Highly efficient activation of endogenous gene in grape using CRISPR/dCas9-based transcriptional activators

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Abstract

Overexpression and knockout (or knockdown) of gene of interest are two commonly used strategies for gene functional study. Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system-mediated gene knockout has been applied in most plant species, including grapevine. However, CRISPR/dCas9 (deactivated Cas9)-based transcriptional activation is still unreported in fruit crops, although a few studies have been documented in Arabidopsis and rice. Here, we tested two transcriptional activators VP64 and TV for transcriptional activation of endogenous genes in grape. Both the dCas9-VP64 and dCas9-TV systems are efficient enough for transcriptional activation of the UDP-glucose flavonoid glycosyltransferases (UFGT) gene in grape cells. The effectiveness of the dCas9-VP64 system in UFGT activation was about 1.6- to 5.6-fold, while the efficiency of the dCas9-TV system was around 5.7- to 7.2-fold. Moreover, in grapevine plants, highly efficient activation of the cold-responsive transcription factor gene CBF4 was achieved by using the dCas9-TV system. The expression of CBF4 was increased 3.7- to 42.3-fold in transgenic plants. Compared with the wild-type plants, the CBF4-activated plants exhibited lower electrolyte leakage after cold treatment. Our results demonstrate the effectiveness of the dCas9-VP64 and dCas9-TV systems in gene activation in grape, which will facilitate application of transcriptional activation in this economically important species.

Introduction

Plant traits are generally controlled by a set of specific genes, and artificial modifications of these genes could improve corresponding traits as designed. For instance, SELF PRUNING (SP) and its paralog SP5G are two regulators of tomato flowering, and simultaneous mutations in the two genes resulted in rapid flowering and enhanced plant compactness [1, 2]. Exploring interactions between phenotypic variations and underlying genes provides solutions for trait improvement of plants, especially agronomically important crops. Due to advances in high-throughput sequencing technologies, the sequencing and assembly of plant genomes highlight plenty of promising genes for plant traits improvement or breeding. However, prior to applications the genes of interest should be cloned and functionally tested, and it remains a big challenge for researchers to clone hundreds of candidate genes rapidly and efficiently. The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system has emerged as a powerful tool for analyzing gene function and crop breeding [3, 4]. To date, the CRISPR/Cas9 system has been widely used for plant genome editing, trait improvement, and development of high-throughput mutant libraries in most crops by taking advantage of endonuclease activity of Cas9 protein [4, 5].

Both loss-of-function and gain-of-function mutations are crucial for dissecting the functions of candidate genes or encoded proteins. CRISPR/Cas9-mediated gene activation has also been reported in plants [6–10]. The native Cas9 protein contains two nuclease domains, HNH and RuvC domain, which can cleave the target DNA when directed by a single guide RNA (sgRNA) in the presence of a protospacer adjacent motif [11]. Introduction of mutations in the HNH (H840A) and RuvC (D10A) domains abolished the endonuclease activity of Cas9 protein, but the binding activity was retained [11]. The nuclease-dead Cas9 (dCas9) can be re-engineered as transcriptional regulator when fused to transcriptional effector domains [12]. There are two major strategies for development of CRISPR activation (CRISPRa) systems. The first one involves the fusion of multiple transcriptional activation domains (TADs) with dCas9, and the other one is
modifying gRNA scaffold to recruit transcriptional activators [12]. The dCas9-VP64 (four tandem repeats of the Herpes simplex viral protein 16) was one of the CRISPRa systems for gene activation in plants [6, 13]. In addition, the TALE and plant-specific EDLL and ERF2m (modified ERF2) TADs were also employed to develop CRISPRa systems [7, 14, 15]. Moreover, potent CRISPRa toolboxes such as CRISPR-Act2.0 and mTALE-Act have been developed in Arabidopsis and rice [6, 7]. Recently, a robust CRISPRa system called dCas9-TV was reported with strong activity in activation of endogenous gene expression in both Arabidopsis and rice [14]. The dCas9-TV system consists of six copies of TALEs and two copies of VP64. The combination of VP64 with additional TADs outperforms the dCas9-VP64 system in gene activation [14].

