**Supplemental Figure 1** 



**Supplementary Figure S1.** Neighbor-Joining Analysis of TM6 and euAP3 lineage proteins. Representative AP3 lineage proteins from core eudicots to gymnosperms were included in the analysis. The two AP3-like proteins from *Fragaria vesca* (FvH4\_1g12260 and FvH4\_2g38970) are represented in bold types. Four *PISTILLATA* genes were used as outgroup. Numbers next to the nodes are bootstrap values from 1000 pseudo-replicates. The protein sequences were obtained from GenBank (see Accession numbers section).

	1	M-domain				l region	
FveTM6	MGRGKTETKL	TENOTNROVT	YSKRRNGTEK	KAOELTVICD	AHVSLTMOSS	TDETHEYTSP	TTTTHKKMFDL
MASAKO B3	MGRGKIEIKL	IENOTNROVT	YSKRRNGIFK	KAOELTVLCD	AOVSLIMOSS	TDKIHEYISP	TTTTKKMFDL
PaTM6	MGRGKIEIKL	IENHTNROVT	YSKRRNGIFK	KAOELTVLCD	ARVSLIMLSN	TGKMHEYISP	TTTTKRMYDD
MdMADS13	MGRGKIEIKL	IENOTNROVT	<b>YSKRRNGIFK</b>	KAOELTVLCD	AKVSLIMLSN	TNKMHEYISP	TTTTKSMYDD
MdTM6	MGRGKIEIKL	IENÕTNRÕVT	<b>YSKRRNGIFK</b>	KAOELTVLCD	AKVSLIMLSN	TSKMHEYISP	TTTT <mark>K</mark> SMYDD
VvTM6	MGRGKIEIKR	IENPTNROVT	<b>YSKRRNGIFK</b>	KAOELTVLCD	AKVSLIMFSN	TGKFHEYTSP	TITT <mark>KK</mark> VYDO
PhTM6	MGRGKIEIKK	IENSTNROVT	<b>YSKRRNGLFK</b>	KAKELTVLCD	AKICLIMLSS	TRKFHEYTSP	NTTTKKMIDL
LeTM6	MGRGKIEIKK	IENSTNROVT	<b>YSKRRNGIFK</b>	KAKELTVLCD	AKISLIMLSS	TRKYHEYTSP	NTTTKKMIDO
FveAP3	MARGKIOIKR	IENVTNROVT	YSKRRNGLFK	KANELTVLCD	AKVSLLMVSS	SGKLHEFISP	STTTKOVYDE
MASAKO euB3	MARGKIOIKR	IENVTNROVT	YSKRRNGLFK	KANELTVLCD	AKVSLLMVSS	SGKLHEFISP	STTTKOVYDE
AtAP3 —	MARGKIÕIKR	IENOTNROVT	YSKRRNGLFK	<b>KAHELTVLCD</b>	ARVSIIMFSS	SNKLHEYISP	NTTT <mark>KEIV</mark> DL
LeAP3	MARGKIQIKK	IENQTNRQVT	YSKRRNGLFK	<b>KANELTVLCD</b>	AKVSIVMISS	TGKLHEFISP	SITT <mark>K</mark> QLFDL
	Lregio	n	1/1				
•	7 <u>1 Tregio</u>	<u></u>	K1			K2	
FveTM6	YQ <mark>K</mark> NLQI <b>DLW</b>	SSHYE AMKEN	LWKLKEVNNK	L <mark>RR</mark> DIR <mark>QR</mark> LG	HDLDGLNLAE	L <mark>QDLEDK</mark> IAQ	SVQII <mark>RERK</mark> Y
MASAKO_B3	YQ <mark>K</mark> NLQIDLW	SSHYEAMKEN	LWKLKEVNNK	LRRDIR QRLG	HDLNGLSYAE	LQDLEETMSQ	SVQIIRDRKY
PaTM6	YQ <mark>K</mark> TMGVDLW	SSHYQAMKDT	LWKLKEINNK	LRREIR <mark>QR</mark> LG	H <mark>DLNGLTHAQ</mark>	L <mark>R</mark> SLEDKMAS	SLEVI RERKY
