mammalian cells [86]. Furthermore, base editors for G-to-Y conversion had also been developed recently [87]. However, the feasibility of these novel base editors in plants remains to be tested in the future.

It is worth noting that multiplex base editing could be accomplished by using different deaminases simultaneously. For instance, a dual adenine and cytosine base editor (A&C-BEmax) was developed by fusing cytosine and adenine deaminases with a Cas9n to achieve C-to-T and A-to-G conversions [88]. Simultaneous and wide editing induced by a single system (SWISS) based on CRISPR/Cas9 was also developed to induce C-to-T and A-to-G substitutions [89]. Likewise, a new dual deaminase-mediated base editor, AGBE, was developed by fusing CGBE (a GBE) with ABE for inducing four types of base conversions (C-to-G, C-to-T, C-to-A, and A-to-G) simultaneously in mammalian cells [90].

2.1.4. Prime Editors

Prime editors (PEs), comprising a Cas9n (H840A) and an engineered reverse transcriptase, can install all 12 possible types of base substitutions, small deletions, and insertions in a precise manner [91]. PEs are guided by an engineered prime editing guide RNA (pegRNA), which contains a spacer for targeting the specific site and an extension carrying the desired edit and a primer binding sequence (PBS) [91]. Once binding to the target, the Cas9n generates a nick at the non-target strand, and the PBS in the pegRNA could hybridize with the 3′ end of the nicked DNA strand to prime reverse transcription to install the carried mutation into the genomic DNA through DNA repair and replication [91]. Several generations of PEs have been developed and characterized. The first developed PE1 is a fusion of Cas9n and Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT). Then the wild-type M-MLV RT in PE1 was replaced by an engineered pentamutant M-MLV RT to generate PE2. Compared to PE2, PE3 harbors an additional sgRNA for nicking the non-edited strand to increase the editing efficiency [91]. However, a high frequency of undesired indel products were detected with PE3, and a PE3b system was therefore developed to reduce unwanted indel mutations [91]. Later on, PE4 and PE5 were developed by fusing an endonuclease-impaired MLH1 protein to the C-terminus of PE2 and PE3, respectively, with an enhanced editing efficiency by an average of 7.7- and 2.0-fold in mammalian cells [92].

The design of pegRNA is a predominant determinant of prime editing efficiency. pegRNA parameters such as the length of PBS and RT template, GC content, secondary structures within pegRNA, and pegRNA stability should be considered [21,93]. In general, efficient PBSs are 8–15 nt in length, while the length of RT templates falls in a range from 10 to 20 nt [21]. A study in rice showed that the optimal melting temperature of PBS was 30 °C [94]. Low GC content generally requires longer PBS to ensure efficient annealing with the nicked non-target strand [91]. Moreover, to avoid undesired base pairing, the last base of RT template should not be a cytidine, which could pair with G81 in the pegRNA scaffold [91]. Modifying the secondary structures of pegRNA is an efficient way to increase prime editing efficiency [95,96]. The instability of pegRNA is thought to be a factor affecting prime editing efficiency because the 3′ end extension of pegRNA is susceptible to exonucleolytic degradation in cells [97]. To enhance the stability of pegRNAs, RNA motifs like evopreQ1 and mpknot were added to the 3′ end of pegRNAs to protect them from degradation, thereby improving the editing efficiency of by 3- to 4-fold in mammalian cells and plants [97]. Recently, several robust methods based on PEs, such as PRIME-Del [98], twinPE [99], PEDAR [100], and GRAND editing [101], were reported to achieve precise large DNA fragment deletions, insertions, or replacement.

2.2. RNA-Targeting Cas Proteins

2.2.1. Cas13 Nucleases

Cas13 nucleases belong to Class 2 type VI CRISPR systems and are known to exclusively cleave RNA molecules [102]. All Cas13 nucleases have RNase activity for pre-crRNA processing and two HEPN (Higher Eukaryotes and Prokaryotes Nucleotide-binding) domains required for RNA degradation [102]. Upon binding to the targets, Cas13/crRNA complexes exhibit conformational changes and cleave both the target RNA molecules (*cis*-cleavage) and bystander RNAs (*trans*-cleavage) [103]. Unlike DNA-targeting Cas nucleases, Cas13 effectors appear not to rely on consensus PAMs; some of Cas13 members prefer a specific sequence termed the protospacer flanking sequence (PFS), which is located just 3′ of the crRNA complementary sequence of the target RNA [103–105]. Cas13 systems have been employed as powerful tools for RNA degradation (gene knockdown) [103,104], RNA editing [106], nucleic acid detection [107], and so on.

Cas13a (formerly C2c2) is the most widely studied Cas13 effector. In addition, Cas13b, Cas13c, Cas13d, and the other Cas13 proteins have also been successively identified and characterized [108]. As for applications of Cas13 systems in plants, CRISPR/Cas13-based RNA interference could protect plants against RNA virus infections. The CRISPR/Cas13-based RNA editing in plants has been reviewed by Kavuri et al. recently [109]. However, the application of CRISPR/Cas13 in plants is still limited.

2.2.2. RNA-Targeting Cas9

Previous studies showed that SpCas9 can also bind and cleave single-stranded RNA [110,111], even though the exact mechanism underlying this action of SpCas9/sgRNA remains unknown. In addition, various Cas9 proteins from other bacteria have been characterized for RNA targeting (RCas9) [108]. For example, the *Francisella novicia* Cas9 (FnCas9) was capable of inhibiting the infection by the hepatitis C RNA virus in human cells by using a small CRISPR-associated RNA [112]. Nevertheless, most of the found RCas9, such as *Staphylococcus aureus* Cas9 and *Campylobacter jejuni* Cas9, were tested *in vitro* [113,114], and their activities for RNA editing *in vivo* have not been reported yet.

