

SUPPLEMENTARY INFORMATION

COMT, CRTZ, and F3'H regulate glycyrrhizic acid biosynthesis in Glycyrrhiza uralensis hairy roots

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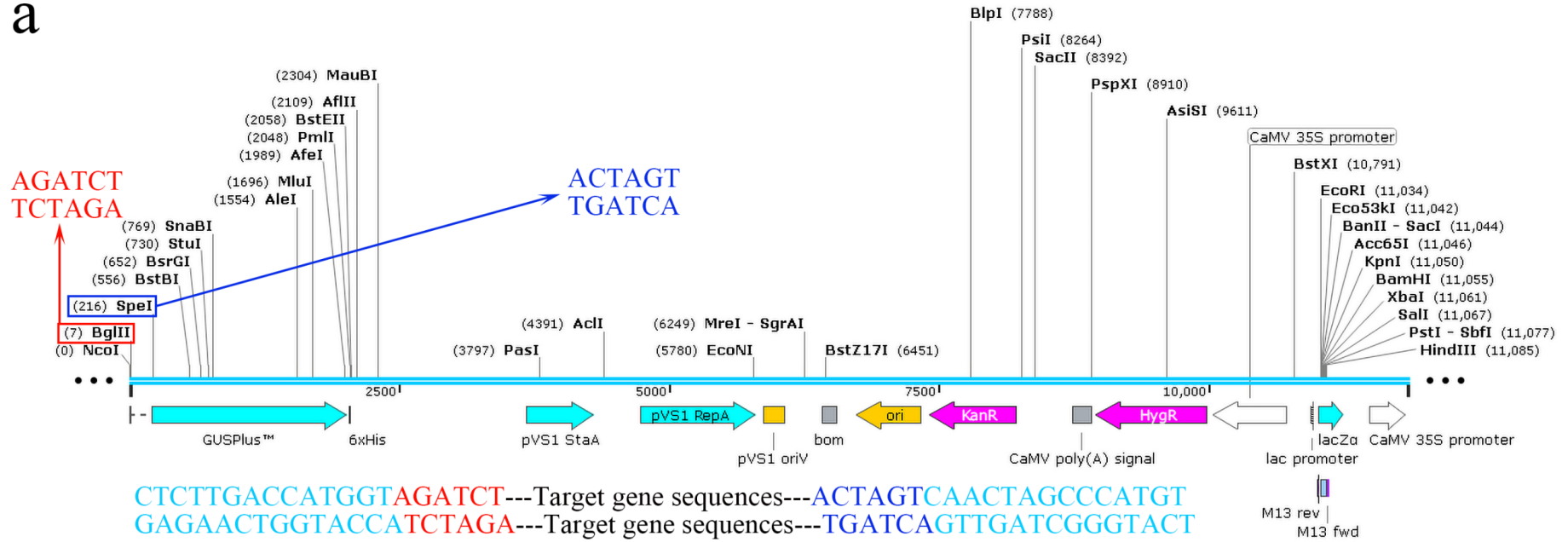
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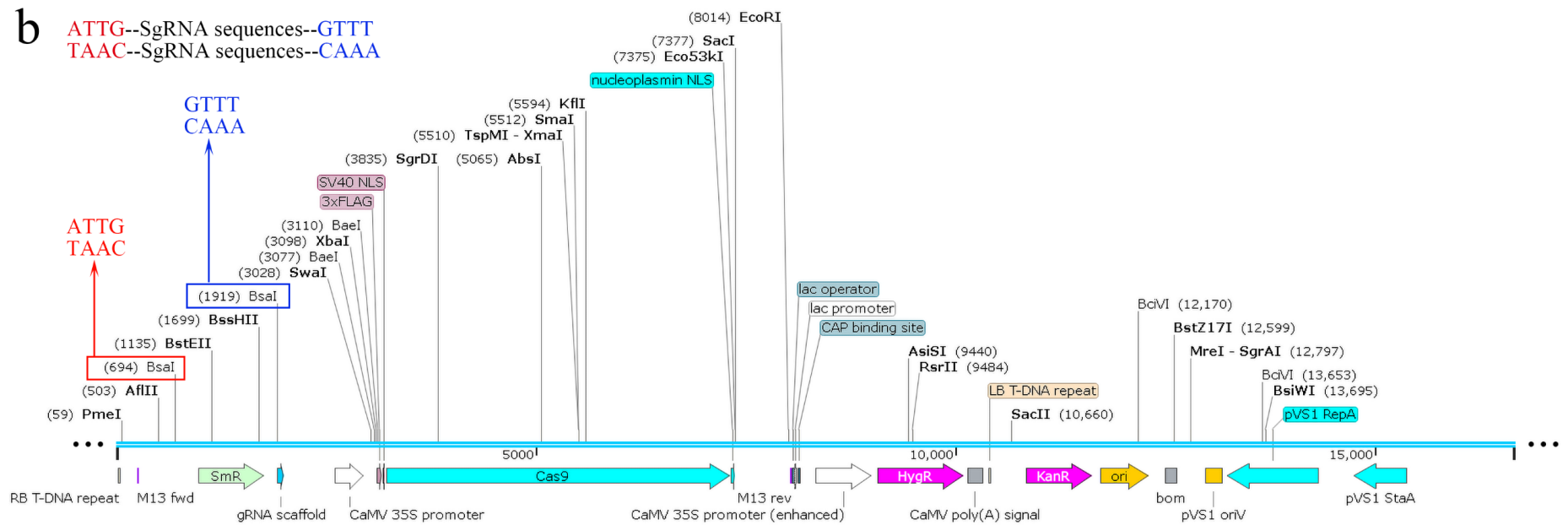