MdMADS13	YQ <mark>K</mark> TM <mark>GIDLW</mark>	RTHEESMKDT	LW <mark>K</mark> L <mark>KEINNK</mark>	LRREIR <mark>QR</mark> LG	HDLNGLSFDE	LA <mark>SLDDE</mark> MQS	SLDAI <mark>RQRK</mark> Y
MdTM6	YQ <mark>K</mark> TM <mark>GIDLW</mark>	RTHYESMKDT	LW <mark>K</mark> L <mark>KEINNK</mark>	LRREIRQRLG	H <mark>DLNGLSYDD</mark>	L <mark>R</mark> SLEDKMQS	SLDAI <mark>RERK</mark> Y
VvTM6	YQ <mark>K</mark> TL <mark>GIDLW</mark>	SSHYERMQEN	L <mark>RK</mark> LKEINNK	LRREIRQRMG	EDLGDLSIED	L <mark>RGLEQ</mark> KMDA	SLGLVRERKY
PhTM6	YQ <mark>R</mark> TL <mark>GVD</mark> IW	NKHYEKMQEN	LNRLKDINNK	<b>LRREIR</b> QRTG	EDM <mark>SGLNLQE</mark>	LCHLQGNVSD	SLAEI <mark>RERK</mark> Y
LeTM6	YQ <mark>S</mark> AL <mark>GVD</mark> IW	SIHYEK MQEN	LKRLKEINNK	<b>LRREIR</b> QRTG	EDMSGLNLQE	LCHLQENITE	SVAEI <mark>RERK</mark> Y
FveAP3	YQKLLGIDLW	<b>KSHYETMQEN</b>	LKKLKETNKS	LKRQIRQRLG	<b>ECLNDLR</b> FAD	LLSLEKEMET	AVEVV <mark>RLRK</mark> E
MASAKO euB3	YQKLLGIDLW	<b>KSHYETMQEN</b>	LKKLKEINKS	LRRQISHRVG	<b>ECLNDLR</b> FAE	LHSLEKEMES	AVEVV <mark>RNRK</mark> L
Atap3 —	YQTISDVDVW	<b>ATQYERMQET</b>	<b>KRK</b> LLETNRN	LRTQIKORLG	ECLDELDIQE	LRRLEDEMEN	TF <mark>K</mark> LV <mark>RERK</mark> F
LeAP3	YQ <mark>K</mark> TIGVDIW	TTHYEK MQEQ	LRKLKDVNRN	LRKEIRORMG	ESLNDLNYEQ	LEELMENVDN	SLKLIRERKF
		1/2					
14	41	КJ					
FveTM6	HVLKTQADTT	WKKVKNQEER	NSQLVHSY	<mark>AGNED</mark> PQY	GYVDDEGDYE	SAVALA <mark>NG</mark> A-	<mark>SNLYLF</mark> N
MASAKO_B3	HVLKTQAETT	RKK <mark>VKNL</mark> EER	NSNLMHGY	<b>GAPGNED</b> PQY	GYVDNEGDYE	SAVALA <mark>NG</mark> A-	<mark>SNLYFFN</mark>
PaTM6	HVIKTQTETS	KKKVKNLEER	RGNMLHGY	- <mark>ELASED</mark> PQY	GYVVNEGEYE	SAVALA <mark>NG</mark> A-	<mark>SNLFTI</mark> H
MdMADS13	HVIKTQTETT	KKK <mark>VK</mark> NLEQR	RGNMLHGYFD	QEAAGEDPQY	GYEDNEGDYE	SALAL <mark>SNG</mark> A-	<mark>NNLYTF</mark> H
MdTM6	HVIKTQTETT	KKKVKNLEER	RGNMLHGY	- <mark>EAASEN</mark> PQY	CYVDNEGDYE	SALVLA <mark>NG</mark> A-	<mark>NNLYTF</mark> Q
VvTM6	HVIKTQTETY	RKKVRNLEEQ	H <mark>GNLLLNF</mark>	-EAKCDDPHY	GLVENDGDYE	<mark>SAVAFANG</mark> A-	<mark>SNLYAFR</mark>
