

Supplementary Figure S1: Additional T-DNA insertions in the *f3h/fls1/ans* mutant identified by loreta. Schematic drawing of two additional T-DNA insertion events in the *f3h/fls1/ans* mutant. (A) The first insertion on chromosome one is located between 3,748,551 - 3,748,573 and carries a 3,238 bp inverted fragment of chromosome three consisting of the sequence from 18,003,546 - 18,000,308 bp. Labelling of fragments smaller than 500 bp was omitted for clarity. (B) The second insertion is followed by a large tandem repeat of the chromosome three fragment ranging from $\sim 14,194 - 14,204$ kbp. Labelling of fragments smaller than 1,000 bp was omitted for clarity. (B) The second insertion is followed by a large indicate genomic fragments matching the TAIR10 reference genome sequence and red boxes indicate the presence of T-DNA (ending with "_tdna") or binary vector sequences (ending with "_vec"). The arrow shape marks the orientation of the fragments.

Α		44/0	FI 04	5011
		AINS	FLS7	F3H
	f3h/fls1/ans-1.bam Coverage	p-323	p-328	v
	13h/fis1/ans-2.bam Coverage	D-323	D-323	v
	f3h/fis1/ans-3.bam Coverage	P - 30	0-20	▼ ■ 20
	f3h/fis1/ans-4.bam Coverage			
	ColO-1.bam Coverage	[-10]	p-rol	h-ual
	ColO-2.bam Coverage	P-69	p- 43	
	ColO-3.bam Coverage	p-31	p-31	
	ColO-4.bam Coverage	p-soj		
	Noe-1.bam Coverage	P-318	p-3ig	p-318
	Noe-2.bam Coverage	р-лл	p-37]	р-лл
	Noe-3.bam Coverage	p-30	p-38	p-39
	Noe-4.bam Coverage	P-334	p-34	
	f3h/fis1/ans.bam Coverage	P-199	p- 194	p- 199
	f3h/fis1/ans.bam			
	Gene	AT4022880.1	AT5608840.2	AT3051238.1
в	Gene	AT4022880.1 ANS	AT5008840.2 FLS1	AT3051238.1
в	Gene f3h/fis1/ans-1.bam Coverage	AT4022880.1 ANS p-389	A1500840.2 FLS1	F3H
в	Gene f3h/fis1/ans-1.bam Coverage f3h/fis1/ans-2.bam Coverage	AT4022880.1 ANS p-381 p-381	A15000040.2 FLS1 P-309 P-309	AT3061238.1
в	Gene 13h/lis1/ans-1.bam Coverage 13h/lis1/ans-2.bam Coverage 13h/lis1/ans-3.bam Coverage	P-38	A15000040.2 FLS1 0-309 0-309	AT3061288.1
в	Gene Sh/lls1/ans-1.bam Coverage Sh/lls1/ans-2.bam Coverage Sh/lls1/ans-3.bam Coverage Sh/lls1/ans-4.bam Coverage	P-301	A15000040.2 FLS1 p-308 p-	AT3051238.1
в.	Gene Bh/Hist/ans-1.bam Coverage Bh/Hist/ans-2.bam Coverage Bh/Hist/ans-3.bam Coverage Bh/Hist/ans-4.bam Coverage Col0-1.bam Coverage	P-301	AT5000040.2 FLS1 p-309 p-309 p-309 p-309 p-309 p-109	F3H □-38
B	Gene Eh/Hist/ans-1.bam Coverage Eh/Hist/ans-2.bam Coverage Eh/Hist/ans-3.bam Coverage Col0-1.bam Coverage Col0-1.bam Coverage	P-30 P-30 P-30 P-30 P-30 P-30 P-30 P-30	A15000040.2 FLS1 P-30 P-	AT305128.1 •