Fig. S1. Construction strategy of plant binary expression vectors (a) and CRISPR/Cas9 vectors (b). The insertion sites of target sequences are shown in red and blue boxes.

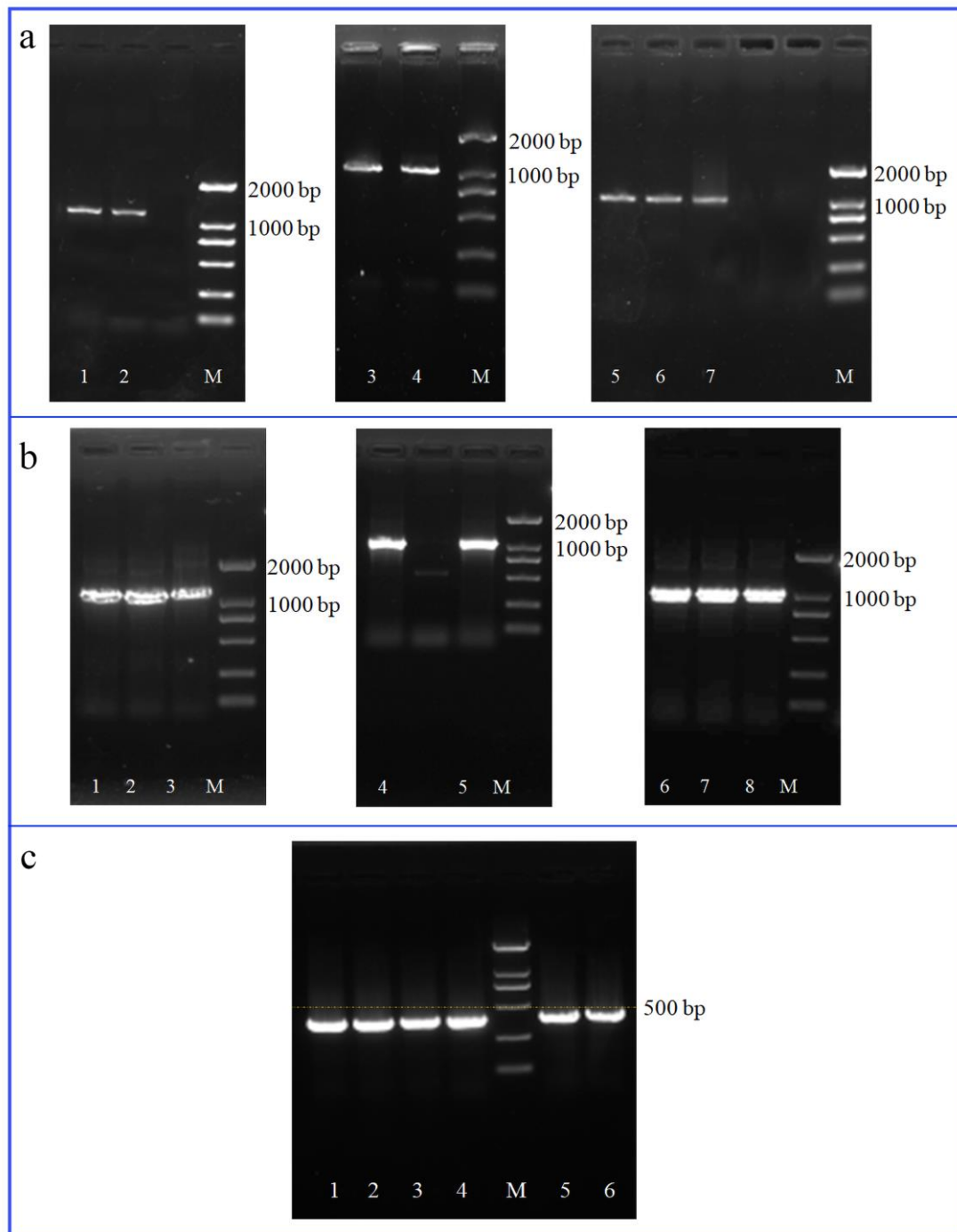


Fig. S2. PCR verification of the recombinant vectors. **a** shows the three genes cloned from *G. uralensis* (Lane 1, 2: *COMT*, 3, 4: *CRTZ*, 5, 6, 7: *F3'H*, M: marker). **b** shows the three genes amplified from pCA-*COMT* (Lane 1, 2, 3), pCA-*CRTZ* (Lane 4, 5), and pCA-*F3'H* (Lane 6, 7, 8), respectively. **c** shows the 400 bp-fragments containing sgRNA sequences amplified from pHSE-*COMT* (Lane 1, 2), pHSE-*CRTZ* (Lane 3, 4), and pHSE-*F3'H* (Lane 5, 6), respectively.