PhTM6	HVIKTQTDTC	RKRVRNLEEQ	H <mark>GSLVHDL</mark>	-EAKSEDPTY	<b>GVVENEGHFN</b>	<mark>SAMAFANG</mark> V-	HNLYAFR
LeTM6	HVIKNQTDTC	KKKAR <mark>NLEEQ</mark>	NGNLVLDL	– <mark>EAK</mark> CEDPKY	GVVENEGHYH	<mark>SAVAFANG</mark> V-	HNLYAFR
FveAP3	KLIMNQIQTK	TKK		––– <mark>DMDD</mark> PHY	<b>GLVEDGVEYY</b>	DSAVI <mark>G</mark> YSAN	EHPHNVFPLR
MASAKO_euB3	KLI SNQIDTK	RKKIRNATEC	NRRL-YEY	<mark>DAMEE</mark> PHY	GLVDDGGDYY	DSAVIGYSAN	EDPHNVFPLR
AtAP3	<b>KSLGNQIETT</b>	KKK <mark>NK</mark> SQQDI	Q <mark>KNLIHEL</mark>	-ELRAEDPHY	GLVD <mark>N</mark> GGDYD	<mark>SVL</mark> GYQIEG-	<mark>SR</mark> AYALR
LeAP3	KVIGNQIETY	RKK <mark>VR</mark> NVEEI	NRNLLLEF	– <mark>DAR</mark> QEDPYG	<mark>GLVEH</mark> DGDYN	<mark>SVL</mark> GFPTGG-	PRILDLR
2.	11					Paleo AP3 M	Actif
۲. ۳.		UNI DUCUDCC	CT VCCTTUT U	NDTNNUUCNU	NTEDCUCCCC		
MACAKO B3			SLVSGITILII		NI ENCUCCCS		
DOUME		CMT UU	оплооттипб	NPNNAGNA			
MdMADC12							
MdmMc							
Matmo		PNLHH			HRGSS	LGSSITHLHD	
					DGG	TOSHD	
FIITMO		PNLQN			GGG	I GSRD	
LeTM6	LOPLH	PNLQN			<mark>E</mark> GG	FGSRD	
FVEAP3	LOPSHHHH	PNLHH			GGG	GGGDRTTYTP	<u></u>
MASAKO_euB3	LQPSHH	PNLHK			GG	GSSDLTTYSL	<u></u>
AtaP3	FHQNH	HHYY <mark>PN</mark> H			GLHAP	SASDITTFHL	
LEAP3	LQPN	NNYHN			HLHSG	GGSDITTFA-	<mark>LG</mark>
						EuAP3 N	Notif

**Supplementary Figure S2.** Alignment of AP3- and TM6-like proteins. Eight TM6- and four AP3-like proteins were selected for the alignment. The M- and K-domain characteristics of MIKC-type MADS transcription factors are boxed. PaleoAP3 and EuAP3 motives are located at the carboxyl end of the TM6- and AP3-like proteins. Red squares mark the region where the sgRNAs were designed for *F. vesca TM6 (FveTM6)*. sgRNA1 is located spanning the M-domain and the I region. sgRNA2 is located at the I region. FveTM6 and FveAP3 (*F. vesca*), MASAKO B3 and MASAKO euB3 (*Rosa rugosa*), PaTM6 (*Prunus avium*), MdMADS13 and MdTM6 (*Malus × domestica*), VvTM6 (*Vitis vinifera*), PhTM6 (*Petunia × hybrid*), LeTM6 and LeAP3 (*Solanum lycopersicum*), AtAP3 (*Arabidopsis thaliana*). The protein sequences were obtained from GenBank (see Accession numbers section).