в	Gene Sh/Hist/ans-1.bam Coverage Sh/Hist/ans-2.bam Coverage Sh/Hist/ans-3.bam Coverage Col0-1.bam Coverage Col0-2.bam Coverage Col0-3.bam Coverage	Ат402280.1 ANS р-38	A15000040.2 FLS1 P-30 P-30 P-30 P-30 P-100	F3H □-33
в	Gene Sh/Nist/ans-1.bam Coverage Sh/Nist/ans-2.bam Coverage Sh/Nist/ans-3.bam Coverage Sh/Nist/ans-4.bam Coverage Col0-1.bam Coverage Col0-2.bam Coverage Col0-3.bam Coverage Col0-3.bam Coverage	P-38	A15000040.2 FLS1 P-30 P-30 P-30 P-30 P-30 P-30 P-10 P-00 P-00 P-00 P-00 P-00 P-00 P-0	AT3061288.1 F3H p-38 p-38 p-38 p-38 p-170 p-88 p-98 p-98
в	Gene Eh/Hist/ans-1.bam Coverage Eh/Hist/ans-2.bam Coverage Eh/Hist/ans-3.bam Coverage ColD-1.bam Coverage ColD-2.bam Coverage ColD-3.bam Coverage ColD-4.bam Coverage Noe-1.bam Coverage	μ-301 μ-302 μ-303 μ-304	AT5000040.2 FLS1 p-301 p-301 p-301 p-301 p-601 p	AT3061288.1 F3H p-39
В	Gene Sh/Hist/ans-1.bam Coverage Sh/Hist/ans-2.bam Coverage Sh/Hist/ans-3.bam Coverage ColO-1.bam Coverage ColO-2.bam Coverage ColO-3.bam Coverage ColO-4.bam Coverage Noe-1.bam Coverage Noe-2.bam Coverage	AT4022800.1 ANS p-38 p-38 p-38 p-781 p-68 p-781 p-781 p-781 p-781 p-781 p-781 p-781	A15000040.2 FLS1 P-38 P-38 P-38 P-38 P-18 P-	AT306128.1 F3H p-38 p-38 p-38 p-38 p-38 p-38 p-38 p-38 p-38 p-18 p-38 p-38 p-18 p-38
в	Gene Sh/Hist/ans-1.bam Coverage Sh/Hist/ans-2.bam Coverage Sh/Hist/ans-2.bam Coverage Col0-1.bam Coverage Col0-2.bam Coverage Col0-3.bam Coverage Noe-1.bam Coverage Noe-1.bam Coverage Noe-3.bam Coverage	P-38	A15000040.2 FLS1 P-30 P-30 P-30 P-50 P-	AT3061288.1 F3H p-30 p-30 p-30 p-30 p-100 p-603 p-703 p-703 p-703 p-703 p-703 p-703 p-703 p-703 p-703 p-704
в	Gene Sh/fist/ans-1.bam Coverage Sh/fist/ans-2.bam Coverage Sh/fist/ans-2.bam Coverage Sh/fist/ans-3.bam Coverage Col0-1.bam Coverage Col0-3.bam Coverage Col0-3.bam Coverage Noe-1.bam Coverage Noe-2.bam Coverage Noe-2.bam Coverage Noe-3.bam Coverage Noe-4.bam Coverage	P-30 P-30 P-30 P-30 P-30 P-30 P-30 P-30	AT5000040.2 FLS1 P-39 P-39 P-39 P-39 P-69 P-69 P-69 P-69 P-69 P-70 P-	AT3061238.1 F3H P-33 P-34 P-35 P-36 P-37 P-38 P-38 P-38
в	Gene Sh/Hist/ans-1.bam Coverage Sh/Hist/ans-2.bam Coverage Sh/Hist/ans-3.bam Coverage ColO-1.bam Coverage ColO-2.bam Coverage ColO-3.bam Coverage ColO-4.bam Coverage Noe-1.bam Coverage Noe-3.bam Coverage Noe-3.bam Coverage Sh/Hist/ans-1.bam Coverage	AT4022800.1 ANS p-38 p-38 p-78 p-78 p-78 p-78 p-78 p-78 p-78 p-78 p-77 p-38	A15000040.2 FLS1 P-30 P-30 P-30 P-30 P-10 P-	AT306128.1 F3H p-38 p-38 p-58 p-58 p-58 p-58 p-58 p-58