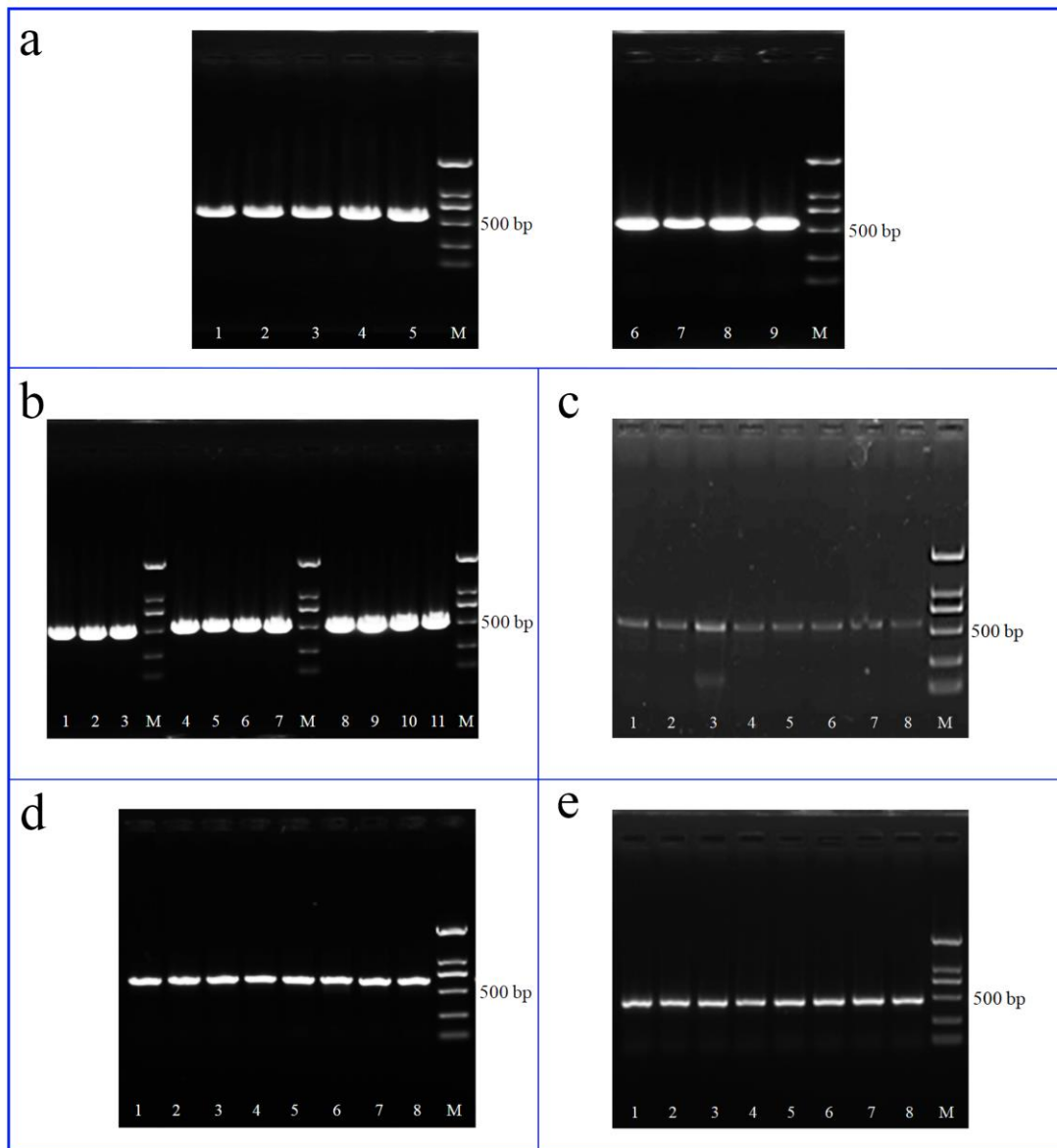