Although the CRISPRa technologies are available now, the applications of transcriptional activation in plants, especially fruit crops, are still scarce. The efficiency of the CRISPR/Cas9 system in grape (Vitis vinifera L.), an economically important fruit crop worldwide, has been demonstrated as early as in 2016 [16]. Since then this technology has been used to generate mutants of specific genes in this species [17–21]. CRISPR/dCas9-mediated gene activation, however, has not been reported in grape to date. In the present study, we tested two different CRISPRa systems, namely dCas9-VP64 and dCas9-TV, in both grape cells and grapevine plants. In grape cells, the UDP-glucose flavonoid glycosyltransferases (UGFT) gene was successfully activated by using the dCas9-VP64 and dCas9-TV system, respectively. The expression level of UGFT gene after transcriptional activation ranged from 2- to 7-fold compared with control cells. In grapevine plants, the transcription factor gene CBF4 was chosen as the target, and the CBF4 expression was strongly induced (over 19-fold using two sgRNAs) by using dCas9-TV/sgRNA complexes. Our results demonstrate the effectiveness of the dCas9-VP64 and dCas9-TV system in activating expression of endogenous genes in grape, which expands the toolbox of CRISPR and will facilitate applications of transcriptional activation in this important fruit crop.

Results and discussion

Construction of dCas9-VP64 vector and target design

To conduct CRISPR/dCas9-mediated gene activation in grape, we first developed the dCas9-VP64 expression vector by modifying the binary vector pBI121 (Fig. 1). To test the activity of the developed dCas9-VP64 system in gene activation in grape, we initially attempted to use this system to activate the expression of the UGFT gene, which is critical for anthocyanin biosynthesis [22], in 41B grape cells. During the culture of 41B cells, no anthocyanin accumulation was observed in the dark or under light conditions. The expression of UGFT in 41B cells was extremely low when compared with the GT (Gamay Fréaux, a somatic variant of V. vinifera cv. Gamay) cells, which accumulated anthocyanins after exposure to light [23] (Supplementary Fig. S1). The low UGFT expression might be explained by the presence of the retrotransposon Gret1 (grapevine retrotransposon...
Figure 2. Target design for UFGT activation and assay of the sgRNAs activities coupled with the dCas9-VP64 system in tobacco leaves. (a) Schematic diagram of designed targets within the UFGT promoter. The position of transcriptional start site (TSS) was set as +1, and the positions of designed sgRNAs relative to TSS are shown in parentheses. The primers UFGTpro-F and UFGTpro-R were used to amplify the promoter of UFGT. (b) Schematic diagram of the UFGTpro-Fluc reporter vector. A 750 bp-fragment of UFGT promoter (UFGTpro) was ligated to the firefly luciferase (Fluc) reporter gene. The directions of designed sgRNAs are indicated by black arrows. (c) Transient expression results of sgRNAs with the dCas9-VP64 effector. The dCas9-VP64 vectors containing designed sgRNAs were co-injected into the tobacco leaves with the UFGTpro-Fluc reporter vector. Three different combinations of sgRNAs, namely sgRNA1 and sgRNA2 (sgRNA12), sgRNA1 and sgRNA3 (sgRNA13), and sgRNA2 and sgRNA3 (sgRNA23), were tested. The dCas9-VP64 vector without sgRNA was used as control (Ctrl).

1) in the promoter of MYBA1 in 41B cells, as indicated by the polymerase chain reaction (PCR) results (Supplementary Fig. S1) conducted according to the previously described method [24]. We hypothesized that activating the UFGT expression directly might promote anthocyanin biosynthesis in 41B cells. Thus the UFGT gene was selected as the target for activation experiment, and the promoter of UFGT was amplified from 41B cells and analyzed by Sanger sequencing (Supplementary Fig. S2). Three sgRNAs were designed to target the UFGT promoter at sites ranging from −28 and −148 relative to the transcription start site (Fig. 2a and Supplementary Fig. S2). Activities of the designed sgRNAs coupled with the dCas9-VP64 were first evaluated by transient expression in Nicotiana benthamiana leaves. The firefly luciferase (Fluc) driven by the UFGT promoter was used as the reporter (Fig. 2b). According to the results, the expression of Fluc was efficiently induced using single sgRNAs, and similar results were observed when using two sgRNAs (Fig. 2c). Notably, the combination of sgRNA2 and sgRNA3 (sgRNA23) outperformed the other combinations (sgRNA12 and sgRNA13) in activating Fluc expression (Fig. 2c). These results suggested that the developed dCas9-VP64 system was effective and can be applied in grape for activation of endogenous gene expression.