3. Genome Editing in Grapevine

Since the efficacy of CRISPR/Cas9 system was demonstrated in 2016, more and more reports on grapevine genome editing by using this CRISPR system have been released in recent years. The optimization of CRISPR/Cas9 was performed to improve the editing efficiency in grapevine. Additionally, several other CRISPR/Cas systems were also tested and applied in grapevine (Figure 3). In summary, the CRISPR/Cas9 and CRISPR/LbCas12a systems are available now for gene knockout in grapevine, while RNA targeting effector like Cas13a has also been developed for RNA editing. Elevated gene expression could be achieved by using CRISPR/dCas9-mediated gene activation. Precise point mutations are expected to be accomplished by using CBE or PE.

3.1. Proof-of-Concept Studies on CRISPR/Cas9

In early 2016, the availability of suitable target sites for CRISPR/Cas9 was thoroughly analyzed in grapevine (*V. vinifera*) genome, and over 7 million highly specific potential targets were found to distribute uniformly in grape genome [115]. Interestingly, the coding regions of grape genes have the highest abundance of predicted target sites [115]. This study showed that CRISPR/Cas9 could be theoretically applicable to grapevine, and gene coding regions might be suitable regions for genome editing. Later on, the efficacy of CRISPR/Cas9 system was experimentally demonstrated by targeting the *L-idonate dehydrogenase* (*IdnDH*) gene in Chardonnay [16]. Successful editing was achieved in both grape calli and regenerated plants with an efficiency as high as 100%, and the targeted mutagenesis of *IdnDH* resulted in a lower tartaric acid content in grape calli [16]. This was the first report on CRISPR/Cas9-mediated genome editing in grapevine, demonstrating the effectiveness of CRISPR/Cas9 in this species. In late 2016, DNA-free genome editing

by using purified CRISPR/Cas9 ribonucleoproteins (RNPs) was reported in Chardonnay protoplasts, and the susceptible gene *MLO7* involved in powdery mildew resistance was successfully targeted, even though the editing efficiency was as low as 0.1% [17]. Based on the experimental methods from the two studies [16,17], a detailed protocol, including plasmid-mediated genome editing and CRISPR/Cas9 RNP-mediated genome editing, was established for grapevine [116]. The development of the protocol, which can be referred to during grapevine genome editing, is expected to prompt applications of CRISPR/Cas9. Subsequently, a research group from Japan reported the editing of *V. vinifera phytoene desaturase* (*VvPDS*) gene in Neo Muscat by using CRISPR/Cas9 system, with an efficiency ranging from 2.7% to 86% [117].

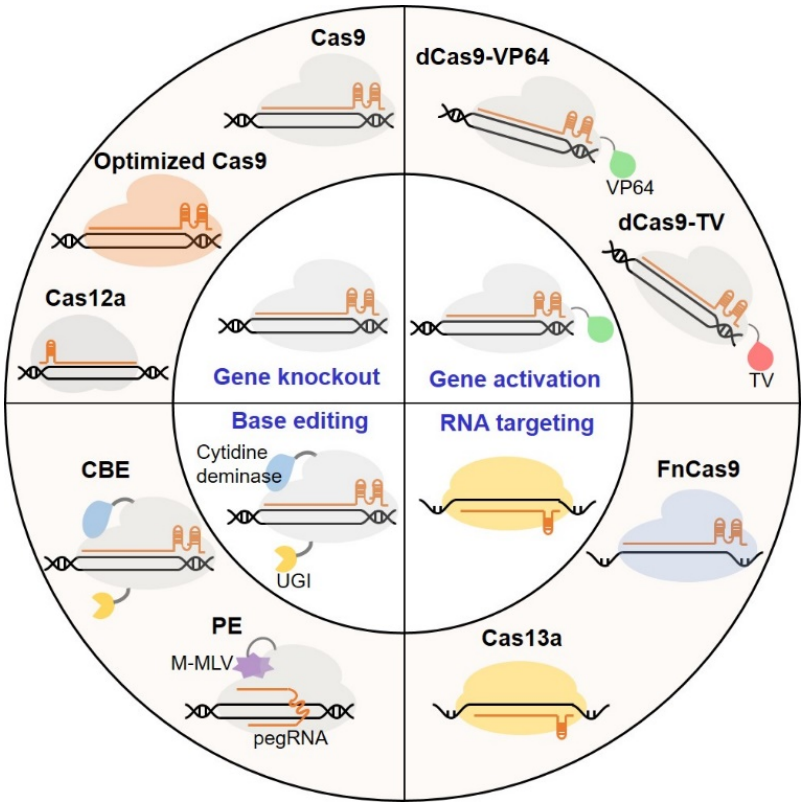

Figure 3. Current CRISPR editing in grapevine. CRISPR/Cas9 (including optimized Cas9 system) and CRISPR/LbCas12a have been employed for gene knockout. CRISPR activation systems based on nuclease-dead Cas9 (dCas9), namely, dCas9-VP64 and dCas9-TV, have been developed for gene activation. Cytosine base editor (CBE) and prime editor (PE) have been used for base editing. RNA-targeting effectors, FnCas9 and LshCas13a, have also been reported in grapevine.

