SUPPLEMENTARY INFORMATION

This file contains the Supplementary Figures and Supplementary Tables corresponding to the manuscript: **"Synthesis of crocin I and crocin II by multigene stacking in Nicotiana benthamiana"**

Lei Xie[1], Zuliang Luo[1], Xunli Jia[1], Changming Mo[2], Xiyang Huang[3], Yaran Suo [4], Shengrong Cui[1], Yimei Zang[1], Jingjing Liao[2]* & Xiaojun Ma[1]*

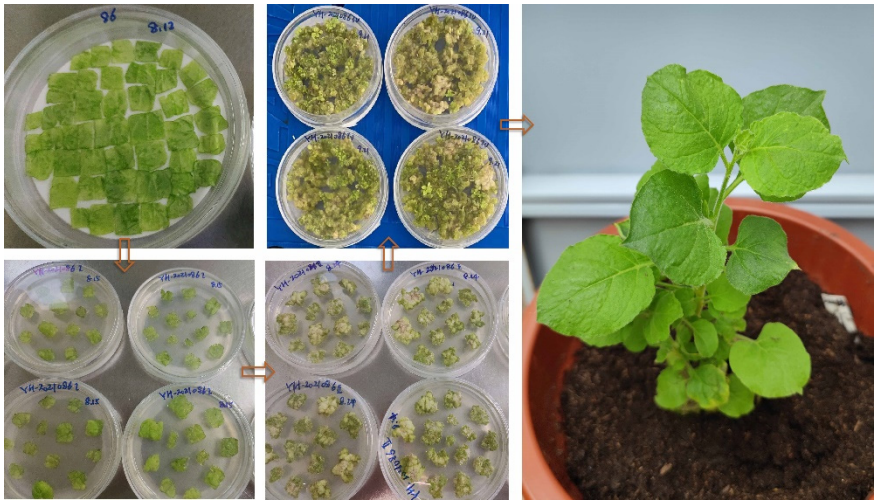

Figure S1. Process of agrobacterium tumefaciens-mediated transformation of multi-gene vector AU-CU into *Nicotiana benthamiana*.

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ATACTCATGGATTTGTAGTTGAGTATGAAAATATTTTTTAATGCATTTTATGACTTGCCAATTGATT
GACAACATGCATCAA

Figure S2. The nucleotide sequence of insertion fragment in the multigene vector AU-CU. The crocins biosynthesis genes *GjALDH2C3* (black), *GjUGT94E13* (blue), *GjCCD4a* (green), and *GjUGT74F8* (orange) were shown in this graph. P2A peptide sequences were highlighted in red. GjALDH2C3-2A-GjUGT9F13 was controlled by CaMV 35S promoter (highlighted in yellow), and the terminator was heat-shock protein 18.2 terminator (Thsp) (highlighted in light blue). GjCCD4a-2A-GjUGT74F8 was controlled by the AtUBQ10 promoter (highlighted in light green) and terminator from the pea rbcS-E9 gene (Te9) (highlighted in gray).

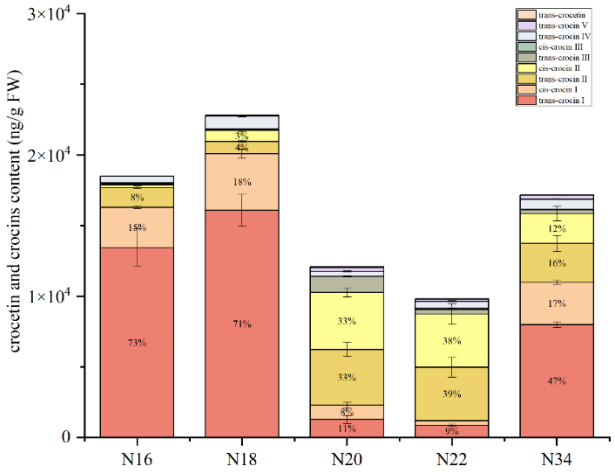


Figure S3. Crocetin and crocins production in transgenic tobacco of T1 generation lines. The data are presented as the mean values ± SDs, n = 3 biologically independent samples.