Efficient activation of UFGT gene in grape cells
Inspired by the results obtained in tobacco, we conducted stable transformation of 41B cells with the dCas9-VP64 (dVP) expression vectors. After antibiotic-dependent selection, kanamycin-resistant cells were obtained (Fig. 3a). However, no anthocyanin accumulation was observed in these cells (Fig. 3a), although exogenous T-DNA insertions were successfully detected by PCR (Fig. 3b). Furthermore, the results of quantitative real-time PCR (qPCR) uncovered that the expression of UFGT in all transgenic cells except dVP-UFGTg3 (dCas9-VP64 vector with sgRNA3) was significantly increased when compared with wild-type (WT) cells and cells transformed with empty dVP vector (EVVP64) (Fig. 3c). The variation in UFGT expression level using different sgRNAs suggested a “position effect” of the designed target on gene activation. Moreover, although the expression of UFGT in dVP-UFGTg2 was comparable with that in dVP-UFGTg12, the highest level (nearly 6-fold) of UFGT was achieved using sgRNA23 (Fig. 3c), which suggested that the use of multiple sgRNAs could boost gene activation. Although the UFGT gene was successfully up-regulated by using the dVP system, the effectiveness of this system in transcriptional activation is limited to 1.6- to 5.6-fold in grape cells (Fig. 3c), which is consistent with the results obtained in Arabidopsis and rice [6, 7, 9].
Figure 3. Transcriptional activation of UFGT gene in 41B cells. (a) Phenotypes of 41B cells transformed with dCas9-VP64-UFGTsgRNA vectors. A total of five expression vectors, including three dCas9-VP64 vectors containing single sgRNA (dVP-UFGTg1, dVP-UFGTg2, and dVP-UFGTg3) and two vectors harboring two sgRNAs (dVP-UFGTg12 and dVP-UFGTg23), were introduced into the 41B cells, respectively. The empty dCas9-VP64 vector (EVVP64) was used as the negative control. Scale bars: 0.5 cm. (b) PCR identification of T-DNA insertions in the 41B cells shown in (a). The specific primers designed for dCas9 gene were used for PCR. The plasmid and wild-type genomic DNA were used as the positive (P) and negative (N) controls, respectively. (c) Expression of UFGT in transgenic cells using the dCas9-VP64 system. The Actin1 and GAPDH genes were adopted as internal controls. Significance of differential expression level was determined using one-way ANOVA. (d) Phenotypes of 41B cells transformed with dCas9-TV-UFGTsgRNA vectors. The empty dCas9-TV vector (EVTV) was used as the negative control. Scale bars: 0.5 cm. (e) PCR identification of T-DNA insertions in the 41B cells shown in (d). (f) Expression of UFGT in transgenic cells using the dCas9-TV system. (g) Phenotypes of VvMYBA1-overexpressing (VvMYBA1-OE) cells. The grape cells and protoplasts shown on the right were observed using a microscope. (h) Expression of UFGT in VvMYBA1-OE cells.
The capacity of the CRISPRa system could be improved by using different combinations of TADs [7, 25], so we supposed that the expression of UF GT could be further enhanced by using an improved CRISPRa system. The dCas9-TV consisting of six copies of TALE and two copies of VP64 TADs is a newly developed CRISPRa system that has been demonstrated to be more robust than the dVP system in plants [14]. The plant codon-optimized TV fragment was synthesized and inserted into the pBl-dCas9-VP64 vector instead of the VP64 via AscI and PacI sites (Supplementary Fig. S3). The newly modified vector was renamed pBl-dCas9-TV (dTV) (Supplementary Fig. S4).