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F. vesca cv. Hawaii 4
                        TATTCGAAGCGACGAAATGGGATCTTCAAGAAGGCTCAAGAGCTCACGGTTCTGTGTGAT
F. vesca cv. RV
                        TATTCGAAGCGACGAAATGGGATCTTCAAGAAGGCTCAAGAGCTCACGGTTCTGTGTGAT
F. × ananassa cv. Camarosa #1
                        TATTCGAAGCGACGAAATGGGATCTTCAAGAAGGCTCAAGAGCTCACGGTTCTGTGTGAT
F. × ananassa cv. Camarosa #2
                        TATTCGAAGCGACGAAATGGGATCTTCAAGAAGGCTCAAGAGCTCACGGTTCTGTGTGAT
F. × ananassa cv. Camarosa #3
                        TATTCGAAGCGACGAAATGGGATCATCAAGAAGGCTCAGGAGCTCACGGTTCTTTGTGAT
F. × ananassa cv. Camarosa #4
                        TATTCGAAGCGACGAAATGGGATCTTCAAGAAGGCTCAAGAGCTCACGGTTATGTGTGAT
F. × ananassa cv. Camarosa #5
                        TATTCGAAGCGACGAAATGGGATCTTCAAGAAGGCTCAAGAGCTCACGGTTCTGTGTGAT
                        ***********************
                        121
                                                      PAM
                                                             sgRNA1
                        GCTCATGTCTCCCTCATCATGCAGTCCTCCACTGATAAAATCCACGAGTATATTAGCCCT
F. vesca cv. Hawaii 4
F. vesca cv. RV
                        GCTCATGTCTCCCTCATCATGCAGTCCTCCACTGATAAAATCCACGAGTATATTAGCCCT
F. × ananassa cv. Camarosa #1
                        GCTCATGTCTCCCTCATCATGCAGTCCTCCACTGATAAAATCCACGAGTATATTAGCCCT
F. × ananassa cv. Camarosa #2
                        GCTCATGTCTCCCTCATCATGCAGTCCTCCACTGATAAAATCCACGAGTATATTAGCCCT
F. × ananassa cv. Camarosa #3
                        GCTCAGGTCTCCCTCATCATGCAGTCCTCCACTAATAGAATCCACGAGTATATTAGCCCT
F. × ananassa cv. Camarosa #4
                        GCTCAGGTTTCCCTCATCATGCAGTCCTCCACTGATAAAATCCACGAGTATATTAGCCCT
F. × ananassa cv. Camarosa #5
                        GCTCAGGTCTCCCTCATCATGCAGTCCTCCACTGATAAAATCCACGATTATATTAGCCCT
                        181
F. vesca cv. Hawaii 4
                        F. vesca cv. RV
                        F. × ananassa cv. Camarosa #1
F. × ananassa cv. Camarosa #2
                        F. × ananassa cv. Camarosa #3
                        ACCACTACGTACGTCCAACATCTCTAGCTAGCTACTCTTTATTATGATCTTTTGTTCAC
F. × ananassa cv. Camarosa #4
                        ACCACTACGTACGTCCAACATCTCTAGCTAGCTACTCTTTATTATGATCTTTTGTTCAC
                        ACCACTACGTACGTCCAACATCTCTAGCTAGCTACTCTTTATTATGATCTTTGTTCAC
F. × ananassa cv. Camarosa #5
                        241
                        TTTTGATCTTTGCTTGATAATTCCATATATAGAACTGATACGGATGTAAATCAGGCACAA
F. vesca cv. Hawaii 4
F. vesca cv. RV
                        TTTTGATCTTTGCTTGATAATTCCATATATAGAACTGATACGGATGTAAATCAGGCACAA
F. × ananassa cv. Camarosa #1
                        TTTTGATCTTTGCTTGATAATTCCATATATAGAACTGATACGGATGTAAATCAGGCACAA
F. × ananassa cv. Camarosa #2
                        TTTTGATCTTTGCTTGATAATTCCATATATAGAACTGATACGGATGTAAATCAGGCACAA