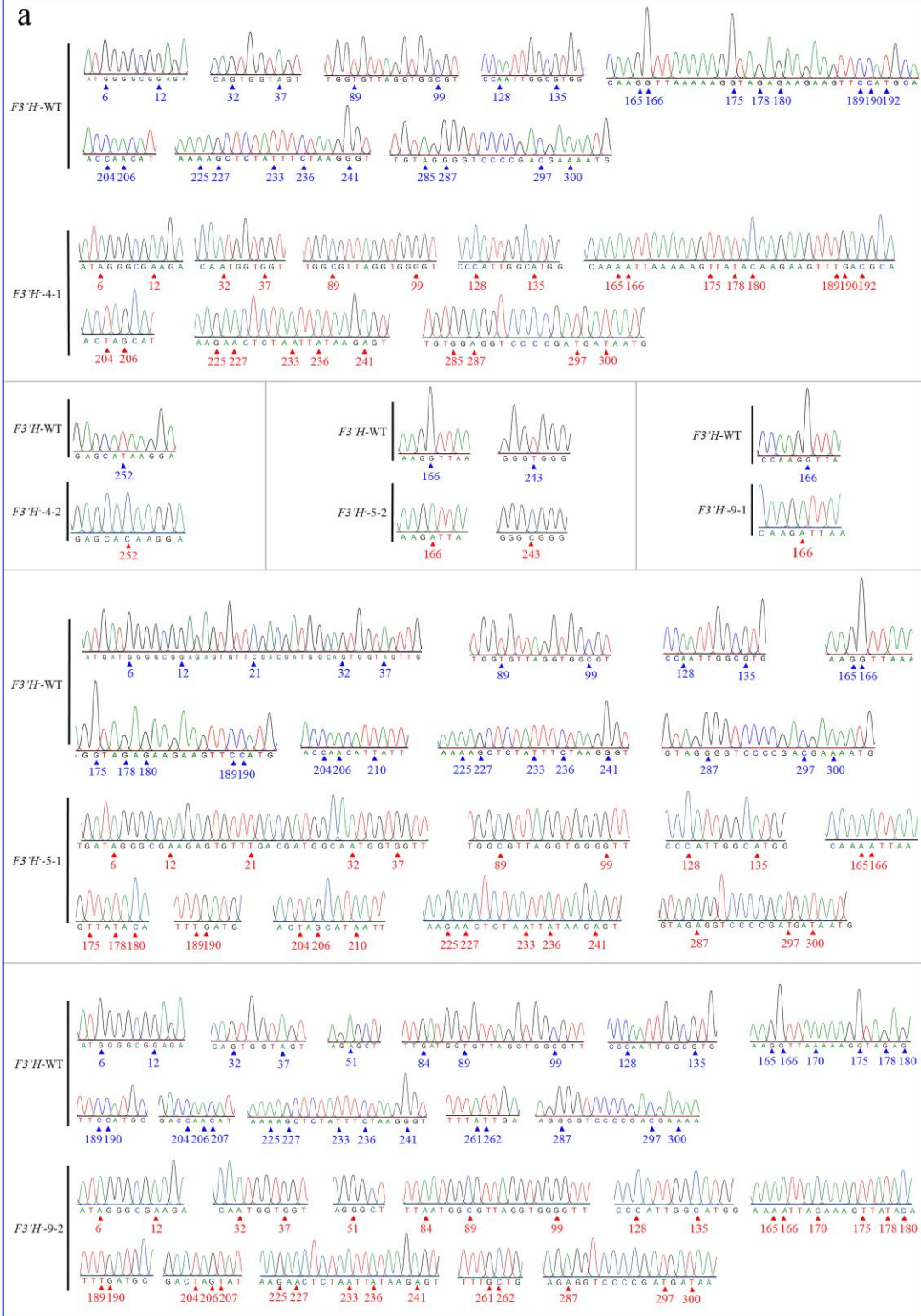