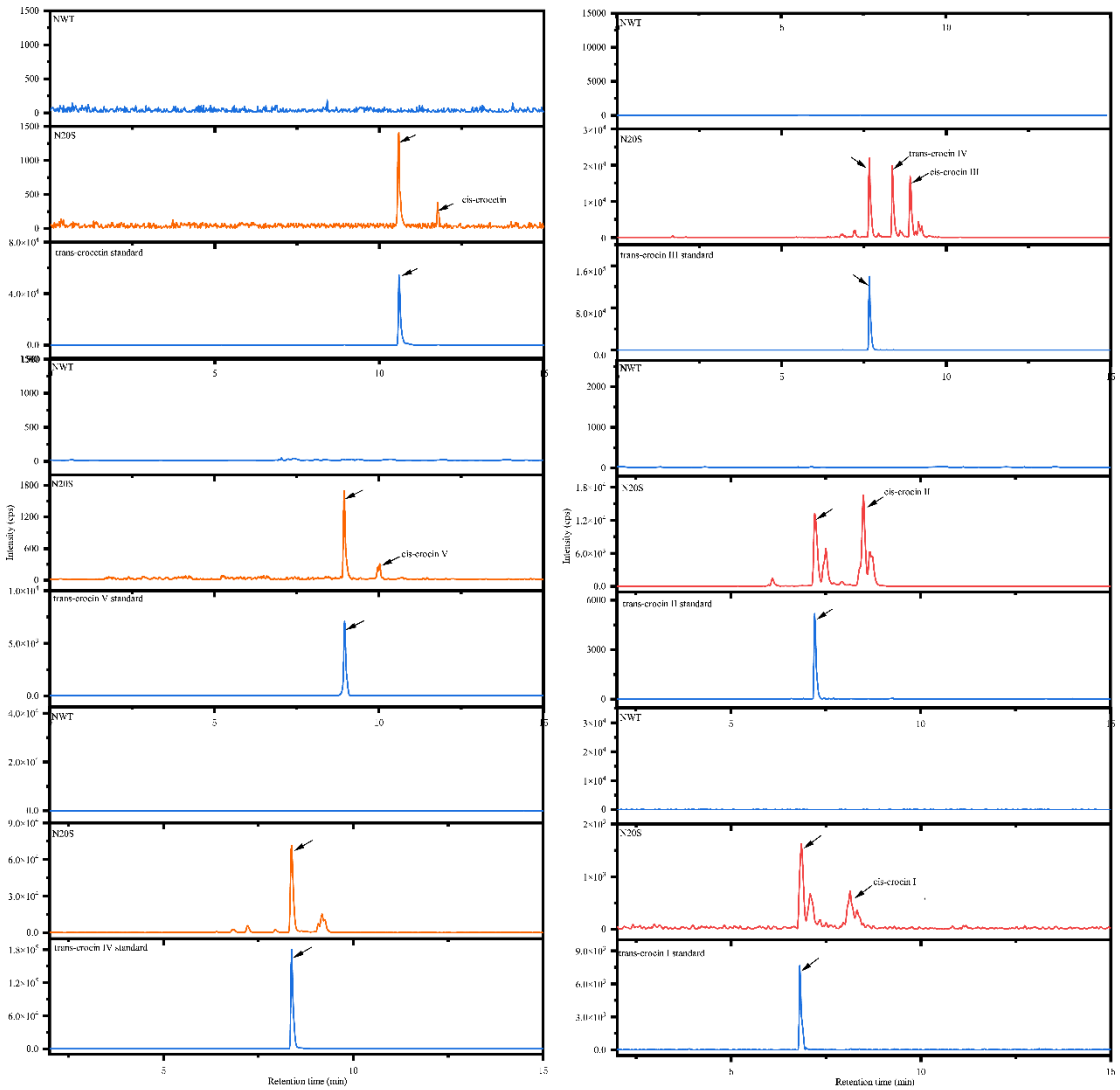


Figure S4. Identification of crocins in the stems of the transgenic tobacco N20 line.

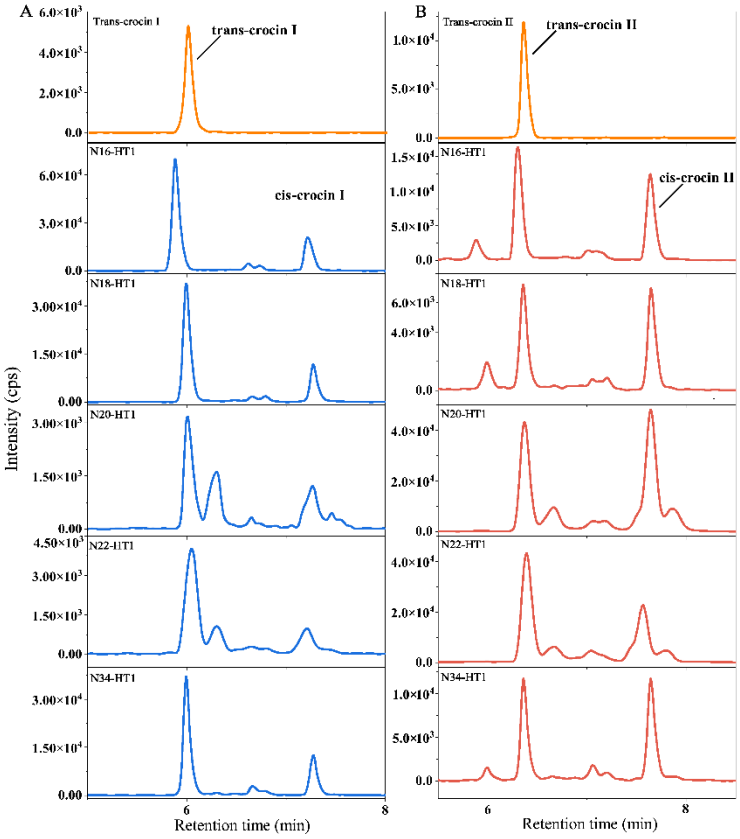


Figure S5. Analysis of crocin I and crocin II in the transgenic tobacco line N16-HT1, N18-HT1, N20-HT1, N22-HT1 and N34-HT1. (A) Ion pair 999.3/675.0 extraction chromatogram for analysis of crocin I in transgenic tobacco line. (B) Ion pair 837.3/675.0 extraction chromatogram for analysis of crocin II in transgenic tobacco line.