                        TTTTGATCTTTGTTTGATTCCATATATAGAACTG---ATACGGATGTAAATCAGGCACAA
F. × ananassa cv. Camarosa #3
F. × ananassa cv. Camarosa #4
                        TTTTGATCTTTGGTTGATTCCATATATAGAACTGATGATACGGATGTAAATCAGGCACAA
F. × ananassa cv. Camarosa #5
                        TTTTGATCTTTGCTTGATTCCATATATAGAACTG---ATACGGATGTAAATCAGGCACAA
                        *****
                                                   *******
                                              *
                        PAM
F. vesca cv. Hawaii 4
F. vesca cv. RV
                        F. × ananassa cv. Camarosa #1
                        F. × ananassa cv. Camarosa #2
                        F. × ananassa cv. Camarosa #3
                        F. × ananassa cv. Camarosa #4
                        F. × ananassa cv. Camarosa #5
                        361
F. vesca cv. Hawaii 4
                        GGTGATTAAAGTTAACCAAAGGAGTCTGTCAATTTTTT-----TTATTCTATAAA
                        GGTGATTAAAGTTAACCAAAGGAGTCTGTCAATTTTTT-----TTATTCTATAAA
F. vesca cv. RV
                        GGTGATTAAAGTTAACCAAAGGAGTCTGTCAATTTTTT-----TTATTCTATAAA
F. × ananassa cv. Camarosa #1
                        GGTGATTAAAGTTAACCAATGGAGTCTGTCAATTTTTT------TTATTCTATAAA
F. × ananassa cv. Camarosa #2
F. × ananassa cv. Camarosa #3
                        F. × ananassa cv. Camarosa #4
                        GGTGATTAAAGTTAACCAAAGGATATAGAAAATAGTTGTA------
                        GGTGATTAAAGTTAACCAAAGGATATAGAAAATATTTGTA-----
F. × ananassa cv. Camarosa #5
                        **************
                                            * ***
                        421
                        F. vesca cv. Hawaii 4
F. vesca cv. RV
                        F. × ananassa cv. Camarosa #1
                        F. × ananassa cv. Camarosa #2
                        F. × ananassa cv. Camarosa #3
                        GAAAATATTTGGAGAGTTTTTGTATGTGTTAATTTTGTTTGCTGATTTTGGTGGA
F. × ananassa cv. Camarosa #4
                        -----GAGTTTCTGTATGTGTTAATTTTGTTTGCTGATTTTGGTGGA
F. × ananassa cv. Camarosa #5
                        -----GAGTTTCTGTATGTGTTAATTTTGTTTGCTGATTTTGGTGGA
```

**Supplementary Figure S3.** Alignment of *TM6* sequences from *F. vesca* and *F.* × *ananassa.* PCR flanking the two target sites (primers P180 and P181; Supplemental Table 3) for *TM6* was performed, purified, cloned and sequenced by the Sanger method for *F. vesca* cv. Hawaii 4, *F. vesca* cv. Reine des Vallées (RV), and *F.* × *ananassa* cv. Camarosa. The aligned region spans from the position 61 after the start codon, to the nucleotide 475, based on the *TM6* sequence in *F. vesca*. Exons are delimited with a black line; red font: sgRNAs; grey background: PAM; green background: synonymous

polymorphisms; red background: non-synonymous polymorphisms; asterisks: conserved nucleotides.