Fig. S3. PCR verification of the transgenic *G. uralensis* hairy root lines. **a** shows the *roIC* genes amplified from different hairy root lines (Lane 1: WT, 2: NC-PCA, 3: *COMT*⁺, 4: *CRTZ*⁺, 5: *F3'H*⁺, 6: NC-PHSE, 7: *COMT*⁻, 8: *CRTZ*⁻, 9: *F3'H*⁻). **b** shows the three genes amplified from the *F3'H*⁺ (Lane 1, 2, 3), *CRTZ*⁺ (Lane 4, 5, 6, 7), and *COMT*⁺ (Lane 8, 9, 10, 11) lines, respectively. **c** shows the 530 bp *COMT* fragments amplified from the *COMT*⁻ lines. **d** shows the 620 bp *CRTZ* fragments amplified from the *CRTZ*⁻ lines. **e** shows the 440 bp *F3'H* fragments amplified from the *F3'H*⁻ lines.

a

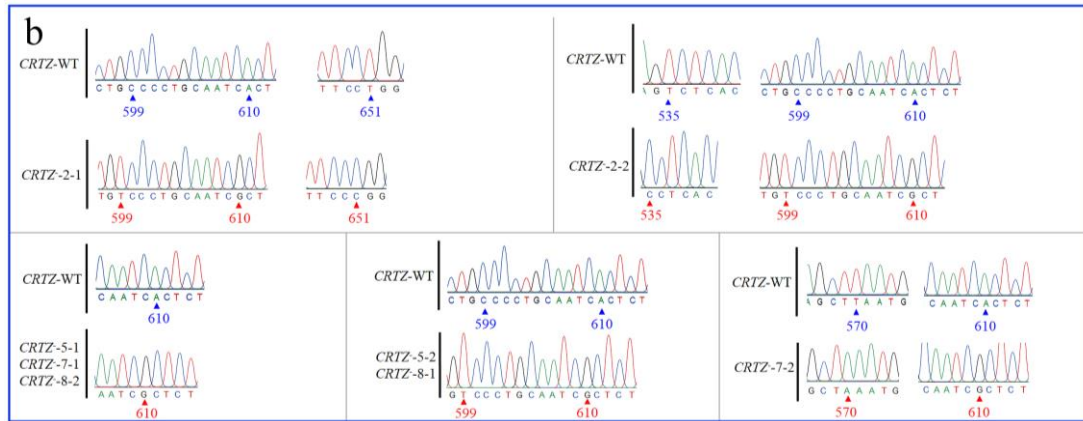


Fig. S4. Sequence analyses show the heterozygous mutations in the *F3'H* (a) and *CRTZ* (b) lines. The mutation sites are marked by the red triangles and the corresponding sites in the wild type sequences are marked by the blue ones.