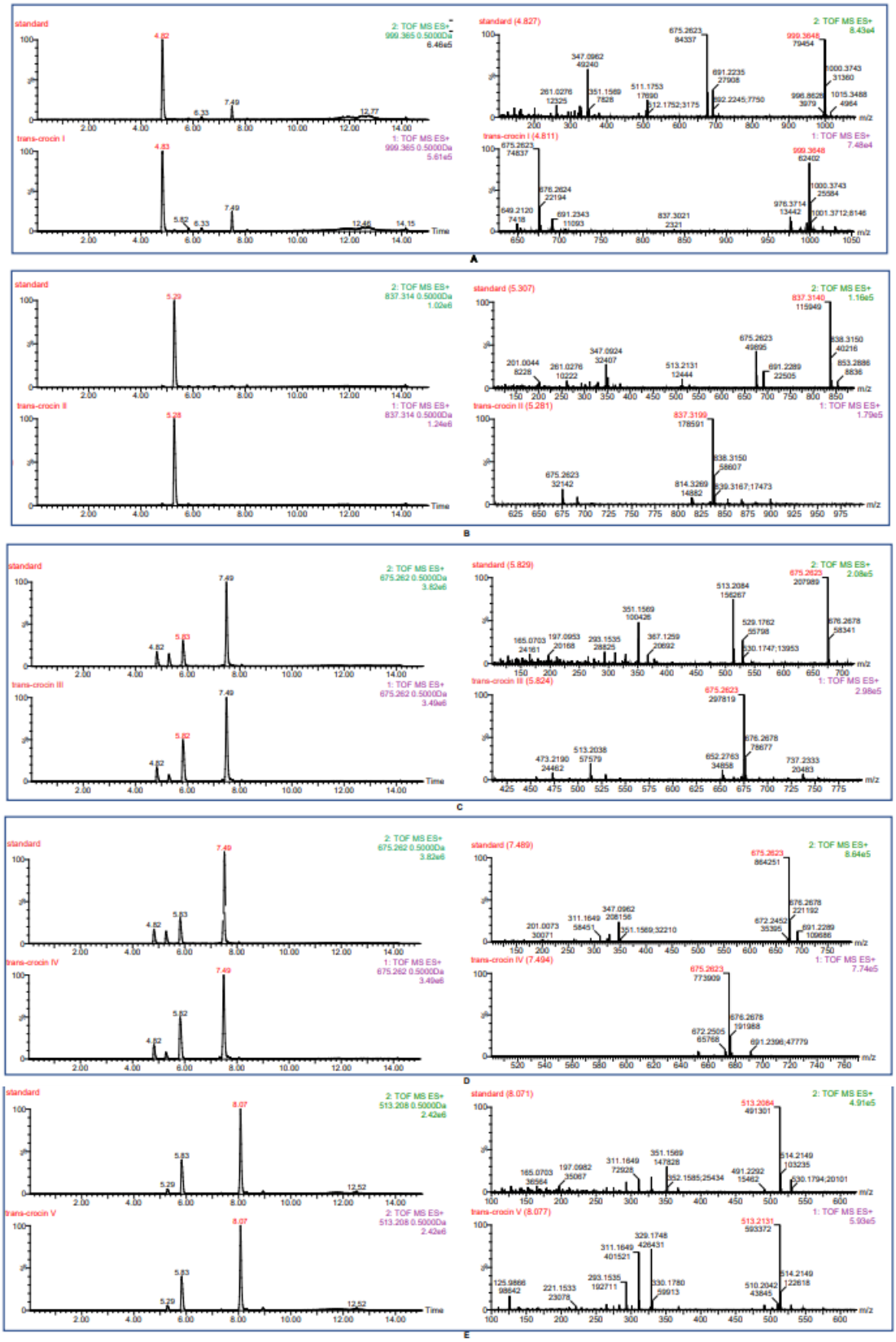


Figure S6. The UPLC-ESI-QTOF-MS/MS analysis of crocins for the standards. (A-E) The ion chromatogram, the MS and MS/MS spectra of crocin V-I.