Supplementary Figure S2: Genomic and transcriptomic characterisation of the mutant loci of the *A. thaliana f3h/fls1/ans* mutant. (A) RNA-Seq read mappings against the TAIR10 Col-0 reference genome sequence. The four RNA-Seq mappings, corresponding to the four biological replicates of the *f3h/fls1/ans* mutant, are shown in row 1-4, while those of Col-0 follow in row 5-8 and Nössen in row 9-12. The 13th row shows the T-DNA and transposon insertion events in the *f3h/fls1/ans* mutant corresponding to the ONT read mapping of the mutant to TAIR10. The last row contains the gene structure based on the TAIR10 annotation. (B) Read mappings against the *f3h/fls1/ans* assembly. The same numeration as in panel A applies. However, in the last row the read mapping of the ONT reads against the *f3h/fls1/ans* assembly is shown.

	-	tig00000294			
		4	965 bp		
	_				
t3h/tis1/ans-1.bam Coverage					p:z-q
t3h/tls1/ans-2.bam Coverage	÷				for-d
t3h/fls1/ans-3.bam Coverage	1				p-223
	-				D -219
13h/fls1/ans-4.bam Coverage	-				
ColO-1.bam Coverage	\$				629 - C
					p-313
ColO-2.bam Coverage	-				
ColO-3.bam Coverage	-				isc) - d
Col0-4.bam Coverage	-				िक्ट - व
No. 4 hora 6 more 1	-	1			D-223
Noe-1.bam Coverage	-				
Noe-2.bam Coverage	÷				tax-d
Noe-3.bam Coverage	-				p-259
Noe-4.bam Coverage		0			612-0
A					
sequence			and the second		0
					_
	end o	of 2'promote		start 5'UTR	ATG

Supplementary Figure S3: The *f3h* **locus of the** *A. thaliana f3h/fls1/ans* **mutant.** RNA-Seq read mappings against the *f3h/fls1/ans* assembly. The four RNA-Seq read mappings corresponding to the four biological replicates of the *f3h/fls1/ans* mutant are shown in row 1-4, while those of Col-0 follow in row 5-8 and Nössen in row 9-12. The last row shows the three reading frames. Per frame the green boxes represent the position of potential start codons. Red boxes mark stop codons. Additionally, the end of the 2'promoter of the T-DNA, the start of the *F3H* 5'UTR and the typically used start codon are marked with black arrows and respective descriptions.



Supplementary Figure S4: *In situ* flavonoid staining of siliques from *A. thaliana* wildtype and 2-ODD mutant plants. Representative pictures of flavonoid accumulation in ethanol bleached and diphenylboric acid 2-aminoethyl ester (DPBA)-stained siliques under (A) white light and (B) UV light. (C) Close-up of different ages *f3h/fls1/ans* siliques under UV light. *please note: for the *ans* single mutant the *tt18-1* allele was used instead of *tds4-4*.

Α

f3h





f3h/ans

chs







fls1/ans















Supplementary Figure S5: In situ flavonoid staining of inflorescences from A. thaliana wildtype and 2-ODD mutant plants. Representative pictures of flavonoid accumulation in ethanol bleached and diphenylboric acid 2-aminoethyl ester (DPBA)-stained (A) inflorescences and (B) f3h stamen under UV light. Red arrows mark the presence of red fluorescence. *please note: for the ans single mutant the *tt18-1* allele was used instead of *tds4-4*.



Supplementary Figure S6: Comparison of the eriodictyol profile between the *A. thaliana f3h/fls1/ans* mutant and Col-0 in UPLC-HDMS^e ESI+. Peak numbers correspond to those given in Supplementary Table S4. E, eriodictyol.



Supplementary Figure S7: Comparison of the eriodictyol profile between the *A. thaliana f3h/fls1/ans* mutant and Col-0 in UPLC-HDMS^e ESI-. Peak numbers correspond to those given in Supplementary Table S4. E, eriodictyol.



Supplementary Figure S8: UPLC-MS/MS profile of one biological replicate from the *A. thaliana f3h/fls1/ans* mutant in ESI- mode. Eriodictyol rutinoside or neohesperidoside (A), eriodictyol-7-O-glucoside (B), eriodictyol di-hexoside (C), eriodictyol tri-hexoside (D).



Supplementary Figure S9: ESI- fragmentation of dihydrokaempferol standard by UPLC-MS/MS and UPLC-TWIM-QToF (HDMS^e) high resolution mass spectrometry.



Supplementary Figure S10: Fragmentation of eriodictyol standard by UPLC-MS/MS and UPLC-TWIM-QToF (HDMS^e) high resolution mass spectrometry.



Supplementary Figure S11: ESI- UPLC-TWIM-QToF (HDMS^e) high resolution mass spectrometry of compound 1 and 8 (Supplementary Table S4) in the *f3h/fIs1/ans* triple mutant bi2logical replicate.

F3	Ή	
----	---	--

f3h/fls1/ans-1.bam Coverage	p-23	 ^
f3h/fls1/ans-2.bam Coverage		
f3h/Hs1/ans-3.bam Coverage	P-131	
f3h/Hs1/ans4.bam Coverage	p- ra	
ColO-1.bam Coverage	P-23	
ColO-2.bam Coverage	p-219	
Col0-3.bam Coverage		
ColO-4.bam Coverage	p-119	
Noe-1.bam Coverage		A
Noe-2.bam Coverage		
Noe-3.bam Coverage	p-193	
Noe-4.bam Coverage		
Gene		AT5608000, 🗸

Supplementary Figure S12: The *F3'H* **locus of the** *A. thaliana f3h/fls1/ans* **mutant.** (A) RNA-Seq read mappings against the TAIR10 Col-0 reference genome sequence. The four RNA-Seq mappings, corresponding to the four biological replicates of the *f3h/fls1/ans* mutant, are shown in row 1-4, while those of Col-0 follow in row 5-8 and Nössen in row 9-12. The last row contains the gene structure based on the TAIR10 annotation.



IMG_1962.JPG



Supplementary Figure S13: Original, unprocessed versions of Figure 4C (top) and Figure 4D (bottom).