Based on the results obtained using the dVP system, the sgRNA1, sgRNA2, and sgRNA23 were used to construct the dTV expression vectors. Similarly, the transgenic cells did not accumulate anthocyanin as expected after a long-time culture under light conditions (Fig. 3d and e). However, the expression of UF GT was improved by using the dTV system combined with sgRNA1 or sgRNA23 (Fig. 3f). The failure in gene activation in dTV-UFGTg2 cells might be explained by the local chromatin structure during exogenous T-DNA integration. For instance, gene transcription in heterochromatin is generally inactive, and the expression of sgRNA and/or Cas9 could be silenced during CRISPR/Cas9-mediated genome editing in plants [16, 26]. As mentioned earlier, the presence of Gret1 retrotransposon inactivates the promoter of MYBA1, resulting in the low expression of UF GT in 41B cells (Supplementary Fig. S1). Here we also overexpressed the VvMYBA1 gene in 41B cells, and the VvMYBA1-overexpressing (VvMYBA1-OE) cells can accumulate anthocyanins under light conditions (Fig. 3g). Anthocyanin accumulation was further confirmed in protoplasts isolated from VvMYBA1-OE cells, whereas no anthocyanin was observed in dTV-UFGTg23 protoplasts (Fig. 3g). We noticed that the expression level of UF GT in VvMYBA1-OE cells was more than 25-fold higher than that in WT cells (Fig. 3h). In contrast, the increase in UF GT transcript abundance by using the dVP and dTV systems was less than 10-fold (Fig. 3c, f). These results suggested that higher fold activation of UF GT might be required for anthocyanins accumulation.

The expression of sgRNAs and Cas9 has been reported to affect genome editing efficiencies in plants [27–29]. The expression profiles of sgRNAs and dCas9 were then characterized in the transgenic cells (Fig. 4). Compared with the dVP-UFGTg2 cells, the failure in gene activation in dTV-UFGTg2 might be explained by the lower expression level of sgRNA2 (Fig. 4b). Notably, the expression levels of sgRNAs and dCas9 in dTV-UFGTg23 were less than those in dVP-UFGTg23 (Fig. 4b–d). However, the dTV-UFGTg23 cells exhibited higher expression of UF GT than the dVP-UFGTg23 cells (Fig. 3). Additionally, the UF GT expression in dTV-UFGTg1 was comparable with that in dVP-UFGTg23 (Fig. 3). These results suggested that the expression of sgRNAs and dCas9 is important, while the type of transcriptional activators is the predominant factor affecting gene activation. Increasing the expression of sgRNAs and dCas9 is therefore a promising strategy for boosting the activation efficiency of CRISPRa systems.

**Highly efficient activation of CBF4 gene in grapevine plants**

In grape cells, the expression of the structural gene UF GT was successfully activated by using the dVP and dTV systems (Fig. 3). Considering the presence of untransformed cells, a prolonged culture (>6 months) of grape cells in liquid selective medium could greatly increase the percentage of transformed cells, which is important for determination of gene expression in cell cultures. Although grape cells have been successfully used for CRISPR experiments [16, 29], it would be better to test the CRISPRa systems in whole plants. We then decided to perform transcriptional activation in grapevine plants by targeting a transcription factor gene. Low temperature is one of the environmental factors that negatively affect the growth and production of grape [30, 31]. CBFs are key transcription factors that play critical roles in cold response in plants [32, 33], and in grape at least four CBFs genes have been identified [34, 35]. Among the four CBFs, the CBF4 was rapidly and strongly induced by cold treatment [35]. Thus, the CBF4 was selected as the target for gene activation by using the dTV system in grapevine plants.