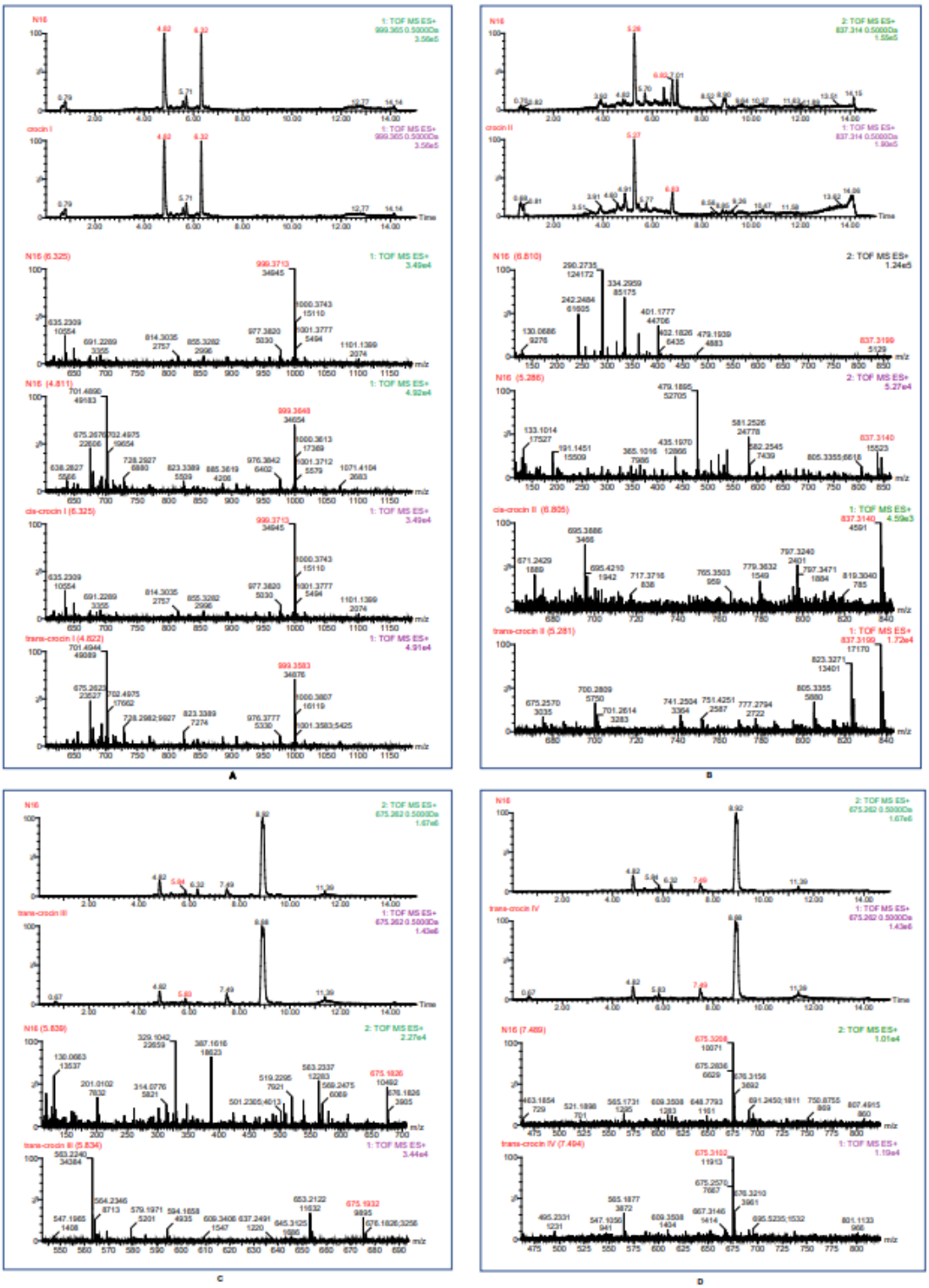
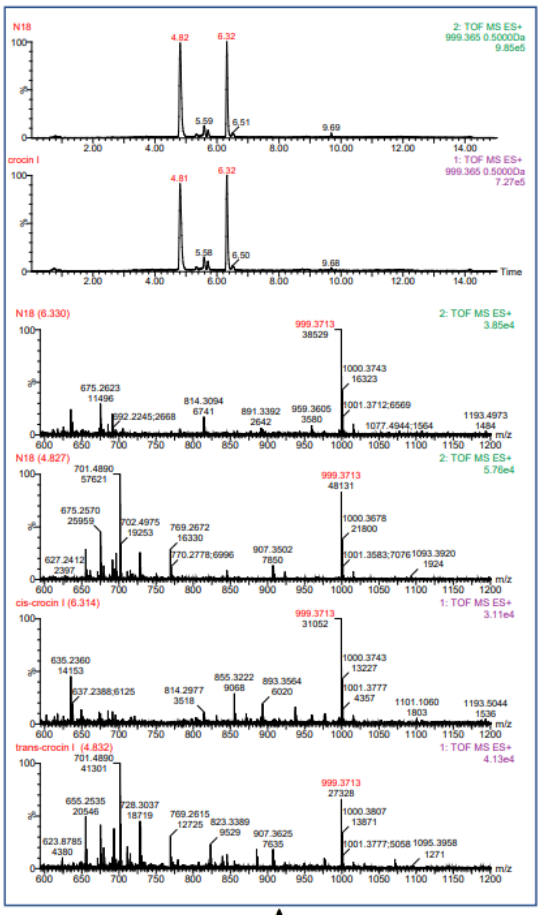
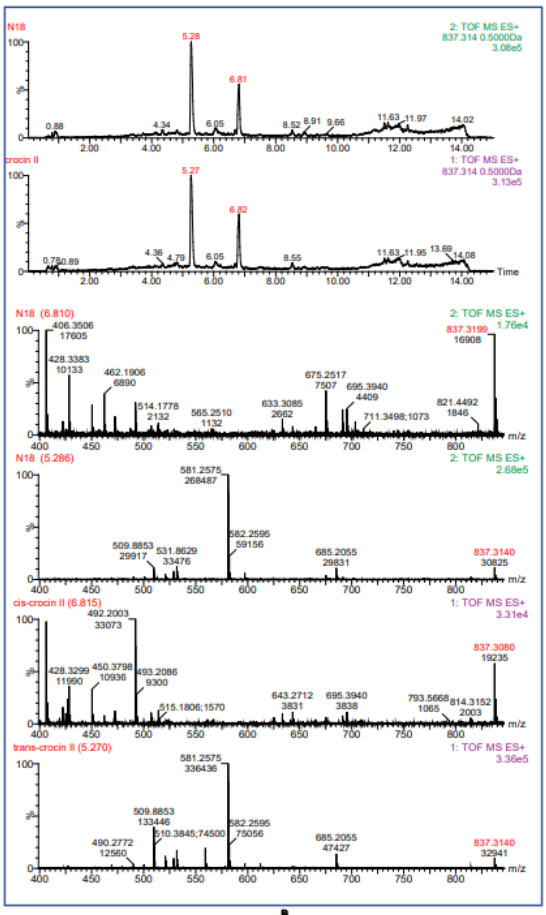