#Alle	les		
Control	#1 #2 #3 #4 #5	33 QELTVLCDAHVSLIMQSSTDKIHE-YISPTTTHKKMFDLYQKNLQIDLWSSHYEAMKENLWKLKEVNN QELTVLCDAHVSLIMQSSTDKIHE-YISPTTTHKKMFDLYQKNLQIDLWSSHYEAMKENLWKLKEVNN QELTVLCDAQVSLIMQSSTNRIHE-YISPTTHKKVFDLYQKNLQIDLWSSHYEAMKENLWKLKEVNN QELTVMCDAQVSLIMQSSTDKIHE-YISPTTHKKMYDLYQKNLQIDLWSSHYEAMKENLWKLKEVNN QELTVLCDAQVSLIMQSSTDKIHD-YISPTTHKKMFDLYQKNLQIDLWSSHYEAMKENLWKLKEVNN	
tm6-1	#1 #2 #3-A #3-B #4-A #4-B #5-A #5-B #5-C	QELTVLCDAHVSLIMQSSTDKIHEAMKENLWKLKEVNN QELTVLCDAHVSLIMQSSTDKIHE <b>TRQ*</b> QELTVLCDAQVSLIMQSSTNRIH <b>D*</b> QELTVLCDAQVSLIMQSSTNRIHESPTTTHKKVFDLYQKNLQIDLWS <b>RGNERELVETEGG*</b> QELTVMCDAQVSLIMQSSTDKIHE-Y* QELTVMCDAQVSLIMQSSTDKIHE-Y* QELTVLCDAQVSLIMQSSTDKIHD-YISPTTHKKMFDLYQKNLQIDLWSSH <b>TRQ*</b> QELTVLCDAQVSLIMQSSTDKIHD-YISPTTHKKMFDLYQKNLQIDLWSS <b>PRONERELVETEGG*</b> QELTVLCDAQVSLIMQSSTDKIHD-YISPTTHKKMFDLYQKNLQIDLWSS <b>PRONERELVETEGG*</b>	-29 PTC PTC PTC PTC PTC PTC PTC wt
tm6-7	#1 #2 #3 #4-A #4-B #5-A #5-B	QELTVLCDAHVSLIMQSSTDKIHE-YEAMKENLWKLKEVNN QELTVLCDAHVSLIMQSSTDKIHE <b>TRQ*</b> QELTVLCDAQVSLIMQSSTNRIHE-YEAMKENLWKLKEVNN QELTVMCDAQVSLIMQSSTDKIHE-YEAMKENLWKLKEVNN QELTVMCDAQVSLIMQSSTDKIHE-YISPTTHKKMYDLYQKNLQIDLWSSH-EAMKENLWKLKEVNN QELTVLCDAQVSLIMQSSTDKIHD-YISPTTHKKMFDLYQKNLQIDLWSSHYEAMKENLWKLKEVNN	-27 PTC -27 -27 -1 PTC wt
tm6-9	#1 #2 #3-A #4-A #4-B #4-C #5-A #5-B #5-C	QELTVLCDAHVSLIMQSSTDKIHE <b>V</b> Y* QELTVLCDAHVSLIMQSSTDKIHE <b>TRQ</b> * QELTVLCDAQVSLIMQSSTNRIHVRTRRCLISTRRICRSIYGARYEAMKENLWKLKEVNN QELTVLCDAQVSLIMQSSTNRILALPLRTRRCLISTRRICRSIYGARTRQ* QELTVMCDAQVSLIMQSSTDSTRRCMISTEAMKENLWKLKEVNN QELTVMCDAQVSLIMQSSTDKIHD-ISPTTHKKMYDLYQKNLQIDLWSSH-EAMKENLWKLKEVNN QELTVLCDAQVSLIMQSSTDKIHE-YEAMKENLWKLKEVNN QELTVLCDAQVSLIMQSSTDKIHD-YISPTTHKKMFDLYQKNLQIDLWSSHLRGNERELVETEGG* QELTVLCDAQVSLIMQSSTDKIHD-YISPTTHKKMFDLYQKNLQIETYS* QELTVLCDAQVSLIMQSSTDKIHD-YISPTTHKKMFDLYQKNLQIDLWSSHYEAMKENLWKLKEVNN	PTC PTC 21 aas subst/-7 PTC 9 aas subst/-23 1 aa subst/-2 -27 PTC PTC wt

**Supplementary Figure S4.** Alignment of TM6 predicted amino acid sequences. TM6 protein sequence from amino acid 33 to 99 in control is aligned with the protein sequences of the *tm6* mutant lines. Red and bold fonts indicate CRISPR/Cas9-induced variants. Red asterisk: premature termination codon (PTC). Information about the amino acid modification is included after the protein sequence.