Two sgRNAs were designed to target the CBF4 promoter at sites ranging from −67 and −350 relative to the transcription start site (Fig. 5a and Supplementary Fig. S5). Activity assay by transient expression in tobacco leaf showed that the designed sgRNAs can successfully direct the dTV protein to activate the expression of Fluc reporter gene (Fig. 5b). The CRISPRa reagents were introduced into 41B cells via Agrobacterium-mediated transformation. The kanamycin-resistant cells (Fig. 5c) were then transferred to the regeneration medium to induce somatic embryos (Fig. 5d). The induced embryos further germinated under light conditions and finally developed into whole regenerated plants (Fig. 5e and f). These plants were identified by PCR using dCas9-specific primers (Supplementary Table S1). A number of 22 plants were used for the identification of transgenic plants for the sgRNA1 (dTV-CBF4g1), sgRNA2 (dTV-CBF4g2), and sgRNA12 (dTV-CBF4g12), respectively. Among the tested plants, 8, 3, and 5 plants were identified as transgenic plants, respectively (Fig. 5g and Supplementary Fig. S6). To check the expression of CBF4, 3 plants randomly selected from dTV-CBF4g1 and dTV-CBF4g12, respectively, together with the 3 dTV-CBF4g2 plants, were used for the measurement of CBF4 expression by qPCR. The plants identified with no exogenous T-DNA were used as the negative controls. The qPCR results showed that the CBF4 expression was successfully activated in these plants, although the effectiveness in transcriptional activation is variable among different plants (Fig. 6a). The combination of the two sgRNAs unsurprisingly resulted in the highest expression level (19.3- to 42.3-fold) of CBF4 (Fig. 6a),
confirming that multiple sgRNAs in one experiment could improve the transcriptional activation [7, 14]. As observed in the experiments conducted in grape cells, the difference in CBF4 expression was also detected between dTV-CBF4g1 and dTV-CBF4g2 plants (Fig. 6a), suggesting that the locations of target sites could affect the efficiencies of gene activation. The results are consistent with the findings reported in previous studies [8, 13, 36, 37]. However, the correlation between sgRNAs locations and gene activation efficiencies has not been established [14]. In addition, the parameters of sgRNAs such as length and guanine and cytosine content should also be systemically studied in the future to optimize the CRISPRa system for a given transcriptional activator.

The dTV-CBF4g12 and control plants were used for cold treatment (−4°C, 0.5 h), and electrolyte leakage of these plants was measured to evaluate their tolerance to cold stress. Among the 3 dTV-CBF4g12 plants, the first two (#12–1 and #12–2) exhibited significantly lower electrolyte leakage when compared with the control plants (WT-1 and WT-2) (Fig. 6b), which suggested that the two plants were more resistant to cold. The plant #12–3 also exhibited a lower electrolyte leakage, although no significant difference was observed when compared with WT plants (Fig. 6b). These results show that activating CBF4 in grapevine plants could improve their cold tolerance.

CBFs could bind to the C-repeat (CRT) elements on the promoters of cold-responsive (COR) genes and induce their expression under cold stress. According to the previous studies, COR genes such as COR15A, KIN2 (KINASE 2), RD29A (Responsive to Desiccation 29A), and GolS3 (Galactinol synthase 3) are the downstream targets of CBFs in Arabidopsis [38, 39]. The counterparts in grapevine of these genes were identified through sequence BLAST (Supplementary Table S2), and the expression profiles of these COR genes in dTV-CBF4g12 plants after cold treatment were investigated by qPCR. The expression of RD29A was not detected in grapevine plants likely due to a very low expression of this gene in these conditions (data not shown). The COR15A and KIN2 genes were instead significantly induced by cold in dTV-CBF4g12 plants when compared with WT plants (Fig. 6c). However, no significant difference was observed in GolS1 expression between WT and dTV-CBF4g12 plants (Fig. 6c).

More recently, several strategies have been successfully adopted for optimization of the dTV system [40]. The employment of the polycistronic tRNA-sgRNA expression cassette (PTG) driven by a Pol II promoter greatly

Figure 4. Expression profiles of UFGT sgRNAs and dCas9 in transgenic cells. The expression level of sgRNA1 (a), sgRNA2 (b), sgRNA3 (c), and dCas9 (d) relative to the internal controls (Actin1 and GAPDH) was measured by qPCR. Significance of differential expression was determined using one-way ANOVA.
Figure 5. Transcriptional activation of CBF4 in grapevine plants. (a) Schematic diagram of the target design for CBF4 activation. The positions of designed sgRNAs relative to the transcription start site (TSS, +1) are shown in parentheses. (b) Transient expression results of sgRNAs with the dCas9-TV effector in tobacco leaf. The empty dCas9-TV vector without sgRNA was co-injected with CBF4pro-Fluc reporter, serving as the negative control (Ctrl). (c) Antibiotic-resistant cells developed on kanamycin-containing medium. (d) Induction of somatic embryos. Somatic embryos were induced from the kanamycin-resistant cells on regeneration medium. (e) Germination of induced embryos. The somatic embryos further germinated on regeneration medium under light conditions. (f) Regenerated plants. The germinated embryos developed into whole plants on regeneration medium. (g) Overview of identified transgenic plants. Scale bars: 0.5 cm.