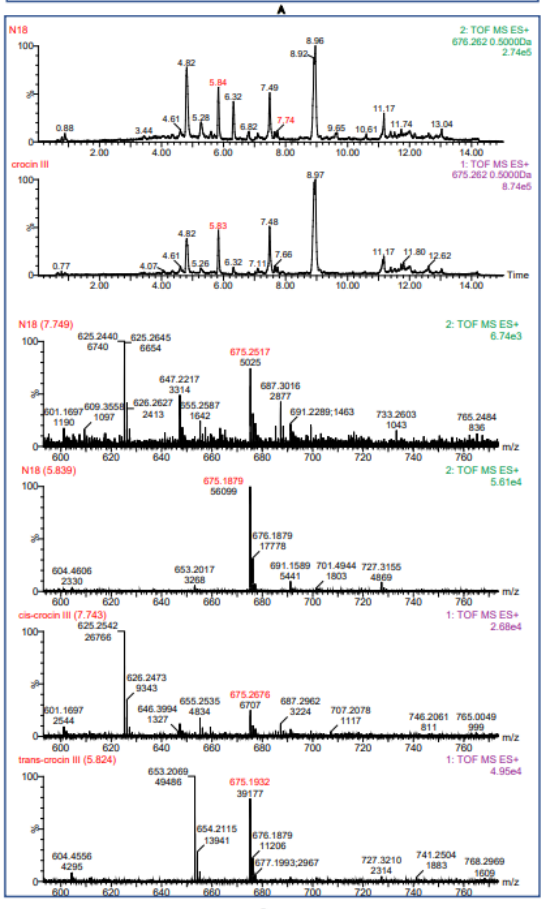
Figure S7. The UPLC-ESI-QTOF-MS/MS analysis of crocins for the N16 line. (A-D) The ion chromatogram, the MS and MS/MS spectra of crocin IV, crocin III, crocin II, and crocin I.


N18
2: TOF MS ES+
999.365 0.5000Da
9.85e5
crocin I
1: TOF MS ES+
999.365 0.5000Da
7.27e5
N18 (6.330)
2: TOF MS ES+
3.85e4
N18 (4.827)
2: TOF MS ES+
5.76e4
cis-crocin I (6.314)
1: TOF MS ES+
3.11e4
trans-crocin I (4.832)
1: TOF MS ES+
4.13e4
m/z
Time

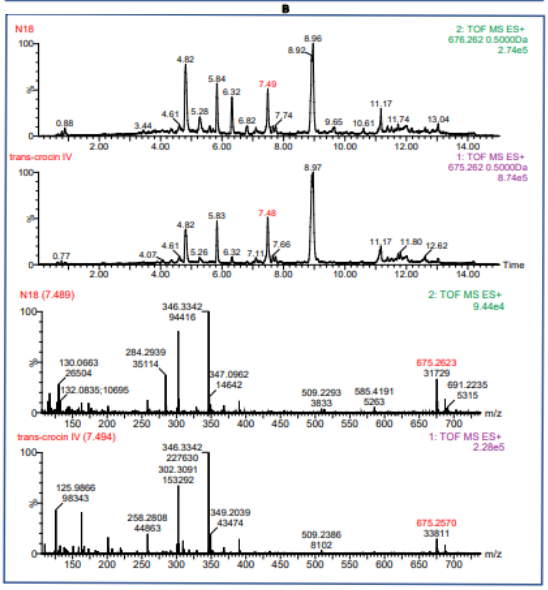
A


N18
2: TOF MS ES+
837.314 0.5000Da
3.08e5
crocin II
1: TOF MS ES+
837.314 0.5000Da
3.13e5
N18 (6.810)
2: TOF MS ES+
1.76e4
N18 (5.286)
2: TOF MS ES+
2.68e5
cis-crocin II (6.815)
1: TOF MS ES+
3.31e4
trans-crocin II (5.270)
1: TOF MS ES+
3.36e5
m/z
Time