Table S1. Primers used for amplifying the three genes

Genes	Primers	Sequences (5' to 3')
<i>COMT</i>	F	ATGGAATATGATCATCACGG
	R	TTAAGCCTTCTTAAGAAATTCC
<i>CRTZ</i>	F	CACTTCACTTCACACGCACC
	R	AACAGCTGTAATGCTAGTTGCT
<i>F3'H</i>	F	TACCAATGCACTGACTAGGGC
	R	TCAGAAAGACGATGAGTACA

Table S2. Primers used for constructing and verifying the plant binary expression vectors

Genes	Primers	Sequences (5' to 3')
<i>COMT</i>	F	<u>CTCTTGACCATGGT</u> AGATCT ATGGAATATGATCATCACGG
	R	<u>TTGATCGGGTACAGACTAGTTT</u> AAGCCTTCTTAAGAAATTCC
<i>CRTZ</i>	F	<u>CTCTTGACCATGGT</u> AGATCT CACTTCACTTCACACGCACC
	R	<u>TTGATCGGGTACAGACTAGTA</u> AACAGCTGTAATGCTAGTTGCT
<i>F3'H</i>	F	<u>CTCTTGACCATGGT</u> AGATCT TACCAATGCACTGACTAGGGC
	R	<u>TTGATCGGGTACAGACTAGTT</u> CAGAAAGACGATGAGTACA

Note: The bold-font sequences indicate the specific restriction enzyme cutting sites, the underlined sequences indicate the homologous sequence of pCAMBIA1305.1, and the left sequences indicate the homologous sequence of the three genes.

Table S3. sgRNA sequences used for constructing the plant CRISPR/Cas9 vectors

Genes	Design basis	Primers	Sequences (5' to 3')
<i>COMT</i>	The third exon	F	ATTGTTCAACAACCTTTTCAACAAGGG
		R	AAACCCCTTGTTGAAAAGGTTGTTGAA
<i>CRTZ</i>	The third exon	F	ATTGCTCTATGGCATGCTTCCTTGTGG
		R	AAACCCACAAGGAAGCATGCCATAGAG
<i>F3'H</i>	The first exon	F	ATTGGGCGTGGCTAGACCTTCAAGGGG
		R	AAACCCCTTGAAGGTCTAGCCACGCC

Note: The bold-font sequences indicate the specific restriction enzyme cutting sites and the left sequences indicate the homologous sequence of the three genes.

Table S4. Primers used for analyzing the gene editing sites in the *COMT*⁻, *CRTZ*⁻ and *F3'H*⁻ hairy root lines

Genes	Primers	Sequences (5' to 3')
<i>COMT</i>	F	CAATTCAGGAGGGTGGCCTT
	R	TGTCTCCACCAATATGCTCCAC
<i>CRTZ</i>	F	GCACATTTGCCCTCTCAGTT
	R	ATGTAGGCCATCCCAAACACC
<i>F3'H</i>	F	AGGTAGCAAGATTGACACGCA
	R	TCCTTTGATCTCGTGTTCGGT

Table S5. Primers used for RT-qPCR analyses of the *COMT*⁺, *CRTZ*⁺ and *F3'H*⁺ hairy root lines

Genes	Primers	Sequences (5' to 3')
<i>COMT</i>	qF	TCCCTTTCCGCTTCTCACA
	qR	TTCAGCAGCAGGATCTCCAT
<i>CRTZ</i>	qF	GCTGTCACCTCTTTGGCTGTCT
	qR	GCTAATCCGAATCCGTCCCT
<i>F3'H</i>	qF	TTGCCTCACTTGCCATACTTG
	qR	TCCATTCTTTTGGGTCACGA
<i>β-actin</i>	qF	CAAAAGGATGCCTATGTGGG
	qR	CAGGAGCAACACGCAATTC

Table S6. The UPLC gradient elution program

Time/min	Mobile phase A (%)	Mobile phase B (%)
0	14	86
1.36	23	77
3.26	30	70
4.08	34	66
4.76	36	64
5.71	42	58
6.53	51	49
9	51	49
9.5	14	86
12	14	86