3.2. Applications of CRISPR/Cas9 in Grapevine

CRISPR/Cas9 is efficient in inducing the targeted mutagenesis of genes of interest, resulting in improved traits in grapevine. Most of the reported applications in grapevine mainly focus on the improvement of disease resistance (Table 1). A transcription factor gene *VvWRKY52* was edited by CRISPR/Cas9 in Thompson Seedless, and 4 out of 22 independent *wrky52* mutants were tested with enhanced resistance to *Botrytis cinerea* [118]. Two grape *MLO* genes, *VvMLO3* and *VvMLO4*, were targeted in Thompson Seedless by CRISPR/Cas9, with the editing efficiency ranging from 0% to 38.5% [119]. Four *VvMLO3*-edited grapevine plants showed enhanced resistance to powdery mildew [119]. In a study reported in 2021, four putative grape susceptibility genes, namely, *auxin induced in root culture 12* (*AIR12*), *sugars will eventually be exported transporter 4* (*SWEET4*), *lesion initiation 2* (*LIN2*), and *dimerization partner-e2f-like 1* (*DEL1*), were successfully edited in Thompson Seedless, respectively [120]. These edited grapevine plants were subjected to *Erysiphe*

necator and *Botrytis cinerea*, and a *DEL1*-edited plant displayed over 90% reduction in symptoms induced by powdery mildew infection [120]. To investigate the function of a *pathogenesis-related protein 4b* (*VvPR4b*) gene in grapevine resistance to downy mildew, the *VvPR4b* gene was knockout in Thompson Seedless by CRISPR/Cas9 system, and the loss of function of *VvPR4b* increased the susceptibility of edited plants to the oomycete pathogen *Plasmopara viticola* [121]. In contrast, the knockout of the *downy mildew resistant 6* (*DMR6*) gene, which encodes a negative regulator of plant immunity, could reduce the susceptibility of grapevine to downy mildew [122,123]. Grapevine contains two copies of *DMR6*: *VvDMR6-1* and *VvDMR6-2*. *VvDMR6-1*-edited biallelic and chimeric plants of 41B rootstock (*V. vinifera* × *V. berlandieri*) exhibited reduced growth as well as susceptibility to *P. viticola* [122]. In another study, Giacomelli et al. developed *dmr6-1*, *dmr6-2*, and *dmr6-1_2* mutants of two different grapevine cultivars and tested their resistance to *P. viticola* by inoculating *P. viticola* to the detached leaf discs of young plants. The expected reduction in susceptibility was only detected in Crimson Seedless but not in Sugraone [123]. Further evaluation with the support of a robust statistical method showed that double mutant *dmr6-1_2* always displayed a significant reduction in susceptibility to *P. viticola* in both cultivars as compared to wild-type plants [123]. Elevated levels of salicylic acid were detected in *dmr6* mutants in both of the two studies [122,123], suggesting that the enhanced resistance to downy mildew may be associated with increased salicylic acid content.

Table 1. CRISPR/Cas genome editing in grapevine.

Effector	Target	Explant	Delivery Method	Editing Type	Trait	Phenotype	Reference
SpCas9	*IdnDH*	EC of Chardonnay	*Agrobacterium tumefaciens*	KO	Tartaric acid synthesis	Decreased TA content	[16]
	MLO7	Protoplasts from Chardonnay EC	PEG	KO	Powdery mildew resistance	ND	[17]
	VvPDS	EC of Neo Muscat	*Agrobacterium tumefaciens*	KO	Carotenoid biosynthesis	Albino phenotype	[117]
	VvPDS	EC of Chardonnay and 41B	*Agrobacterium tumefaciens*	KO	Carotenoid biosynthesis	ND	[124]
	VvPDS	Protoplasts from EC of 101-14, Cabernet Sauvignon, Chardonnay, Merlot, Thompson Seedless, Colombard, GRN1, *V. arizonica*, and Pixie	PEG	KO	Carotenoid biosynthesis	ND	[125]
	VvPDS	EC of Nebbiolo	PEG and lipofectamines	KO	Carotenoid biosynthesis	Albino phenotype	[126]
	VvWRKY52	EC of Thompson Seedless	*Agrobacterium tumefaciens*	KO	*Botrytis cinerea* resistance	Enhanced resistance	[118]
	CCD8	EC of 41B	*Agrobacterium tumefaciens*	KO	Shoot branching	Increased branches	[127]
	VvPR4b	EC of Thompson Seedless	*Agrobacterium tumefaciens*	KO	Downy mildew resistance	Decreased resistance	[121]
	VvMLO3, *VvMLO4*	EC of Thompson Seedless	*Agrobacterium tumefaciens*	KO	Powdery mildew resistance	Enhanced resistance	[119]
	TAS4, *MYBA7*	EC of rootstock 101-14	*Agrobacterium tumefaciens*	KO	Anthocyanin accumulation	No accumulation	[128]