B

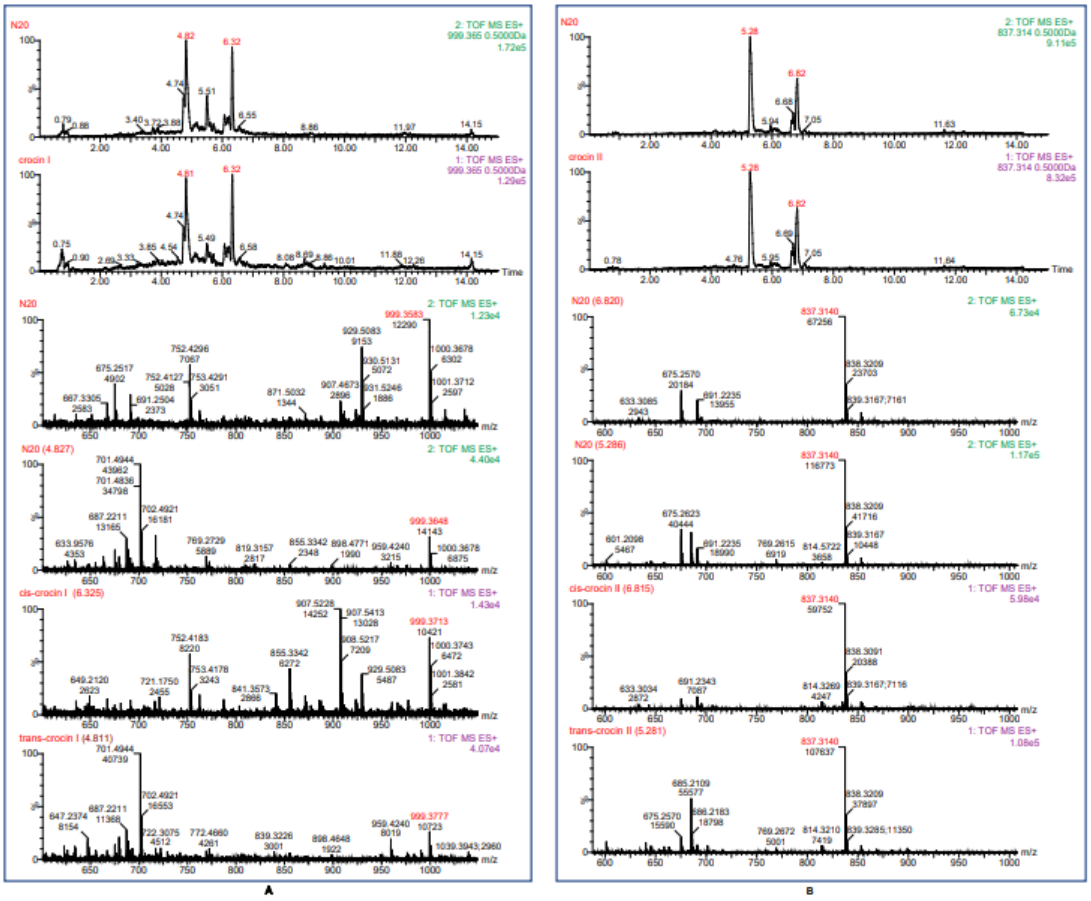

N18
2: TOF MS ES+
676.262 0.5000Da
2.74e5
crocin III
1: TOF MS ES+
675.262 0.5000Da
8.74e5
N18 (7.749)
2: TOF MS ES+
6.74e3
N18 (5.839)
2: TOF MS ES+
5.61e4
cis-crocin III (7.743)
1: TOF MS ES+
2.68e4
trans-crocin III (5.824)
1: TOF MS ES+
4.95e4
m/z
Time

C


N18
2: TOF MS ES+
676.262 0.5000Da
2.74e5
trans-crocin IV
1: TOF MS ES+
675.262 0.5000Da
8.74e5
N18 (7.489)
2: TOF MS ES+
9.44e4
trans-crocin IV (7.494)
1: TOF MS ES+
2.28e5
m/z
Time

D

Figure S8. The UPLC-ESI-QTOF-MS/MS analysis of crocins for the N18 line. (A-D) The ion chromatogram, the MS and MS/MS spectra of crocin IV, crocin III, crocin II, and crocin I.



