

Fig. S1 Sequence alignment of the coding sequence of the *DFR2* gene in different genome annotations. The gene sequences from commonly used version 2 (V2) and newly annotated version 4 (V4) grape genomes were used for alignment.



Fig. S2 PCR identification of transgenic plants regenerated from 41B embryogenic grape cells transformed with LbCas12a-TMT1 without (a) or with (b) heat treatment. The constructed plasmid and genomic DNA from wile-type plant were used as positive (P) and negative (N) controls, respectively. Lanes 1-25 in (a) and Lanes 1-29 in (b) represent independent regenerated plants.

(a) TMT1-F2 TMT1-F1 Target1 Target2		
437 bp		
1136 bp		
(b)	N	o of clones
ета аст <u>тт сресалее атое е аталт</u> аттеса еет стте а <u>ттте са ла еса стета е атт</u> е те ест	АТСТ Т	0. 01 010103
Anna Mana an Anna Anna Anna An	AAAA	4/6
	<u>VVVV</u> ⊦39 bp	
οτλας <u>τητ στα σα απο απο απο απο απο τα στα</u> τη στη σα <u>τητοίο αλλά σα στα τα σ</u> α α Α	TAGCC	
*2 MMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMM	MM	2/6
	ттете	
		c/c
Ammand Mandaland Mandaland	WW	6/6
WT « ۵ ۵ ۲ ۸ ۸ ۵ <u>۲ ۲ ۲ ۵ ۲ ۵ ۵ ۸ ۲ ۵ ۵ ۵ ۸ ۲ ۸ ۵ ۵ ۸ ۲ ۸ ۲</u>	, ТТ <mark>СТС</mark>	
#5 1 4	A A	6/6
	WW	
↓ -7 bp		
	GGCTA	3/6
MMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMMM	\mathcal{M}	0,0
#12 6 6 T A A C T T T C T 6 C A A 6 6 A T A 6 6 A T A 6 C A C T 6 A T T 6 C A 6 6 T T 6 A T T 6 C A 6 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 A 6 C A C T 6 T A 6 C A C T 6 C A C T 6 C A C T 6 C A C T 6 C A C A C T 6 C A C A C T 6 T A 6 C A C A C A C A C A C A C A C A C A	ATGTC	
		3/6
Main Main Mark Mark Mark Mark Mark Mark Mark Mark	MM	
Target 1 Target 2	Mutation	No. of clones
#3 TAACTTTCTGCAAGGATGGGATACTAT/290bp/ATTTGGAAGGCACTGTAGAA	-6/-13 bp -6 bp/WT	2/6 4/6
TAACTTTCTGCAAGGATGGGATAATGCTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTG	-3/-3 bp	3/6
LTAACTTTCTGCAAGGATGGGATACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTGTG	-6/-4 bp -8/-6 bp	3/6
#6 TAACTTTCTGCAAGGATGGGATACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTG	-6/-3 bp	3/6
LTAACTTTCTGCAAGGATGGGATAACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTGTG	-5/-4 bp -5/-4 bp	2/6
#7 TAACTTTCTGCAAGGATGGGATACTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTG	-3/-3 bp	1/6
#8	-7/-4 bp -3/-5 bp	3/6 3/6
TAACTTTCTGCAAGGATGGGATAACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTG	-5/-3 bp	2/6
LTAACTTTCTGCAAGGATGGGATATACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGTGTG LTAACTTTCTGCAAGGATGGGATAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG	-4/-5 bp -9 bp/WT	4/6 2/6
#10_TTAACTTTCTGCAAGGATGGGATACTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGTTGTG	-3/-6 bp	4/6
TAACTTTCTGCAAGGATGGGATACTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTGTG	-3/-4 bp -6 bp/WT	1/6 2/6
TAACTTTCTGCAAGGATGGGATAAACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTG	-4/-3 bp	2/6
#13 TAACTTTCTGCAAGGATGGGATAATGCTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG	WT/WT	6/6
#14 TAACTTTCTGCAAGGATGGGATAAT/2900p/ATTTGGAAAGCACTGTAGAAGTTGTG TAACTTTCTGCAAGGATGGGATAATT/2900p/ATTTGGAAAGCACTGTAGAAGGGTGTG	-8/-6 bp -7/-5 bp	2/6 4/6
TAACTTTCTGCAAGGATGGGATACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG #15	-6 bp/WT -3/-5 bp	5/6 1/6
TAACTTTCTGCAAGGATGGGATAAACTAT/290bp/ATTGGAAAGCACTGTAGAAGGGCTTGTG	-4/-3bp	4/6
#16 TAACTTTCTGCAAGGATGGGATAATGAT/290bp/ATTTGGAAAGCACTGTAGAAGTTGTG TAACTTTCTGCAAGGATGGGATAATGCTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG	-5/-6bp -3 bp/WT	1/6 1/6
TTAACTTTCTGCAAGGATGGGATAATT/290bp/ATTTGGAAAGCACTGTAGTTGTTGTG	-7/-6bp	3/6
LTAACTTTCTGCAAGGATGGGACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGTTGTG LTAACTTTCTGCAAGGATGGGATGCTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGTTGTG	-8/-4 bp -3/-6bp	3/6 5/6
#18 TAACTTTCTGCAAGGATGGGATAATGCTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGTTGTG	WT/-5 bp	1/6
TAACTTTCTGCAAGGATGGGATAATGCTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG	WT/WT -7/-6bp	1/6 4/6
TAACTTTCTGCAAGGATGGGATACTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGTTGTG	-3/-5 bp	1/6
<pre>#20 TAACTTTCTGCAAGGATGGGATAACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTG #20 TAACTTTCTGCAAGGATGGGATATACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGTGTG</pre>	-5/-3 bp -4/-5 bp	3/6 3/6
TTAACTTTCTGCAAGGATGGGATAACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGTTGTG	-5/-5 bp	1/6
LTAACTTTCTGCAAGGATGGGATATAT/290bp/ATTTGGAAAGCACTGTAGAAGGGTGTG TTAACTTTCTGCAAGGATGGGATACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTGTG	-7/-5 bp -6/-4 bp	5/6 3/6
#22 TAACTTTCTGCAAGGATGGGATAACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTG	-5/-3 bp	3/6
#23 TAACTTTCTGCAAGGATGGGATACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTGTG TAACTTTCTGCAAGGATGGGATAATGAT/290bp/ATTTGGAAAGCACTGTAGTTGTTGTG	-6/-4 bp -5/-6 bp	4/6 2/6
#24 TAACTTTCTGCAAGGATGGGATAT/290bp/ATTTGGAAAGCACTGTAGAATTGTG	-9/-7 bp	2/6
LIAACIITUTGCAAGGATGGGATAATGTAT/290Dp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG [TAACTTTCTGCAAGGATGGGATACTACTAT/290Dp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG	-4 bp/WT -3 bp/WT	4/6 2/6
TAACTTTCTGCAAGGATGGGATAACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGTTGTG #26 maactmmcmccaaccamcaaccamaa	-5/-4 bp	2/6
TAACITTCTGCAAGGATGGGATAT/290Dp/ATTTGGAAAGCACTGTAGAAGGTTGTG TAACTTTCTGCAAGGATGGGATTAT/290Dp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG	-э/-5 бр -8 bp/WT	1/6
TAACTTTCTGCAAGGATGGGATAAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG TAACTTTCTGCAAGGATGGGATG	-8 bp/WT -7/-4 bp	1/6
#27 TAACTTTCTGCAAGGATGGGATAATAT/230bp/ATTTGGAAGGCACTGTAGAAGGGCGTG	-6/-5 bp	3/6
LTARCITTCTGCAAGGATGGGATAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG TTAACTTTCTGCAAGGATGGGATACTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGTTGTG	-9 bp/WT -3/-5bp	1/6 5/6
#28 TAACTTTCTGCAAGGATGGGATAAACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGTCCGCCTCA	-4/-14,+11	bp 1/6
TAACTTTCTGCAAGGATGGGATAATG-ACTAT/290bp/ATTTGGAAAGCACTGTAGAATGTG TAACTTTCTGCAAGGATGGGAT/290bb/ATTTGGAAAGCACTGTAGAAGGGTTGTG	-2/-8 bp -11/-4 bp	2/6 1/6
#29 TAACTTCTGCAAGGATGGGATAACTAT/200b/ATTTGGAAGCACTGTAGCTGTG	-5/-10 bp	2/6
LIANCIIICIGCAAGGAIGGGAIAAACTAT/290DD/ATTTGGAAAGCACTGTAGAAGTGTG	-4/-/ bp	1/0