Table 1. *Cont.*

Effector	Target	Explant	Delivery Method	Editing Type	Trait	Phenotype	Reference
	PDS *TMT1*, *TMT2*	EC of 41B	*Agrobacterium tumefaciens*	KO	Carotenoid biosynthesis sugar accumulation	Albino phenotype Decreased sugar content	[129]
	VvAIR12, *VvSWEET4*, *VvLIN2*, *VvDEL1*	EC of Thompson Seedless	*Agrobacterium tumefaciens*	KO	*Botrytis cinerea* resistance	Enhanced resistance	[120]
	VvPLATZ1	EC of microvine, genotype 04C023V0006 (H/H)	*Agrobacterium tumefaciens*	KO	Female flower morphology	Reflex stamens	[130]
	VvbZIP36	EC of Thompson Seedless	*Agrobacterium tumefaciens*	KO	Anthocyanin accumulation	Increased anthocyanin content	[131]
SpCas9	*VvDMR6*	EC of Crimson seedless and Sugraone	PEG	KO	Downy mildew resistance	ND	[132]
	VvMLO6				Powdery mildew resistance	ND	
	VvMYBA1	EC of Chardonnay EC of Shine Muscat	*Agrobacterium tumefaciens*	KO	Anthocyanin accumulation	ND ND	[133] [134]
	VvEPFL9-1	EC of Sugraone	*Agrobacterium tumefaciens*	KO	Stomata formation	Reduced stomatal density	[135]
	VvDMR6-1	EC of 41B	*Agrobacterium tumefaciens*	KO	Downy mildew resistance	Enhanced resistance	[122]
	GFP	Protoplasts from Thompson Seedless EC	PEG	KO	GFP fluorescence	Loss of GFP fluorescence	[136]
	VvDMR6-1, *VvDMR6-2*	EC of Crimson seedless and Sugraone	*Agrobacterium tumefaciens*	KO	Downy mildew resistance	Enhanced resistance	[123]
zCas9i	*LysM receptor-like kinase gene* (*Vitvi05g00623*)	EC of Chardonnay	*Agrobacterium tumefaciens*	KO	Immune response	ND	[137]
LbCas12a	*TMT1*, *TMT2*, *DFR1*	EC of 41B	*Agrobacterium tumefaciens*	KO	Sugar accumulation flavonoid accumulation	Altered sugar and flavonoid contents	[138]
PE	*VvDXS1*	EC of Scarlet Royal	*Agrobacterium tumefaciens*	Base editing	Monoterpenes biosynthesis	Increased monoterpenes content	[19]

EC, embryogenic calli; KO, gene knockout; ND, not detected.

In addition to the studies on pathogen resistance, the targeted mutagenesis of grape genes involved in plant development and secondary metabolite accumulation was also reported. The biosynthesis of strigolactones, which play a key role in controlling axillary bud outgrowth, is affected by two enzymes: carotenoid cleavage dioxygenase 7 (CCD7) and CCD8. Grapevine *CCD7* and *CCD8* were knocked out in 41B by using CRISPR/Cas9, and the obtained *ccd8* grapevine mutants exhibited increased shoot branches [127]. Epidermal patterning factor like 9 (EPFL9) induces stomata formation in vascular plants, and the targeted mutation of *VvEPFL9-1*, one of the two grape *EPFL9* genes, resulted in

reduced stomatal density in the edited Sugraone plants [135]. Intrinsic water-use efficiency was also improved in *epfl9-1* mutants as compared to the wild-type control [135]. The CRISPR/Cas9-mediated editing of *plant AT-rich sequence- and zinc-binding protein1* (*VvPLATZ1*) in a hermaphrodite genotype showed that homozygous, edited lines produced flowers with reflex stamens, suggesting a role for VvPLATZ1 in controlling female flower morphology in grapevine [130]. A grapevine bZIP family gene named *VvbZIP36* was edited in Thompson Seedless, and corresponding mutants with monoallelic mutations were created with the increased accumulation of anthocyanins in leaves [131]. Moreover, the *trans-acting small-interfering locus4* (*TAS4*) and *MYBA7* genes are thought to be involved in pathogen-induced anthocyanin accumulation, and grapevine mutants of the two genes were developed in the rootstock 101-14, but no visible anthocyanin accumulation was observed in these mutants [128].

3.3. Optimization of CRISPR/Cas9 for Grapevine Genome Editing

The function of CRISPR/Cas9 system relies on two components: sgRNA and Cas9. The efficient cleavage of the target is associated with the expression of sgRNA and *Cas9* in cells. During the editing of *IdnDH* gene in grape calli and plants, the edited lines exhibited a higher expression level of sgRNA as compared to the control [16]. Previous studies showed that increasing sgRNA expression could improve CRISPR/Cas9 genome editing [139,140]. To increase the expression of sgRNA and *Cas9*, four VvU3 and VvU6 promoters and two ubiquitin (UBQ) promoters were identified and amplified from grape genome; the use of grape promoters significantly promoted the expression of sgRNA and *Cas9* in grape, thereby resulting in a higher editing efficiency [129]. Furthermore, CRISPR/Cas9 editing efficiency was surveyed with different parameters: sgRNA GC content, different varieties, and the expression level of *Cas9* [124]. The results showed that genome editing in 41B was more efficient than in Chardonnay, and sgRNA with a high GC content (50–65%) yielded higher editing efficiency independent of the grape varieties [124]. The *Cas9* expression level also had an effect on editing efficiency [124,137]. In a recent study, a maize-codon-optimized Cas9 containing 13 introns (zCas9i) was used to achieve up to 100% biallelic mutations in Chardonnay [137]. Another effective method is using geminivirus-derived vectors for the expression of sgRNA and *Cas9*. After transformation, a large number of geminivirus replicons could be produced through rolling-circle replication in plant cells, so as to greatly improve the expression level of sgRNA and *Cas9* [141]. Luckily, the commonly used bean yellow dwarf virus (BeYDV) had been successfully modified and used for grape genome editing [120], which provides an alternative to common vectors during grapevine genome editing.

3.4. Multiplex Genome Editing in Grapevine

An advantage of CRISPR/Cas9 system over ZFNs and TALENs is that it is easier to conduct multiplex genome editing. In general, multiple sgRNAs driven by different U3 or U6 promoters could be simply stacked in one CRISPR vector for achieving multiplex genome editing [142]. In addition, self-cleaving ribozymes (RZ), tRNA, and Csy4 have also been employed to produce different sgRNAs from a single transcript array [143,144]. In fact, in several studies, researchers used over two sgRNAs to target the same gene to guarantee efficient cleavage during the editing in grapevine [118,119,137]. Importantly, the use of multiple sgRNAs could likely generate large DNA fragment deletion, which is exemplified recently by the removal of a 10-kb *Gret1* transposon from *VvMYBA1* promoter in grapevine [133,134]. Moreover, in addition to the multiplex genome editing system based on multiple sgRNA expression cassettes, the polycistronic tRNA-sgRNA cassette (PTG) was also reported to target the grape *tonoplastic monosaccharide transporter 1* (*TMT1*) and *TMT2* genes simultaneously, resulting in reduction in the contents of maltose, glucose, and fructose [129].