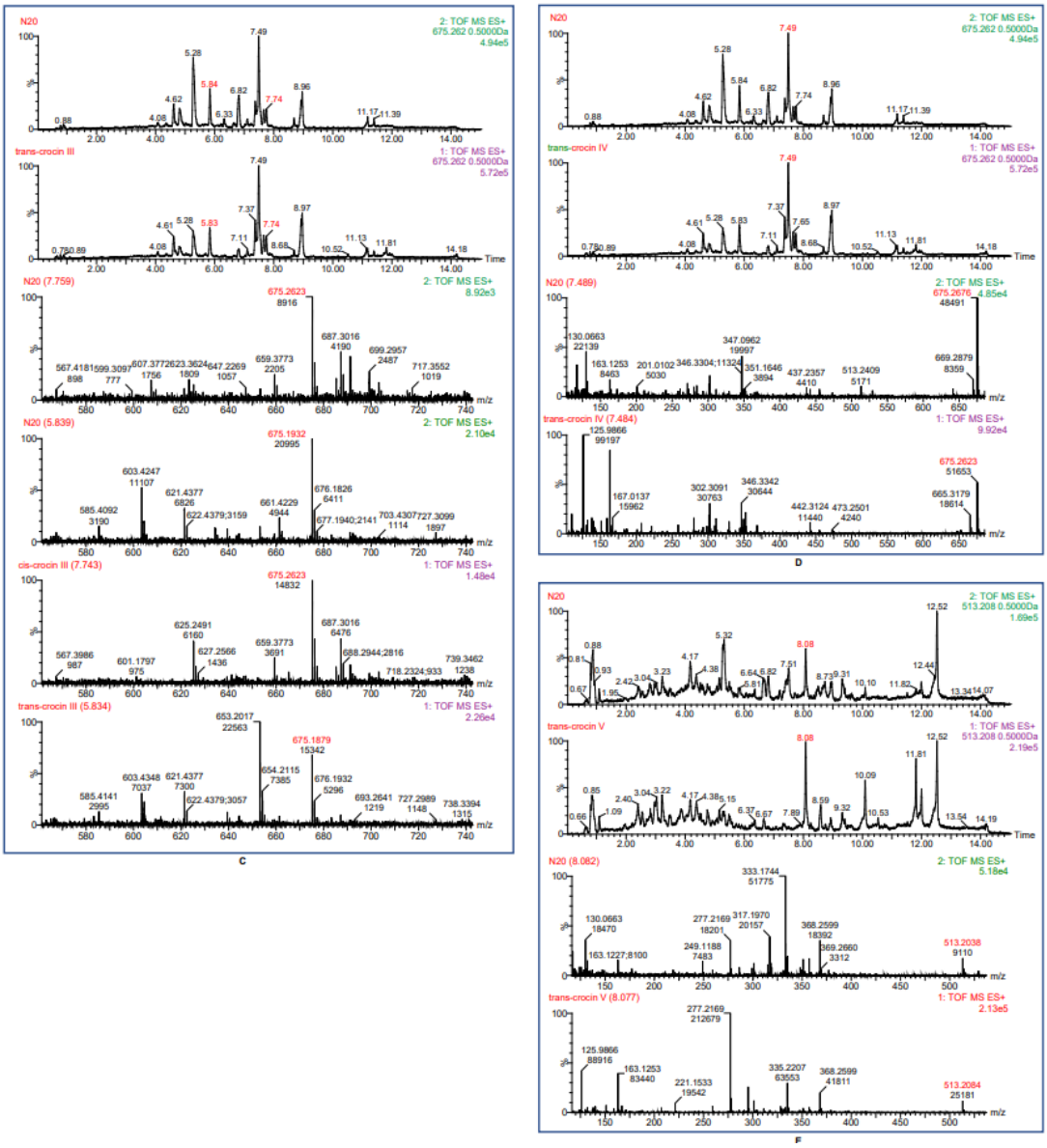


Figure S9. The UPLC-ESI-QTOF-MS/MS analysis of crocins for the N20 line. (A-E) The ion chromatogram, the MS and MS/MS spectra of crocin V, crocin IV, crocin III, crocin II, and crocin I.

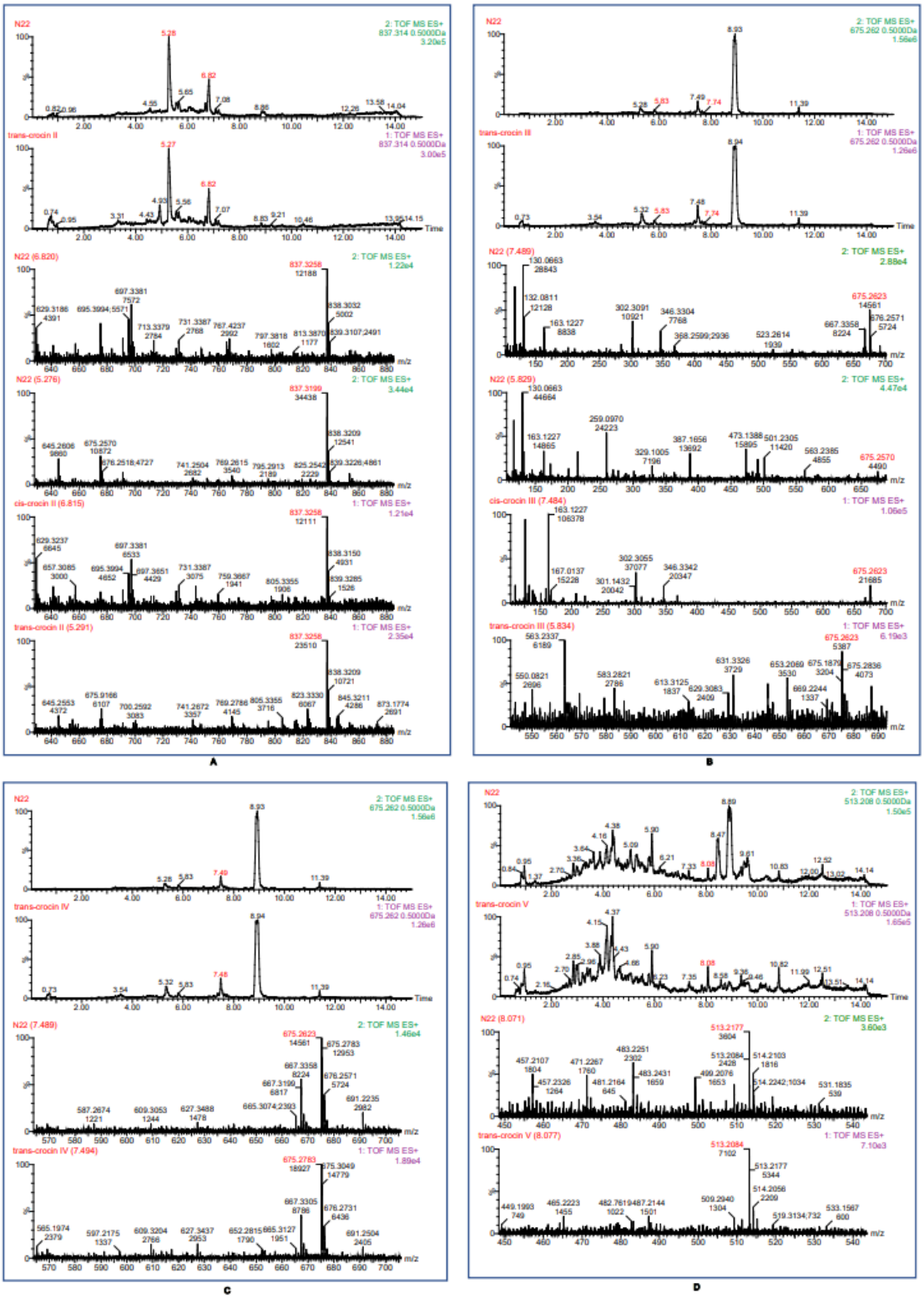


Figure S10. The UPLC-ESI-QTOF-MS/MS analysis of crocins for the N22 line. (A-D) The ion chromatogram, the MS and MS/MS spectra of crocin V, crocin IV, crocin III, and crocin I.

