



**Figure S1** The compared genome editing efficiency in Cas9-edited plants. (a) The editing efficency in different Cas9-edited plants. (b) The compare genome editing efficiency of At and Dt-subgenome based on WGS data.

## (a) AP2 D13 homologous sgRNA1

		58 193.320					50	402	220				43	bp	50.4	00.0	240			58,192,350							_,				
		[0-25]	58,193,320		_		58	,193,	,330		_			-	58,1	92,3	340					_	58,	192,	350		_			_	_
WT	s79	[0-23]																													
WT	s195	[0-48]																													
WT	s199	[0-46]																													
Ne	s65	[0-57]																													
Ne	s66	[0-31]																													
Ne	s67	[0-31]																													
AP2	s1	[0-42]																													
AP2	s3	[0-52]																													
AP2	s4	[0-35]														+															
AP2	s15	[0-56]															2														
Ref gff	Seđ 3	G T	T G G A	G C	C A	A	P2	2 D	)13	5 h	or	no	log	go	us	د د د	sg	RN	١A	1	P	Å	M	T (	C A	G	G	T C	T T	C (	A

## (b) AP2 D12 homologous sgRNA1

			43 bp	10.007.100	
	12,897,460	12,897,470	12,89,7,480	12,89,7,490	12,897,500
WT s79					
WT s195	[0-47]				
WT s199	. [0-43]	¢			
Ne s65	[0-41]			A	
Ne s66	[0-34]				
Ne s67	[0-43]	C			
AP2 s1	[0-40]				
AP2 s3	[0-34]				
AP2 s4	[0-20]				
AP2 s15	[0-44]				
RefSeq gff3	• 6 T T 6 6 A 6 C C	AP2 D12 hom	ologous sgRNA1	PAM	T T C A

# (c) AP2 D13 homologous sgRNA2

		58,192,610	58,192,620	3 bp	58,192,630	58,192,640
WT s79	[0-42]					
WT s195	[0-47]					
WT s199	[0-44]					
Ne s65	[0-43]					
Ne s66	[0-29]					
Ne s67	[0-25]					
AP2 s1	[0-33]					
AP2 s3	[0-50]	· · · · · · · · · · · · · · · · · · ·				
AP2 s4	[0-27]					
AP2 s15	[0-56]	A		T		τ κ
RefSeq → gff3		PAM	AP2 D13 h	omolo	gous sgRNA2	A C T G G A A T C C

# (d) AP2 A12 homologous sgRNA1

					43 hn			
			17,278,130	17,278,1	40	17,278,140	17,278,150	
		[0-27]						
F	62		I I G T I G	Ţ	G			G G
5	5		T T G T T G	A I				G G
		[0-40]	T T G	Ţ				G
			I I G T T G	Y				G G
Ž	195							
>	ò							
		[0-37]	T T G	ĩ				G
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	0)		T T G T T G	Ţ				G G
		[0-31]						
Ð	35							
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		[0-32]						
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	_	[0-27]						
<u>N</u>	67		-					
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		[0-51]	=====					
P2	2		T C T 6 T J G	I				G G
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		[0-48]						
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			Ť Ť Ğ T T G	i T				G G
~		[0-33]						
D	54 2		c					
$\triangleleft$	37							
		[0-41]					c	
P	15							
◄	S							
Ref	Seri	T T C C		GTCCGAT	G G T A T A			A G
aff	3	PA	M AP2 A12 h	omologous	sgRNA2			

**Figure S2** Confirmation of the *AP2* on-target mutation in two sgRNAs from WT, negative and CRISPR/Cas9 plants based on the WGS data. The orange box represents the target sites, and the red box represents the PAM sequence. Whole genome sequencing analysis at sgRNA2 target region of *MYB44* in 3 wild-type, 3 negative plants and 4 CRISPR/Cas9-edited *AP2* plants by Integrative Genomics Viewer (IGV). The number in the square brackets e.g. [0-56] represents the WGS supporting sequence reads with on-target sites and the pileup strip represent Cas9-edited *MYB44* plants a heterozygous deletion of 43-bp of *AP2*. The *AP2* sgRNA1 and sgRNA2 homologous loci reads were showed in (a-b) and (c-d) , respectively.

(a) [		A06		43 hn		
		1,366,580	1,366,590	1,366,600	1,366,610	1,366,620
	WT s79	[0-35]				
ţ	WT s195	[0-80]				
÷.	WТ s199	[0-86]				
	Ne s65	[0-74]				
	Ne s66	[0-40]				
	Ne s67	[0-55]	2			
	MYB44 s62	[0-50]				
	MYB44 s75	[0-53]				
	MYB44 s76	[0-29]				
	MYB44 s77	[0-47]				
R	efSe <del>q</del> gff3		PAM	MYB44 sgl	RNA1	T A G T C T C A



**Figure S3** Confirmation of the *MYB44* on-target mutation in two sgRNA loci from WT, negative and CRISPR/Cas9 plants based on the WGS data.