3.5. CRISPR/dCas9-Mediated Gene Activation

Both loss-of-function and gain-of-function mutations are necessary for gene functional study. The native Cas9 protein is efficient in generating loss-of-function mutations, whereas dCas9 can be used to develop CRISPR activation (CRISPRa) systems for gene activation. Two major strategies are commonly used to develop CRISPRa systems. One is the fusion of transcriptional activation domains (TADs) to dCas9, and the other is modifying sgRNA scaffold to recruit TADs [145]. The grape CRISPRa systems were developed by using dCas9-VP64 (four tandem repeats of the Herpes simplex viral protein 16) and dCas9-TV (six copies of TALEs and two copies of VP64) fusion proteins, and their efficiency in gene activation was tested by targeting *UDP-glucose f lavonoid glycosyltransferases* (*UFGT*) and *C-repeat binding factor 4* (*CBF4*) genes in grapevine [18]. The effectiveness of the dCas9-VP64 system in the transcriptional activation of *UFGT* gene was limited to 1.6- to 5.6-fold in grape cells; in grapevine plants, the expression of *CBF4* was activated by 19.3- to 42.3-fold [18]. Notably, the genetic manipulation of gene promoters has emerged as a robust approach for altering gene expression in crops [146]. Promoter editing can be performed in a designed manner or be exploited to introduce random mutations to generate novel genetic variations with altered expression patterns [146]. Additionally, the genome editing of upstream open reading frames (uORFs) has also been demonstrated to be an effective way to regulate mRNA translation [147]. All these strategies could be tested and applied in grapevine for the regulation of gene expression in the near future.

3.6. Base Editing in Grapevine

Increasing evidence suggests a relationship between single nucleotide polymorphisms (SNPs) and grapevine traits [148,149]. The modification of single nucleotide has great potential in grapevine genome editing. An initial attempt using CBE in grapevine was made by targeting *gibberellin insensitive1* (*GAI1*) in 41B [150]. Though successful C-to-T substitutions were achieved, the editing efficiencies were only 2.4–15% [150]. Moreover, PE was adopted for the creation of a single-point substitution of a lysine with an asparagine at position 284 in *VvDXS1* recently, and most of the edited plants were identified with one allele being successfully edited [19]. These edited grapevine plants had higher contents of monoterpenes in their leaves than the control [19]. The applications of base editors and PEs in grapevine remain to be reinforced in the future.

3.7. Other CRISPR/Cas Systems Used in Grapevine

To expand the editing scope in grapevine, the CRISPR/LbCas12a was developed and applied for the targeted mutagenesis of *TMT1* and *dihydrofavonol-4-reductase 1* (*DFR1*) genes in 41B [151]. Short-term heat treatment could improve the performance of CRISPR/LbCas12a in genome editing, and truncated crRNAs with 20 nt guide sequences were efficient enough to induce targeted mutagenesis as original crRNAs with 24 nt guides [151]. The knockout of *DFR1* gene by CRISPR/LbCas12a led to the alteration in flavonoid accumulation in *dfr1* mutant cells [151]. Moreover, a method based on CRISPR/Cas12a had been developed for the detection of grapevine red-blotch virus (GRBV) [138], which could be deployed for the rapid detection of viral infections in the vineyard. The CRISPR/xCas9 system was also tested by targeting 12 designed targets with different PAMs within *GAI1*, *TMT1*, and *CCD8*. The results showed that no desired mutations were detected except for the first target of *GAI1* (GAI1-g1) with TGG PAM, but the editing efficiencies were less than 1.89% [150]. The optimization of the CRISPR/xCas9 system should be conducted prior to further applications in grapevine.

RNA-targeting Cas effectors, CRISPR/FnCas9 and CRISPR/LshCas13a, have been used to interfere with grapevine leafroll-associated virus 3 (GLRaV-3) in grapevine plantlets [152]. The transient expression of CRISPR/FnCas9 and CRISPR/LshCas13a reagents in *in vitro* Cabernet Sauvignon plantlets inhibited viral accumulation, and LshCas13a outperformed FnCas9 in virus inhibition [152]. This study provides novel antiviral

strategies and serves as a successful example for the applications of RNA-targeting Cas effectors in grapevine.

3.8. Online Tools for Guide RNA Design

Early efforts to identify potential targets for Cas9 in grape genome gave birth to the Grape-CRISPR, which is a database containing all the predicted protospacers and detailed information about the target sites [115]. An upgraded version named Plant-CRISPR was developed later by incorporating 138 plant genomes and potential protospacers for Cas9 and Cas12a editing [153]. The target design and test of the effectiveness of designed guide RNA are supported by the web tool of Plant-CRISPR [153]. In addition, other CRISPR websites such as CRISPR-P v2.0 [154] and CRISPR-GE are also good online tools for target design [155].

4. Challenges for Grapevine Genome Editing

Though a number of encouraging studies have been reported in recent years, grapevine genome editing is still facing challenges that restrict the extensive applications of CRISPR technology in this species.