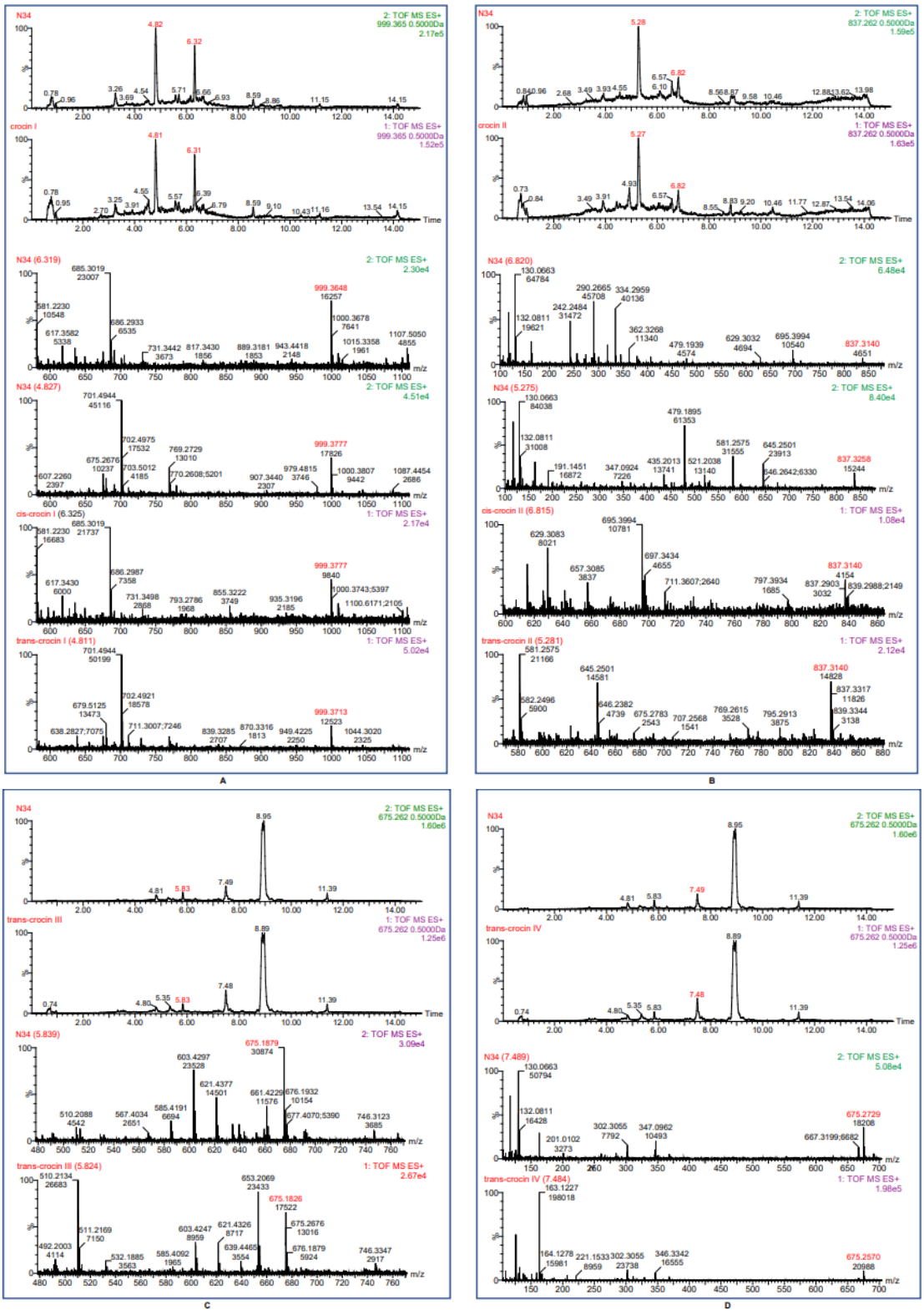


Figure S11. The UPLC-ESI-QTOF-MS/MS analysis of crocins for the N34 line. (A-D) The ion chromatogram, the MS and MS/MS spectra of crocin IV, crocin III, crocin II, and crocin I.

Primers	Sequences (5' to 3')
***GjCCD4a*-F**	ATGACTGATTTATTGTGTTTCCTATACG
***GjCCD4a*-R**	TTACAATTTATTGAGCTCACTTTCTCTG
***GjALDH2C3*-F**	ATGGCCGTCCAAAGCAACG
***GjALDH2C3*-R**	TTACAGCCAAGGTGAATTATAAATGG
***GjUGT74F8*-F**	ATGAATTCCAGCAAAGTTCATGTT
***GjUGT74F8*-R**	CTAGTTCTGGGCCTTCTGGCC
***GjUGT94E13*-F**	ATGAAGGTACTAATGTTGCCATGG
***GjUGT94E13*-R**	TTAACAGCCCCCGTTGTTCTT

Table S1. Primers for PCR detection

Primers	Sequences (5' to 3')
***GjCCD4a*-qF**	CAAGATTGATCTCAAGACTGG
***GjCCD4a*-qR**	GTCTACAACTGCTGCATATAC
***GjALDH2C3*-qF**	GGATTCTCATGTTAAGGTTCC
***GjALDH2C3*-qR**	GGATCTATTGTCTCAAACGTC
***GjUGT94E13*-qF**	CAATAGAACCAGTTGAGCTAC
***GjUGT94E13*qR**	GATATTAGAAAAGTCGGGAGC
***GjUGT74F8*-qF**	CAAAGGACTCGGTAGTTTATG
***GjUGT74F8*-qR**	CTTCTCAAATGATCTCACGAC
***NbActin*-qF**	TATTCCCTAGTATTGTTGGC
***NbActin*-qR**	CTGGGGTATTAAAAGTCTCA

Table S2. Primers for qRT-PC