**Supplementary Figure S5.** Expression analysis of two putative off-targets. Expression of FvH4\_5g20380 and FvH4\_2g29560 was analyzed using the eFP browser for *F. vesca* (Hawkins *et al.*, 2017). Expression data from the flower and fruit stages were obtained from Hollender *et al.* (2014) and Kang *et al.* (2013) respectively. All stage numbering follows Hollender *et al.* (2011).



**Supplementary Figure S6.** Sequence analyses of two putative off-targets. Spanning region of the putative off-targets #1 (2nd exon of FvH4\_5g20380) (A), and #3 (4th exon of FvH4\_2g29560) (B) for the sgRNA1 were amplified and Sanger sequenced. No variation in the sequence was found between the control and the *tm6-9* mutant line. Off-target sequences are boxed in grey; frequency of each sequence is indicated in parenthesis; arrow indicates a single nucleotide polymorphism detected at the off-target #3, indicating that FvH4\_2g29560 contains at least two alleles in *F*. × *ananassa* cv. Camarosa.



**Supplementary Figure S7.** Pollen yield quantification and germination assay. (A) Pictures of pollen grains stained with acetocarmine. (B) Quantification of pollen amount using the Neubauer chamber. Error bars denote the standard deviation (s.d.) of three biological replicates. (C) Pollen germination assay.

Α



**Supplementary Figure S8.** Fruit phenotype quantification. Chart showing the percentage of fruits with mutant, intermediate (Int.) and *wild-type* phenotype in control and *tm6* lines. Fruits with partial receptacle enlargement were considered to have an intermediate phenotype. Numbers of fruits analyzed for each genotype are indicated above the bars.

# Supplementary Table S1

	Sequence	CFD score	Position (Strawberry Genome v4.0.al chromosomes/maker standard CDS)
sgRNA1	TGGTAGGGCTAATATACTCG TGG		Fvb1_v4.0.al: 6715277 - 6715299 (+) / FvH4_1g12260 (1st exon)
Off-target #1	TG <b>CTG</b> GGGC <b>A</b> AATATACTC <b>A</b> TGG	0.222527	Fvb5_v4.0.al: 12055720 - 12055742 (+) / FvH4_5g20380 (2nd exon)
Off-target #2	TGGT <b>T</b> GGGCTAATATA <b>TA</b> C <b>A</b> <i>TGG</i>	0.106061	<pre>Fvb2_v4.0.a1: 25934976 - 25934954 (-) / intergenic region (putative promoter of FvH4_2g35080 (279 nt upstream of start codon) and/or FvH4_2g35070 (2593 nt upstream of start codon)</pre>
Off-target #3	TG <b>CA</b> AAGGCTAATAAACTCG <b>G</b> GG	0.106034	Fvb2_v4.0.al: 22903461 - 22903439 (-) / FvH4_2g29560 (4th exon)
Off-target #4	TG <b>T</b> T <b>G</b> GGGCTAAT <b>C</b> TA <b>T</b> TCG <b>C</b> GG	0.036000	Fvb4_v4.0.al: 19433168 - 19433146 (-) / intergenic region
Off-target #5	TGG <b>G</b> AG <b>T</b> GCTAATATA <b>G</b> T <b>G</b> G <i>TGG</i>	0.002626	Fvb4_v4.0.a1: 10677003 - 10677025 (+) / intergenic region
sgRNA2	TCTATGGAGCTCGCACTACG AGG		Fvb1_v4.0.a1: 6715121 - 6715099 (-) / FvH4_1g12260 (2nd exon)
Off-target #1	TCGACGGAGCTAGCACTACT CGG	0.250000	Fvb3_v4.0.al: 18665022 - 18665044 (+) / intergenic region
Off-target #2	TATATGGACCTCG <b>TT</b> CTACG GGG	0.057436	Fvb5_v4.0.al: 27110536 - 27110514 (-) / intergenic region

**Supplementary Table S1.** Off-target analysis for sgRNA1 and sgRNA2. Sequences, Cutting Frequency Determination (CFD) score (Doench *et al.*, 2016), and position in the *F. vesca* v4.0.a1 reference genome (Edger *et al.*, 2018) are displayed. CFD score is predictive of off-target potential of sgRNA:DNA interactions. Off-targets are ranked by CFD score from most to least likely. Mismatches compared with the sgRNA sequence are shown in bold type. Off-targets located within coding sequences (CDS) are marked in grey.