4.1. Lack of Efficient System for Testing Cas Effectors

For a novel CRISPR/Cas system, its editing efficacy should be tested before further applications. However, CRISPR/Cas9 genome editing conducted with grape embryogenic calli requires ~9 months to obtain transgenic calli, and the experimental period would be prolonged to at least 12 months to get regenerated plants [118] (Figure 4). Thus, a rapid and efficient system for testing Cas nucleases in grapevine is important. In rice, for example, protoplasts have been used as an efficient system to verify the functions of CRISPR/Cas systems, with the transfection rates reaching as high as 90% [156,157]. In contrast, the transfection rates of grape protoplasts with CRISPR/Cas9 RNPs were ~20% [136,158], and successful editing was tightly related to the amount of RNPs [136]. In the first report on CRISPR/Cas9 editing in grape protoplasts, the editing efficiency was only 0.1% [17]. These results suggest that the current transfection method of grape protoplasts is not suitable for the evaluation of the editing efficiencies of CRISPR/Cas systems, given that the editing mediated by CRISPR/Cas systems with low activities may not be detected in grape protoplasts.

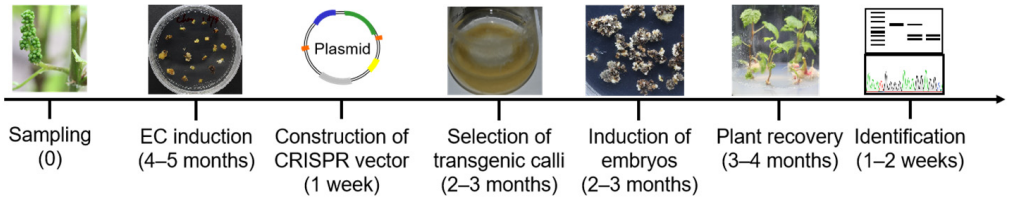

Figure 4. Time schedule of Agrobacterium-mediated CRISPR editing in grapevine.

As with grapevine, the plant transformation of soybean is also laborious and time-consuming, and soybean hairy root system is therefore developed as an efficient and rapid research tool for assessing genome editing efficiency within 14 days [132,159]. Importantly, the editing efficiency detected in soybean hairy roots is totally comparable to that detected in stable transformed plants [159]. For grapevine, hairy root cultures had been used to produce secondary metabolites, such as resveratrol and cyanogenic glucoside [160–162]. However, the use of hairy roots for grapevine genome editing has not been reported yet. According to the previous results, the transformation efficiency in grape hairy roots could be further improved [162,163] if this system is applied to genome editing in the future.

4.2. Limited Available Explants

Almost all the studies on grapevine genome editing are conducted using embryogenic calli (EC) or protoplasts isolated from EC as the explants, which are expected to generate stable grapevine plants via somatic embryogenesis. EC could be induced from anthers, ovaries, filaments, or whole flowers [117,164,165]. Nevertheless, EC are only successfully induced in limited varieties, such as Chardonnay [16,165], Thompson Seedless [118,119], Crimson Seedless [123], and Sugraone [123,135]. Furthermore, the induction efficiency was usually low and varied in different cultivars [166,167]. Meanwhile, the frequency of EC induction changed according to the adopted induction media [165]. In theory, it is better to establish an optimal system for EC induction for a given grape variety to get plenty of EC more easily.

4.3. Delivery of CRISPR/Cas Reagents and Plant Regeneration

A prerequisite for successful editing is to deliver CRISPR/Cas reagents into plant cells. The lack of efficient transformation system is a bottleneck that restricts the applications of CRISPR systems in grapevine. Some proven genetic transformation methods, including *Agrobacterium* infection, particle bombardment, and polyethylene glycol (PEG) or lipofectamine-mediated delivery, have been successfully applied in grapevine. The *Agrobacterium tumefaciens*-mediated transformation of EC is the commonly used method for grape transformation. This approach is cheap and user-friendly, but the transformation rate, however, is generally not high. Efficient transformation requires a relatively long antibiotic-dependent selection process to enrich stably transformed cells from a pool of untransformed cells [118,127,129]. To facilitate the selection of transformed plants, fluorescent markers like EGFP and DeRed2 were used to help to enrich transformed cells during the selection process [137,151]. The biolistic method can deliver biomolecules to a wide range of plant materials, but the transformation rate is low, and plant tissue is often damaged under high bombardment pressures [126,168]. The high cost and requirement of specialized equipment also restrict its application. Though the biolistic transformation of grapevine had previously been reported [169,170], this method is now rarely used during grapevine transformation. As discussed above, CRISPR/Cas reagents can also be delivered into plant cells by protoplast transfection [17,136,158]. PEG is commonly used to mediate the delivery of RNPs [17,136,158]. Very recently, lipofectamine-mediated protoplast transformation was reported in grapevine, and edited grapevine plants were obtained 5 months after the transfection [167]. The biggest advantage of PEG/lipofectamine-mediated delivery is its support for DNA-free genome editing. However, this delivery method is high skilled, and the stability of protoplasts after transfection is important for efficient transformation. Unfortunately, the transformation rate of protoplasts is low (~20%) [17,136], and the editing efficiency reported so far in grapevine protoplasts is about 0.1–42.3% [17,136,158,167], much lower than that obtained with *Agrobacterium*-mediated transformation. Additionally, it is difficult to get calli from protoplasts, and plant regeneration efficiency from protoplasts is less than 30% [158]. Other transformation methods such as electroporation and microinjection have not been used in grapevine, and the presence of the thick cell wall of plant cells limits the applications of the two delivery methods [126,168].