Table S7. GA contents in the *G. uralensis* hairy root lines

Groups	Lines	Repetition	Peak area (mAU*s)	Contents (mg·g ⁻¹)	Group average contents (mg·g ⁻¹)
Wild Type	WT	1	15622	3.2201	3.3242
		2	16691	3.4193	
		3	16230	3.3334	
Negative Control	NC-PCA	1	14922	3.0897	3.5188
		2	21123	4.2451	
		3	15629	3.2214	
	NC-PHSE	1	15979	3.2867	3.3459
		2	15581	3.2125	
		3	17331	3.5386	
<i>F3'H</i> Over-expression	<i>F3'H</i> ⁺ -1	1	9816	2.1383	2.3654
		2	13362	2.7990	
		3	12142	2.5717	
	<i>F3'H</i> ⁺ -3	1	12866	2.7066	
		2	10307	2.2298	
		3	10156	2.2017	
	<i>F3'H</i> ⁺ -4	1	13390	2.8043	
		2	12061	2.5566	
		3	7804	1.7634	
	<i>F3'H</i> ⁺ -5	1	11337	2.4217	
		2	12902	2.7133	
		3	10563	2.2775	
	<i>F3'H</i> ⁺ -6	1	10863	2.3334	
		2	12745	2.6841	
		3	12416	2.6228	
	<i>F3'H</i> ⁺ -7	1	11091	2.3759	
		2	11883	2.5235	
		3	7338	1.6766	
	<i>F3'H</i> ⁺ -10	1	9349	2.3211	
		2	9255	2.7905	
		3	10088	3.1268	
<i>F3'H</i> Knockout	<i>F3'H</i> ⁻ -2	1	35235	2.0513	5.4744
		2	34542	2.0338	
		3	28047	2.1890	
	<i>F3'H</i> ⁻ -7	1	22062	6.8746	
		2	20175	6.7454	
		3	18266	5.5352	
	<i>F3'H</i> ⁻ -8	1	29807	4.4201	
		2	32511	4.0685	
		3	32037	3.7128	
	<i>F3'H</i> ⁻ -10	1	33682	6.5852	
		2	28785	5.6728	
		3	17497	3.5695	
<i>CRTZ</i> Over-expression	<i>CRTZ</i> ⁺ -1	1	7689	1.7420	2.2050
		2	11439	2.4407	
		3	10363	2.2402	

<i>CRTZ</i> Knockout	<i>CRTZ</i> ⁺ -2	1	11490	2.4502	5.3651
		2	10467	2.2596	
		3	9033	1.9924	
	<i>CRTZ</i> ⁺ -3	1	8364	1.8678	
		2	8699	1.9302	
		3	8394	1.8734	
	<i>CRTZ</i> ⁺ -4	1	12744	2.6839	
		2	13702	2.8624	
		3	13387	2.8037	
	<i>CRTZ</i> ⁺ -5	1	5286	1.2943	
		2	9964	2.1659	
		3	11588	2.4685	
	<i>CRTZ</i> ⁻ -1	1	47746	9.2057	
		2	33414	6.5353	
		3	22832	4.5636	
	<i>CRTZ</i> ⁻ -3	1	18038	3.6703	
		2	17668	3.6014	
		3	22571	4.5149	
	<i>CRTZ</i> ⁻ -4	1	20373	4.1054	
		2	21915	4.3927	
		3	18838	3.8194	
	<i>CRTZ</i> ⁻ -6	1	44316	8.5666	
		2	46585	8.9894	
		3	27496	5.4326	
	<i>CRTZ</i> ⁻ -7	1	15217	3.1447	
		2	15282	3.1568	
		3	35798	6.9795	
<i>COMT</i> Over-expression	<i>COMT</i> ⁺ -1	1	9958	2.1648	2.3509
		2	8792	1.9475	
		3	10341	2.2361	
	<i>COMT</i> ⁺ -4	1	9819	2.1389	
		2	11851	2.5175	
		3	9984	2.1696	
	<i>COMT</i> ⁺ -7	1	12220	2.5862	
		2	13024	2.7361	
		3	14912	3.0878	
	<i>COMT</i> ⁺ -10	1	13328	2.7927	
		2	9653	2.1079	
		3	8580	1.9080	
	<i>COMT</i> ⁺ -11	1	9961	2.1653	
		2	11810	2.5099	
		3	10118	2.1946	
<i>COMT</i> Knockout	<i>COMT</i> ⁻ -5	1	24364	4.8490	4.2746
		2	23633	4.7128	
		3	23436	4.6761	
	<i>COMT</i> ⁻ -6	1	25829	5.1220	
		2	21247	4.2682	
		3	15636	3.2227	