## (a) ARC A-genome sgRNA1

	A06 I		40.1		
	15,589	,950 15,589	43 bp	15,589,970	15,589,980
	[0-16]				) A
WT 879					
WT s195	[0-50]		6		1         4
WT s199	[0-44]	¢		1 A	
Ne s65	[0-32]				
Ne s66	[0-11]			=	
Ne s67	[0-35]				
ARC s84	[0-24]				
ARC s85	[0-24]				
ARC s91	[0-12]				
ARC 894 RefSec	[0-10]	та 6 тстасс	A T C A G T	6 T T C C C A C G	
gff3			ARC sgR	NA1 PA	M

## (b) ARC D-genome sgRNA1

	<b>D</b> 06	1			
		11,882,100	43 bp	11,882,120	11,882,130
	[0-48]				
WT s79	A	T		K	
WT s195	[0-98]				C G
WT s199	[0-81]				
Ne s66	[0-56]				G
Ne s67	[0-77]	T			2 2 2
ARC s84	[0-34]	T			
ARC s85	[0-60]				
ARC s91	[0-39]				
ARC s94	[0-51]	-			
RefSeq gff3		AR	C D-genome sgl	RNA1 PAM	чні і А G Т А G С . ссссссссссссс

## (c) ARC A-genome sgRNA2

		A06										
			15.589.200			15.589.220	8	3 bp	15,589,240		15.589.260	
		[0-43]	-,-,-,-			-,-,-, -						
F	6											
$\leq$	s7			-								
										-		
			-									
	ß	[0-60]										
5	10		-							-		
>	`ω											
	_	[0-44]				_						
F	00											
$\leq$	5					-						
				1								
		10,401										
		[0-42]					-					
Ð	65											
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		[0-35]										
a	ဖ											
Ž	s6			_								
			_									
		[0-27]										
0	67					_			_			
~	S		_			-						
		[0-37]					-					
8	<b>4</b>											
A	ŝ					-						
							-					
		[0-41]					Ξ					
S S	35						-					
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		[0-25]										
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R	94						-					
A	Ś						·					
R≏f	Sert	TCTTA		TCATCA	TTCCAC	T C A T T C C A	сааатсат	C G A G	G A T T A T G A T G A	ACTTTCCCT	C T T T C A G T G	с <u>та са с са</u> та
af	f3		e e e e e		i i i i i	PAI	M ARC	sal	RNA2	<u></u>	<del>.</del>	. <u></u>
							/ 11 (0	-9				

### (d) ARC D-genome sgRNA2

	<ul><li>▲</li><li>▲</li><li>▲</li><li>11,881,360</li></ul>	<b>11,88</b> 1,	370	<b>43 bp</b> 11,88,1,380	11,88,1,390	11,881,400
WT s79	[0-38]					
WT s195	[0-37]					
WT s199	[0-51]	c			1	
Ne s66	[0-56]				¢	A
Ne s67	[0-77]					
ARC s84	[0-26]					
ARC s85	[0-38]	A				•
ARC s91	[0-21]					
ARC s94	[0-46]	A			A	
RefSeg aff3		ΡΔΝ	ARC I	)-genome s	a tota tota tota a	

**Figure S4** Confirmation of the *ARC* on-target mutation in two sgRNA loci from WT, negative and CRISPR/Cas9 plants based on the WGS data. The *ARC* sgRNA1 and sgRNA2 homologous loci reads were showed in (a-b) and (c-d) , respectively.



**Figure S5** Scatterplot of on-target site correlation of sanger sequencing (x axis) and Whole genome sequencing (y axis). The Correlation Coefficient was calculated based on Pearson's method (Pearson's correlation coefficient).



#### Tissue culture process (TSP) mutations in AP2 Cas9-edited plants





#### Tissue culture process (TSP) mutations in MYB44 Cas9-edited plants





#### Tissue culture process (TSP) mutations in ARC Cas9-edited plants



**Figure S6** The somaclonal variation or/and inherent genetic variation were detected in *AP*2, *MYB44*, *ARC* Cas9-edited plants and WT by IGV. The red box and arrow represent the SNPs and indels, respectively.



#### Common and private SNPs in different Cas9-edited lines

Common and private Indels in different Cas9-edited lines



**Figure S7** The common and private SNPs/indels in four Cas9-edited lines of *AP2, MYB44, ARC*.



**Figure S8** Genome-wide prediction off-target sites. (a) Genome-wide circos plot representing off-target site scores for AP2 sgRNA1 (A) and sgRNA2 (B), MYB44 sgRNA1 (C), and sgRNA2 (D), ARC sgRNA1 (E), and sgRNA2 (F). The different off-target PAM scores were highlighted circos center. (b) The off-target sequences logos were showed.