improved gene activation in rice. In the previous study, we had demonstrated the efficacy of the PTG in grape, and the identified grape promoters can also be used to optimize the CRISPRa systems. In summary, both the dVP and dTV systems are efficient for transcriptional activation of endogenous genes in grape, and the two CRISPRa systems, especially the dTV system, can be utilized to efficiently activate the expression of genes of interest for basic and applied research in this species.

Materials and methods
Target design and construction of CRISPRa vectors
The promoters of UFGT (VIT_16s0039g02230) and CBF4 (VIT_16s0100g00380) genes were amplified from 41B cells using their specific primers (Supplementary Table S1), respectively. The amplified promoter fragments were cloned into the pLB vector (TIANGEN, China) and analyzed by Sanger sequencing. The verified sequences of promoters were used as inputs for target design using the online tool CRISPR-GE [41].

To generate Fluc reporter vectors, the UFGT and CBF4 promoters were amplified from the pLB vectors and ligated into the pCAMBIA1302-Fluc vector, respectively, via homologous recombination (HR) using the ClonExpress II One Step Cloning Kit (Vazyme, China).

To develop the dCas9-VP64 expression vector, the dCas9-VP64 coding sequence was amplified from the pcDNA-dCas9-VP64 plasmid (Addgene: #47107) using the primers dVP-121-F and dVP-121-R (Supplementary Table S1) and ligated into the pBI121 vector in place of β-glucuronidase (GUS) gene through BamHI and SacI sites via HR. To construct the dCas9-TV vector, the plant codon-optimized TV fragment (Supplementary Fig. S3) was commercially synthesized (Tsingke, China) and inserted into the dCas9-VP64 vector in place of the VP64 via the AscI and PacI sites. The pCACRISPR/Cas9 vector [16] was used as the template to assemble AtU6-sgRNA expression cassettes by PCR. The individual sgRNAs expression cassettes were developed according to the method as previously described [16]. Briefly, two pairs of primers were designed to amplify the AtU6 promoter and sgRNA scaffold, respectively, and the 20-bp target sgRNA was used as the adaptor to ligate the two elements by PCR. To combine two sgRNAs expression cassettes, adaptors were added to both the reverse primer for the first sgRNA cassette and the
Figure 6. Expression of *CBF4* and downstream cold-responsive genes in transgenic plants and electrolyte leakage assay after cold treatment.

(a) Expression level of *CBF4* in transgenic grapevine plants using the dCas9-TV system. A number of 3 transgenic plants randomly selected from dTV-CBF4g1, dTV-CBF4g2, and dTV-CBF4g12, respectively, were used for expression measurement by qPCR. The non-transgenic plant (or wild-type, WT) was used as control. (b) Electrolyte leakage assay of *CBF4*-activated plants after cold treatment. Two WT plants and the three dTV-CBF4g12 plants were used for cold treatment (−4 °C, 0.5 h), and electrolyte leakage of these plants was measured to evaluate their cold tolerance. (c) Expression profiles of downstream cold-responsive genes in WT and dTV-CBF4g12 plants after cold treatment. Significant difference was determined by Student’s t-test. *P* < 0.05, ***P* < 0.001.