The bottleneck of grapevine transformation and regeneration is species and genotype dependence. To promote genotype-independent transformation, several strategies have been developed in recent years. One strategy is using morphogenic regulators, which are typically *baby boom* (*BBM*) and *Wuschel2* (*Wus2*) isolated from maize [171,172]. The overexpression of the two genes could produce high transformation frequencies in recalcitrant maize inbred lines [171,172]. Similarly, the overexpression of *BBM1* gene from apple could significantly improve transgenic plant production in apple [173]. These morphogenic regulators might be experimentally tested in grapevine, expecting to increase the transformation as well as regeneration frequencies. However, the constitutive overexpression of morphogenes causes negative phenotypic and reproductive outcomes [172], making it necessary to remove these morphogenes after inducing embryogenesis. Alternatively, the

use of tissue-specific promoters or inducible expression systems would be good choices when using morphogenic regulators. The growth regulating factor 4 (GRF4) and GRF interacting factor 1 (GIF1) chimeric protein was reported to enhance transformation and regeneration efficiency in wheat, and grape GRF4-GIF1 chimeric protein was also revealed to improve the transformation and regeneration in citrus [174], which suggests that the grape chimeric protein might be used to enhance the transformation and regeneration efficiency in grape, too. To sidestep the need for tissue culture, Maher et al. [175] reported a method to directly induce transgenic or edited shoots from *in vitro* plants by the de novo induction of meristems using developmental regulators (DRs). A proof-of-concept study had been conducted in grapevine, and transgenic plants with luciferase luminescence were successfully produced [175]. This approach promises to overcome the bottleneck in grape varieties that are recalcitrant to *Agrobacterium* transformation. However, the occurrence of genetic chimeras is a big concern when using this approach. Alternatively, nanoparticles could penetrate plant cell wall and can be employed as genotype-independent transformation method for exogenous biomolecule delivery [176,177].

5. Regulation of Gene-Edited Grapevine Plants

Public concern is often triggered by the cultivation and release of genetically modified (GM) organisms. The regulation of GM crops and foods is positive and necessary to protect human safety, the environment, and the economy [178]. However, one issue is that the current legislation might no longer be fit for the regulation of GM crops due to the rapid advances in molecular biology. Should the gene-edited (GE) plants or products produced by using CRISPR/Cas9 be regulated under the same regulatory framework as conventional GM organisms? In general, GM regulations can be categorized as process- and product-oriented [125,179]. According to process-oriented regulations, new breeding technologies could be regarded as novel techniques when compared with conventional methods, thus requiring specific legislation for the regulation. In contrast, the novel characteristics of produced products were emphasized under the product-oriented regulations [180]. In fact, the mutations introduced by editing generally rely on the formation of DSBs, which also occur naturally. The role of Cas nucleases is to generate DSBs at specific sites, and the DSBs are repaired by cellular repair mechanisms. In theory, the specific changes in GE plants cannot be discerned from the same mutations occurring naturally in unedited plants [181,182] if the editing components are not integrated or removed from plant genome after the editing.

Some countries have been reevaluating their legislation and considering how to regulate GE plants or products. In USA, the updated Coordinated Framework for the Regulation of Biotechnology allows for a modernized regulatory system [178,183], and GE crops without recombinant DNA, pesticidal activity, or food safety attributes are exempt from the regulation [178]. Recently, genome-edited mustard greens (*Brassica juncea*) approved by the Department of Agriculture (USDA) had been released to market [184]. In Japan, the GE products with SDN (site-directed nuclease)-1 type of modifications, which are characterized as mutations without using a DNA template, are not regarded as "living modified organisms" [185]. GE tomato with a high content of γ-aminobutyric acid was approved by the Japan government in 2021 [178,186]. As well-known wine countries, France and Italy, for example, are bound by European Union (EU) GM legislation. In July 2018, the European Court of Justice ruled that plants generated by NGTs (new genomic techniques) should be strictly regulated according to the Directive 2001/18/EC, which is previously applied to GM organisms [187]. This judgment is totally contrary to what EU scientific and breeder community expect, and as a consequence, research into plant genome editing in Europe has been greatly hampered in recent years [182]. However, the good news is that a regulatory proposal was published by the European Commission in July 2023, and Category 1 NGT plants (NGT1) are considered to be equivalent to conventionally bred plants [187]. Even though there is still a long way to go before the proposal is officially

approved to be a law, exciting changes have emerged in how GE plants, including non-crops, are to be regulated in the future.

6. Future Perspectives

6.1. Taking Advantage of the Model System

A stable and efficient system for studying grapevine genome editing is always important and indispensable. Notably, grapevine has a relatively long juvenile stage, and it is impractical to harvest grape berries in a short time after plant regeneration, which undoubtedly restricts the investigation of the genes of interest associated with fruit quality traits. In this case, the grape microvine, a *gai1* mutant allele that confers a dwarf stature, short generation cycles and continuous flowering [188], could be an ideal model system for grapevine genome editing. Using the microvine model, many grapevine fruit attributes can be investigated easily. The great potential of multiplex genome editing could be further exploited by targeting multiple genes involving different traits of not only stress resistance but also fruit quality.

6.2. Exploiting the Precise Genome Editing

Most of the state-of-the-art CRISPR tools have not yet been applied in grapevine, and some efficient genome editing methods like virus-induced plant genome editing are not applicable to grapevine due to the lack of valid virus vectors. All these issues should be addressed by researchers in the near future. The CRISPR toolbox in grapevine needs to be expanded, and precise genome editors like BEs and PEs should be used more for grapevine genome editing. Moreover, the knockout of a developmentally important gene could sometimes result in developmental defect or even death. The conditional or tissue/cell-type-specific knockout of genes of interest could serve as tailored solutions to address specific biological questions [189,190]. Genome editing in grapevine could be more flexible and versatile, and the target regions can be expanded to promoters or uORFs to regulate the gene expression level or protein abundance [146,147]. Novel alleles can be created by manipulating the specific elements in gene promoters [146]. Precise knock-in of large DNA fragment has been accomplished by using PE-based tools like twinPE [99] and GRAND [101]. The targeted insertion of DNA fragments at a designed site in the grapevine genome could be tested with these tools in the future, and a robust promoter or regulatory element may be integrated into the grape genome to regulate the expression of the genes of interest instead of cloning gene coding sequences laboriously. The application of precise genome editing would simplify the gene functional research in grapevine.