Fig. S3 Sequencing results of *TMT1* editing in LbCas12a-TMT1 grapevine plants. The results obtained with transgenic plants after HT are shown as examples. (a) Schematic illustration of primers design for amplification of target sequences of *TMT1* gene. (b) Sanger sequencing results of the two targets in grapevine plants. Representative sequencing chromatograms as well as identified sequences are shown. The PAM sequences are underlined and the targets are shown in red box in chromatograms. For each plant, 6 clones of amplicons were randomly selected and analyzed by Sanger sequencing. Mutation types and corresponding number of clones are shown on the right.



Fig. S4 Knockout of *TMT1* and *TMT2* genes in grape. (a) Schematic illustration of the targets design for *TMT2*. (b) Representative mutated sequences detected at the four targets in *TMT1* and *TMT2*. (c) Sugar content in *tmt1tmt2* cells. The cells transformed with empty vector were used as the control (CK). The significant differences are determined by Student's *t*-test. * P < 0.05; ** P < 0.01. Suc, sucrose; Glc, glucose, Fru, fructose.

(a)	↓-7 bp TMT1 Target 1 TMT1 Target 2 WT	No. of	clones
	A G A A CA A G TA C CT G CAA <mark>TA G A T C C C A T C C T T G C A G A A A A G C C C T T T A C A G G C C T T T A C A G G C C T T T A C A G G C C T T T A C A G G C C T T T A C A G C C C T T T A C A G C C C T T T A C A G C C C T C T A C A G C C A C A A C A A G C C A C A A C A A G C C A C A</mark>	<u>1 CAA</u> 2	6
		MM	
	↓-5 bp TMT1 Target 1 /M11 larget 2↓-18 bp A 6 A A C A A 6 T A C T C 6 A A T A T C C C A T C A C C A C T T C C A A C A C	CA A G	
	in March March March March March March March March	4/ MM	6
Plant 3		TATC	
		1/	6
	WT 7////////////////////////////////////	<u>MW</u>	
		ATA 6	6
	Marinethether and the second of the second se	<u> </u>	•
	7///7 Target 1 WT 7//7/1 Target 2 WT T 10 0 ΤΛΛ C T T 1 C C Λ 0 0 Λ Τ 1 0 C Λ 0 0 Λ 0 C T 1 0 Λ Λ 0 C Λ C T 1 Λ Λ 0 C Λ C T 1 0 C Λ 0 0 Λ 0 C T 1 0 Λ Λ 0 C Λ C T 1 0 C Λ 0 0	CTAT	
	France and a second and a second and a second and a second s	6/	6
Plant 7	7MT2 Target 1 WT 7MT2 Target 2 WT		
i idirit i	CATCCCTATTTTATTTT GTCA 6 G C C TT 6 T 6 A T 6 C T 6 T 6 G C A C C C C C C A T 6 T T A T T A G C C T 6 C T 6 C C A 6 G C T T A C C C C C C C C C C C C C C C C C C C	C T G G	
	- Manana Ma	M 6/	6
(b)		Mutation	No. of clones
	TMAT Target 1 TMAT Target 2 TMAT Target 2 TMAT Target 2 TTACTTTCTGCAAGGATGGGATACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG	-6 bp/WT	2/6
Plant	TAACTTTCTGCAAGGATGGGATAAT/290bp/ATTTGGAAAGCACTGTAGAAGTTGTG TAACTTTCTGCAAGGATGGGATAATGCTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG	-8/-5 bp WT/WT	1/6 3/6
Plant	TAACTTCTGCAAGGATGGGATAATGCTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG	WT/WT	4/6
Plant	TAACTTICTGCAAGGATGGGTATCTACTAT/290bp/ATTTGGAAGGCACTGTAGAAGGCTTGTTGG	-3/WT	3/6
Flant	LTAACTTTCTGCAAGGATGGGATAATGCTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG TAACTTTCTGCAAGGATGGGATAATGCTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG	WT/WT	3/6
Plant	5 TAACTTTCTGCAAGGATGGGATAATGCTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGTTGTG	WT/-4 bp	1/6
Plant	6 TAACTTTCTGCAAGGATGGGATAATGCTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG	WT/WT	6/6
Plant	8 TAACTTCTGCAAGGATGGGATAATGCTACTAT/290bp/ATTTGGAAGGACTGTAGAAGGG	-8/-60 bp WT/-15 br	1/6 p 1/6
		WT/WT	4/6
Plant	TAACTTICTGCAAGGATGGGATAAAT/290bp/ATTTGGAAAGCACTGTAGAAGGGTGTG 9 TAACTTICTGCAAGGATGGGATAAT/290bp/ATTTGGAAAGCACTGTAGAAGGGGTTGTG	-7/-5 bp -8/-3 bp	1/6 2/6
	LTAACTTTCTGCAAGGATGGGATAATGCTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGGG	WT/WT	3/6
Plant 1	⁰ TAACTTTCTGCAAGGATGGGATAACTAT/290bp/ATTTGGAAAGCACTGTAGAAGTTGTG	-5/-6 bp	2/6
Plant 1	TAACTTTCTGCAAGGATGGGATGCTACTAT/290bp/ATTTGGAAAGCACTGTAGAATTGTG	-3/-7 bp -3 bp/WT	1/6 3/6
	TAACTTTCTGCAAGGATGGGATAACTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG	-2 bp/WT	2/6
Plant 1	2 TAACTTTCTGCAAGGATGGGATA7/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG TAACTTTCTGCAAGGATGGGATAT/290bp/ATTTGGAAAGCACTGTAGAAGGGCTTGTTGTG	-10 bp/W1 -9 bp/WT	T 2/6 4/6
Plant 1	TAACTTTCTGCAAGGATGGGATA-TGCTACTAT/290bp/ATTTGGAAAGCACTGTAGAAGGGTTGTG	-1/-4 bp	3/6
(c)		-3/-3 bp	5/0
Plant	TMT2 Target 1 TTTATTTGTCAGTGGCCTTGTGATGCTGGGGCTCCCCAATGTTTATGTGCGGCTGTGGCAAGGCTTCTGGA	Mutation N WT/WT	lo. of clones 6/6
Plant	2 TTTATTTTGTCAGTGGCCTTGTGATGCTGTGGGTCTCCCAATGTTTATGTGCTGCTGCTGGCAAGGCTTCTGGA	WT/WT	6/6
Plant	TTTATTTGTCAGTGGCCTTGTGATGCTGTGGGTCTCCCAATGTTTATGTGCTGCTGGTGGCAAGGCTTCTGGA TTTATTTTGTCAGTGGCCTTGTGATGCTGTGGGTCTCCCAATGTTTATGTGCTGCTGCTGGCAAGGCTTCTGGA	WT/WT	6/6
Plant	6 TTTATTTGTCAGTGGCCTTGTGATGCTGTGGGTCTCCCAATGTTTATGTGCTGCTGTTGGCAAGGCTTCTGGA	WT/WT	6/6
Plant	8 TTTATTTTGTCAGTGGCCTTGTGATGCTGTGGGCCTCCCAATGTTTATGTGCTGCTGTTGGCAAGGCTTCTGGA	WT/WT	6/6
Plant	3 TTTATTTTTGTCAGTGGCCTTGTGATGCTGTGGGCCTCCCAATGTTTATGTGCCGCGCTGTGGCAAGGCTTCTGGA AD TTTATTTTTGTCAGTGGCCGCTTGTGGATGCTGGCGGATGGCCGAATGTTTATGTGCCGCGCGCG	WT/WT	6/6
Plant '	WITTATTTTGTCAGTGGCCTTGTGATGCTGTGTGGCCCCAATGTTTATGTGCTGCTGTTGGCAAGGCTTCTGGA	WT/WT	0/0
Plant '	12 TTTATTTTGTCAGTGGCCTTGTGATGCTGTGTGGGCCCCCAATGTTATGTGCCGCTGCTGGCAAGGCTTCTGGA	WT/WT	6/6
	TTTATTTGTCAGTGGCCTTGTGATGCTGTGGTGGTCTCCCAATGTTTATGTGCTGCTGTTGGCAAGGCTTCTGGA	WT/WT	4/6
Plant 1	JTTTATTTTGTCAGTGGCCTTGTGGTCTCCCAATGTTTATGTGCTGCTGTTGGCAAGGCTTCTGGA	-8 bp/WT	2/6