# Supplementary Table S2.

Oligo	Sequence (5' -> 3')	Amplicon size	Purpose	
P131	GATTGGGTAGGGCTAATATACTCG	24 bp	sgRNA1	
P132	AAACCGAGTATATTAGCCCTACCC			
P133	GATTGCTATGGAGCTCGCACTACG	24 bp	sgRNA2	
P134	AAACCGTAGTGCGAGCTCCATAGC			
P177	ATTGCCCGGGAGCTTCGTTGAAC	600 bp	Cloning sgRNAs into the final vector	
P179	GCCGTCTAGATGATGGATATCTGC			
P443	ATCACCACAGCAACCACAAC	85 bp	qRT-PCR <i>FaTM6</i>	
P444	AGGCGGAGATCATGGAGATG			
P476	TCAGATTCAAACCAAGACGAAA	97 bp	qrt-PCR FaAP3	
P477	AGCCAATGACTGCAGAATCA			
FaCHP1-F	TGCATATATCAAGCAACTTTACACTG	91 bp	qRT-PCR CHP1 (housekeeping)	
	A			
FaCHP1-R	ATAGCTGAGATGGATCTTCCTGTGA			
P180	AGCTGATTGAGAACCAGACGA	512 bp	FveTM6 and FaTM6 alleles characterization.	
P181	CGAGTCATCAAATGGCCAAAC		sgRNA1-2/Cas9-mediated mutagenesis detection	
P248	GAGATCGTGTGGGATAAGGG	717 bp	Cas9 detection	
P297	GATATTCTCGGCCTGCTCTC			
P445	TCGTCGGCAGCGTCAGATGTGTATAA	472 bp	FveTM6 amplicon sequencing (NGS) gDNA	
	GAGACAGTATTCGAAGCGACGAAAT			
	GG			
P446	GTCTCGTGGGCTCGGAGATGTGTAT			
	AAGAGACAGTCCACCAAAATCAGCAA			
	ACA			
P445	TCGTCGGCAGCGTCAGATGTGTATAA	314 bp	<i>FveTM6</i> amplicon sequencing (NGS) gDNA	
	GAGACAGTATTCGAAGCGACGAAAT			
D. /	GG			
P475	GICICGIGGGCICGGAGAIGIGIAI			
	AAGAGACAGICCICAGCITATIGITA			
DE 40		1001		
P546		46200	UIT-target #1 located at the 2nd exon of	
			FVH4_5g20380	

P547	CCTATGGCAAAGGCAGAAAG		
P544	TAGGCGAGAAGGGATTGTCA	389bp	Off-target #3 located at the 4th exon of FvH4_2g29560
P545	TAGCTTCCCCTGCATTGTTC		

**Supplementary Table S2.** List of oligonucleotides used in this study.

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