6.3. Accelerating the Improvement of Grapevine Traits

With the development of sequencing technology, increasing data of grapevine genomes, resequencing, and transcriptomes are available now and provide us with a lot of candidate genes based on bioinformatics-assisted analyses. For example, over 44 grape genomes have been released to date and whole-genome resequencing projects containing almost 5000 accessions have been published; more than 900 genes involved in grapevine resistance, quality, and development were identified from these datasets using the genome-wide association studies (GWASs) and other methods [191,192]. CRISPR/Cas editing, combined with a grape transformation system, could serve as a rapid and efficient approach to preliminarily confirm the functions of selected candidate genes without the cloning of their coding sequences (Figure 5). The verified results enable researchers to improve the grapevine traits more accurately and rapidly. In the future, new grapevine elite cultivars with improved fruit quality and increased tolerance to challenging environments such as cold, high temperatures, and drought should facilitate the sustainable development of viticulture and wine production.

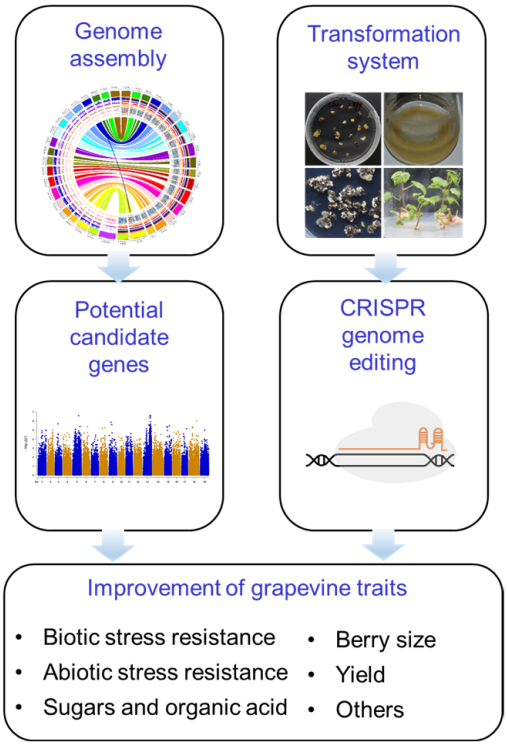

Figure 5. A strategy for accelerated improvement of grapevine traits. Bioinformatics analysis based on sequencing data and grapevine phenome provides candidate genes, and CRISPR editing, together with grape transformation system, enables a rapid verification of gene functions. The results pave the way for improvement of different grapevine traits.

6.4. Generating Transgene-Free, Edited Grapevine Plants

Considering that CRISPR editing, particularly conventional plasmid-mediated genome editing, may raise the concern of GM vines, which are morally unacceptable by consumers according to the online survey results conducted in the USA recently [193]. Furthermore, grapevine is in general vegetatively propagated, and it is unlikely to remove transgenic elements via genetic segregation. The use of the inducible Flp/FRT system or Cas9-based synthetic cleavage target sites (CTS) close to T-DNA borders can produce transgene-free genetically modified grapevine plants [194]. To guarantee the removal of transgenes, FRT sequences or Cas9 CTS are put next to T-DNA borders in designed binary vectors, and site-specific recombinase Flippase or Cas9 is driven by a heat-shock inducible promoter; the integrated T-DNA could be removed from the grapevine genome after the editing by heat-shock treatment [194]. It should be noted that a minimal trace of exogenous DNA sequence was still left in grapevine genome after the excision by using this strategy [194]. DNA-free genome editing using CRISPR/Cas9 RNPs followed by protoplast regeneration is a promising approach to obtain transgene-free, genome-edited grapevine plants [136,158,167,195]. The CRISPR/Cas9 components, sgRNA and Cas9, were expressed and preassembled *in vitro*, avoiding the use of DNA (plasmid) during the transformation [136,158,167]. This direct delivery of preassembled CRISPR/Cas9 RNPs into protoplasts may address public concerns about GM vines. Notably, it is almost impossible to get transgene-free grapevine plants in all grape varieties at present, because only limited varieties have been successfully used for the induction of EC, which serve as a source of protoplasts for transformation.

7. Conclusions

The emergence of CRISPR/Cas genome editing has initiated a new era in which genetic manipulation becomes precise and predictable. The application of CRISPR/Cas has advanced the pace of research in plants, including the grapevine. Targeted modifications

of grapevine traits are highlighted by the improvement of plant resistance to the major diseases, namely, powdery mildew, gray mold, and downy mildew. Plant development, which includes shoot branching, stomata development, and female flower morphology, has also been modified in grapevine by using CRISPR/Cas technologies. The accumulation of important secondary metabolites such as anthocyanins and flavonoids was also improved through the targeted editing of specific genes. Even if in the past 8 years there have been some advances in grapevine genome editing, researchers are still expected to promote the application of CRISPR/Cas technologies in grapevine. We believe that CRISPR technology will play an increasingly important role in grapevine genome engineering due to the predicted innovation in CRISPR technology in the decade ahead.

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Conflicts of Interest: The authors declare no conflicts of interest.

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