forward primer for the second sgRNA cassette, and introduction of adaptors to the two different sgRNA cassettes allows the ligation of the two cassettes by overlapping PCR. For example, the primer sets AtU6-UFGTgR-F/AtU6-UFGTg1-R1 and AtU6-UFGTg1-F2/AtU6-UFGTgR-R (Supplementary Table S1) were designed to develop the AtU6-UFGTg1 expression cassette by PCR with the pCACRISPR/Cas9 being used as the template. The AtU6-UFGTg2 expression cassette was constructed similarly. The AtU6-UFGTg1 or AtU6-UFGTg2 cassette could be directly ligated into the BsaI-digested dCas9-VP64 (or dCas9-TV) vector through HR. To construct the AtU6-UFGTg12 expression vector, the AtU6-UFGTg1 and AtU6-UFGTg2 cassettes were amplified from the well-constructed dCas9-VP64 vectors by using the primer sets AtU6-UFGTg12/23-F1/AtU6-UFGTg12/23-R1 and AtU6-UFGTg12/23-F2/AtU6-UFGTg12/23-R2, respectively. Then the two amplified cassettes were ligated together by overlapping PCR using the primers AtU6-UFGTg12/23-F1 and AtU6-UFGTg12/23-R2. The fused sgRNAs expression cassette was finally ligated into the dCas9-VP64 vector via the BsaI site. All the primers for the construction of vectors are listed in the Supplementary Table S1. The pCAMBIA2300-VvMYBA1 overexpression vector was kindly provided by Dr W. Liu (Institute of Botany, Chinese Academy of Sciences).

**Plant materials, transformation, and regeneration**

The 41B (*V. vinifera* cv. Chasselas × *V. berlandieri*) embryogenic cells were cultured in glycerol-maltose (GM) medium containing 1 mg L⁻¹ naphthyox acetic acid (NOA). The transformation of 41B cells and plant regeneration, as well as identification of exogenous T-DNAs, were conducted as previously described [19, 29]. For the transformation, around 1 mL of 41B cell cultures were used as the explants for *Agrobacterium*-mediated transformation, and three independent transformation experiments were conducted as three technical replicates. For regeneration, the 41B cells were cultured in liquid selective medium (GM supplemented with 200 mg/L timentin and 5 mg/L paromomycin) for two months and then transferred to regeneration medium (GM without NOA) to generate plants [19].
Transgenic expression experiment

Five- to six-week-old *N. benthamiana* plants were used for transgenic expression experiment. To test the activities of designed sgRNAs, the bacterial cells containing sgRNAs and those cells harboring the Fluc reporter vector were mixed at a ratio of 5:1, and the mixture was incubated at room temperature (RT) for 2 h before injection. The fluorescence was detected using a CCD camera (Tanon 5200, China) 3 days after injection.

qPCR assay

Total RNA was prepared using the HiPure HP Plant RNA Mini Kit (Magen, China) following the manufacturer’s protocols. The first strand of cDNA was synthesized from 1 μg of total RNA using the HiScript II Q RT SuperMix for qPCR Kit (Vazyme, China) according to the instructions. qPCR assays were conducted using AceQ qPCR SYBR Green Master Mix (Vazyme, China) with the CFX Maestro system (Bio-Rad, USA) as previously described [42], and grape Actin 1 and GAPDH were used as internal controls. The relative expression levels of genes were calculated using the 2−ΔΔCT method [43]. The data were obtained from three biological and three technical replicates. The significance of gene expression was determined by using one-way ANOVA or Student’s t-test.

Electrolyte leakage assay

The in vitro plants of grapevine grown under normal conditions (25°C, 16 h light/8 h dark) were transferred to the incubator set to 4°C and cultured for 12 h. Then the temperature was decreased to −4°C slowly (1°C/min) and kept for 0.5 h. After treatment, the plants were cultured at 4°C for 12 h and then recovered at 26°C for 1 d. The leaves of each plant were pooled and sampled as discs with a diameter of 0.5 cm, and three leaf discs collected as a biological replicate were immersed in 4 ml of deionized water (S0). After shaking at 180 rpm and RT for 20 min, the initial conductivity (S1) was measured using FE30 (Mettler Toledo, Switzerland). The samples were then boiled for 20 min and shaken at 180 rpm and RT for another 20 min. The conductivity was remeasured as S2. Electrolyte leakage was calculated as (S1−S0)/(S2−S0). Three biological replicates were considered for each plant.

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Author contributions

C.R. and Z.L. designed the experiments. C.R., H.L. and Y.L. performed the experiments. C.R. and S.L. wrote the manuscript. S.L. and Z.L. edited the manuscript. All of the authors approved the final manuscript.

Data availability

The data supporting the results of this study are available in the article and supplementary material.

Conflict of interest statement

The authors declare no conflict of interest.

Supplementary data

Supplementary data is available at Horticulture Research Journal online.

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