Fig. S5 Sequencing results of *TMT1* and *TMT2* editing in LbCas12a-TMTs plants. Representative sequencing chromatograms (a) and identified sequences for each plant (b and c) are shown. The PAM sequences are underlined and the targets are shown in red box in chromatograms. For each plant, 6 clones of amplicons were randomly selected and analyzed by Sanger sequencing. Mutation types and corresponding number of clones are shown on the right.



Fig. S6 Schematic illustration of the targets design for PDS1.



Fig. S7 Predication of the secondary structures of *PDS1* and *TMTs* crRNAs. The RNA secondary structures were predicted using *RNAfold* web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) with default parameters.



Fig. S8 Heatmap of the determined compounds in dfr1 and EV cells. The log2-transformed values were visualized as the heatmap using R.

VvU3. 1-TMT1 crRNAs:

VvU3. 1-TMTs crRNAs:

The reverse *TMTs*-crRNAs:

TTTTTTT<mark>AAGCTT</mark>

The reverse *PDS1-TMTs-*crRNAs:

Fig. S9 Sequences of *TMT1*, *TMTs* (*TMT1* and *TMT2*), reverse *TMTs* and reverse *PDS1*-*TMTs* crRNAs expression cassettes. The VvU3.1 promoter is indicated in blue and DR sequences are denoted in red. The two designed *TMT1* crRNA targets are indicated in green and purple, and the two crRNA targets for *TMT2* are indicated in orange and light blue, respectively. The two crRNA targets for *PDS1* are indicated in black and pink, respectively. The *Hin*dIII recognition sites are highlighted in yellow.

VvU3. 1-DFR1 crRNAs:

Fig. S10 Sequence of *DFR1* crRNAs expression cassette. The VvU3.1 promoter is indicated in blue and DR sequences are denoted in red. The two designed crRNA targets for *DFR1* are underlined. The *Hin*dIII recognition sites are highlighted in yellow.