**Figure S9** The potential off-target mutations in *MYB44* Cas9-edited cotton plants. The data show the variations in Cas9-s62, Cas9-s75 and WT plants.



	Off-target sites mutation in non-coding regions (GhARC) OFTM4
WT s79	Ä01:4,382,006-4,382,026
ARC_T0 s91	
RefSeq	Off-target site PAM

**Figure S10** The potential off-target mutations in ARC Cas9-edited cotton plants. The data show the variations in Cas9-s91 and WT Plants.

### AP2 sgRNA1 site1 D13: 58,193,326-58,193,348 (Gh\_A12G0630)

	▲ 326 bp 58,193,328 bp	58.193.330 bp 58.193.332 b 	p 58,193,334 bp	58,193,336 bp 5 1	) (8,159,338 bp 58,159,340 bp 	18,193,342 bp	58,193,344 bp	58,193,346 bp I	58,193,348 bp
TW 79	11		G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A		T         G         C           T         G         C				G         G           G         G
AP2_T0 S1 (Cas9+)	<b>A T G A T G A T</b> <th></th> <th>G         C         A           G         C         A</th> <th></th> <th>I         G           I         G</th> <th></th> <th></th> <th></th> <th>G         G           G         G</th>		G         C         A           G         C         A		I         G           I         G				G         G           G         G
AP2_T1 S20 (Cas9-)	P • • • • •           A         Y         G	G         Y         Y           G         Y         Y	G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A           G         C         A	T C C C C C C C C C C C C C C C C C C C	T         6         C           T         6         C				6         6           6         6
AP2_T1 S23 (Cas9+)	0**40           A         T         G	G         Y         Y           G         T         T	G         C         A           G         C         A		I         G           I         G				G         G           G         G
ReSeq → gff3		G T T « « « « « « « «	G C A C C C C C	ÁP2 sgR	NA1	C T « « « « « «	A A : c c c c c		PAM

#### AP2 sgRNA1 site2 D12: 12,897,468-12,897,490 (Gh\_D12G0647)



## AP2 sgRNA2 site1 D13: 58,192,611-58,192,633 (Gh\_D13G2207)

	<b>-</b> 58.192,612 bp − 1	58,192,614 bp	58,192,616 bp	58,192,618 bp 	58,192,620 bp 		58.192.624 bp	58,192,626 bp I I	58,192,628 bp 	58,192,630 bp I	58,192,632 bp	58,192
WT s79	***				A         G           A         G	T C T C T C T C T C T C T C T C T C T C	C G C G C G C G C G C G C G C G C G C G		G         G           G         G			
AP2_T0 S1 (Cas9+)	p-30           C         C		6 C 6 C 6 C 6 C 6 C 6 C 6 C 6 C		A         G           A         G			A         Y           A         T	G         G           G         G			
AP2_T1 S20 (Cas9-)	p0         -           c         -		6 C 6 C 6 C 6 C 6 C 6 C 6 C 6 C		A         G           A         G	T C C T C C C T C C C T C C C T C C C T C C C T C C C T C C C T C C C T C C C T C C C T C C C T C C C T C C C T C C C T C C C T C C C T C C C C T C C C C T C C C C T C C C C T C C C C T C C C C T C C C C T C C C C T C C C C T C C C C T C C C C T C C C C C T C C C C C T C	C G G G G G G G G G G G G G G G G G G G	A         T           A         T				
AP2_T1 S23 (Cas9+)	C         C           C         C		G         C           G         C		A         G           A         G           A         G           A         G           A         G           A         G           A         G           A         G           A         G           A         G           A         G           A         G           A         G           A         G           A         G           A         G           A         G           A         G           A         G	T C T C T C T C T C T C T C T C T C T C	C G G G G G G G G G G G G G G G G G G G	A T A T A T A T A T A T A T A T A T A T	G         G           G         G           G         G           G         G           G         G           G         G           G         G           G         G           G         G           G         G           G         G           G         G           G         G           G         G           G         G           G         G           G         G           G         G			
gff3	PAM	Τ Α < < < < <	G C	A A < < < <	A G < < < <	T C < < < < <		P2 sgRNA	<u>6</u> <u>6</u> <u>6</u>	ΤΑ	T A A	A

#### AP2 sgRNA2 site2 A12: 17,278,125-17,278,147 (Gh\_A12G0630)



**Figure S11** Confirmation of the *AP2* mutations of inheritance from T0 to T1 plants in two sgRNA loci by WGS data. The orange box represents the sgRNA target sites, and the red box represents the PAM sequence.