<i>COMT</i> -8	1	23274	3.0180
	2	17623	3.7092
	3	13988	4.8933

Table S8. Lignin contents in the WT, NC-PCA, NC-PHSE, *COMT*⁺ and *COMT*⁻ hairy root lines

Groups	Lines	Repetition	Relative absorbance	Contents (mg·g ⁻¹)	Group average contents (mg·g ⁻¹)
Wild Type	WT	1	0.1017	44.4226	45.0050
		2	0.1097	47.9170	
		3	0.0977	42.6754	
Negative Control	NC-PCA	1	0.0957	41.8018	44.1314
		2	0.0967	42.2386	
		3	0.1107	48.3538	
	NC-PHSE	1	0.1057	46.1698	45.4418
		2	0.1047	45.7330	
		3	0.1017	44.4226	
<i>COMT</i> Over-expression	<i>COMT</i> ⁺ -1	1	0.1567	68.4466	72.2322
		2	0.1437	62.7682	
		3	0.1537	67.1362	
	<i>COMT</i> ⁺ -4	1	0.1897	82.8610	
		2	0.1807	78.9298	
		3	0.1707	74.5618	
	<i>COMT</i> ⁺ -7	1	0.1267	55.3426	
		2	0.1427	62.3314	
		3	0.1307	57.0898	
	<i>COMT</i> ⁺ -10	1	0.1907	83.2978	
		2	0.1947	85.0450	
		3	0.2067	90.2866	
	<i>COMT</i> ⁺ -11	1	0.1587	69.3202	
		2	0.1647	71.9410	
		3	0.1697	74.1250	
<i>COMT</i> Knockout	<i>COMT</i> ⁻ -5	1	0.0697	30.4450	37.2266
		2	0.0647	28.2610	
		3	0.0637	27.8242	
	<i>COMT</i> ⁻ -6	1	0.0976	42.6290	
		2	0.0946	41.3186	
		3	0.0917	40.0658	
	<i>COMT</i> ⁻ -8	1	0.0957	41.8130	
		2	0.0929	40.5602	
		3	0.0964	42.1234	

Table S9. Total flavonoid contents in the WT, NC-PCA, NC-PHSE, *F3'H⁺* and *F3'H⁻* hairy root lines

Groups	Lines	Repetition	Relative absorbance	Contents (mg·g ⁻¹)	Group average contents (mg·g ⁻¹)
Wild Type	WT	1	0.046	34.3969	36.1425
		2	0.063	36.5682	
		3	0.07	37.4623	
Negative Control	NC-PCA	1	0.058	35.9296	35.3761
		2	0.052	35.1632	
		3	0.051	35.0355	
	NC-PHSE	1	0.056	35.6741	36.0147
		2	0.064	36.6960	
		3	0.056	35.6741	
<i>F3'H</i> Overexpression	<i>F3'H⁺</i> -1	1	0.136	45.8923	50.9953
		2	0.131	45.2537	
		3	0.146	47.1696	
	<i>F3'H⁺</i> -3	1	0.186	52.2786	
		2	0.184	52.0232	
		3	0.185	52.1509	
	<i>F3'H⁺</i> -4	1	0.193	53.1727	
		2	0.187	52.4064	
		3	0.192	53.0450	
	<i>F3'H⁺</i> -5	1	0.208	55.0886	
		2	0.171	50.3627	
		3	0.139	46.2755	
	<i>F3'H⁺</i> -6	1	0.193	53.1727	
		2	0.164	49.4687	
		3	0.201	54.1946	
	<i>F3'H⁺</i> -7	1	0.252	60.7086	
		2	0.175	50.8737	
		3	0.178	51.2568	
	<i>F3'H⁺</i> -10	1	0.157	48.5746	
		2	0.171	50.3627	
		3	0.146	47.1696	
<i>F3'H</i> Knockout	<i>F3'H⁻</i> -2	1	0.017	30.6928	31.7040
		2	0.013	30.1819	
		3	0.008	29.5432	
	<i>F3'H⁻</i> -7	1	0.056	35.6741	
		2	0.051	35.0355	
		3	0.049	34.7801	
	<i>F3'H⁻</i> -8	1	0.007	29.4155	
		2	0.013	30.1819	
		3	0.008	29.5432	
	<i>F3'H⁻</i> -10	1	0.032	32.6087	
		2	0.03	32.3532	
		3	0.015	30.4373	