Electronic Supplementary Information

C-methylation controls the biosynthetic programming of alternapyrone

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Experimental

Plasmid construction and site directed-mutagenesis

The alternapyrone polyketide synthase gene, *mtaltA* (7.98 kb), was amplified from genomic DNA of *Menisporopsis theobromae* BCC 4162 using a primer pair containing regions of overlapping sequences with the *E. coli*-yeast shuttle vector, pEYA2eGFP (Table S1). This vector provided an *egfp* sequence fused with the *mtaltA* at the 3′-end. In this work, KOD-Plus-Neo DNA polymerase (TOYOBO, Japan) was used for both gene amplification and site-directed mutagenesis as described below. The programme used for amplification was as follows: initial denaturation at 94 °C for 2 min followed by 35 cycles of 98 °C for 10 s, 63 °C for 30 s and 68 °C for 8 min with the final extension at 72 °C for 5 min. This was subsequently followed by homologous recombination in *Saccharomyces cerevisiae* YPH 499 between pEYA2eGFP linearised upstream of *egfp* and amplified *mtaltA* using the TRAFO method.[1] The resulting plasmid was purified using Zymoprep Yeast Plasmid Miniprep II (Zymo Research, USA) and named pEYA2-MtAltAeGFP. The plasmid was then amplified in *Escherichia coli* DH5α and purified prior to cloning into the expression vector, pTYGSarg, using the Gateway® cloning system (Invitrogen, USA). The resulting expression vector, containing *mtaltA* fused to *egfp* in the *amy*B expression cassette, was named pTYarg-MtAltAeGFP. For auxiliary genes, *mtaltB* and *mtaltC*, both genes were amplified using the above thermal cycle except annealing temperature, 63 °C for *mtaltB* and 52 °C for *mtaltC*, and extension temperature at 68 °C for 2 min. Both genes were then cloned to pTYGSarg under the *adh* and *eno* expression cassettes by homologous recombination in yeast and the Gateway® cloning was performed with pEYA2-MtAltAeGFP to obtain the expression vector

containing the *mtalt* gene cluster. Mutation of the *C*-MeT domain at the catalytic dyad residues E1604A and H1578A/Q was performed on pEYA2-MtAltTeGFP, and the mutated coding regions transferred to pTYGSarg as above. The primers used for site-directed mutagenesis are shown in Table S1 and the thermal cycles were as follows: initial denaturation at 94 °C for 2 min followed by 35 cycles of 98 °C for 10 s, 70 °C for 30 s and 68 °C for 14 min with the final extension at 72 °C for 5 min. The PCR products were digested with *Dpn*I and then introduced to *E. coli* competent cells. The mutant plasmids were subsequently purified from the *E. coli* transformants. The nucleotide sequences of wild-type and mutated *mtaltA* were checked by Barcode Tag sequencing using Illumina MiSeq platform from Celemics, Inc., South Korea.

Amino acid sequence analysis

Multiple amino acid sequence alignment of *C*-MeT domains of fungal polyketide synthases was performed on Bioedit software[2] and rendered using ESPript 3.0.[3]

***Aspergillus oryzae* NSAR1 transformation**

The expression plasmids were introduced into *A. oryzae* NSAR1 by protoplast-mediated transformation as described by Heneghan et al., using lysing enzymes from *Trichoderma harzianum* for cell wall removal.[4] Transformants were serially subcultured until genetically pure on Czapek-Dox plates containing 4.9 % w/v Czapek-Dox agar, 1 M sorbitol, 0.05 % w/v adenine, 0.1 % w/v ammonium sulphate, 0.15% w/v methionine prior to fermentation for metabolite production.

Fermentation and extraction of transformants

mtaltA and its *C*-MeT mutants were expressed from the amyB promoter. For *mtaltB* and *mtaltC*, they were expressed from the constitutive promoters, P*adh* and P*eno*, respectively. These genes were expressed by culturing in CMP medium (3.5% w/v Czapek-Dox, 2% w/v maltose and 1% peptone). After 7 days, mycelia were separated from broth by vacuum filtration, soaked in acetone for 3 hours and then removed by vacuum filtration. The liquid broth was also extracted with ethyl acetate for metabolite analy-

sis of *C*-MeT mutant transformants. Extraction was also performed under acidic condition by adjusting the pH of culture to 2 and left for 20 min with shaking. The mycelia were then homogenized prior to extraction with ethyl acetate. Subsequently, mycelia were separated from broth and soaked in acetone for 3 hours. Solvent was then evaporated to dryness to yield both acetone and ethyl acetate crude extracts which were subsequently dissolved in HPLC-grade methanol prior to metabolite screening by HPLC-UV and UHPLC-QTOF-MS/MS.

Metabolite screening of transformants by HPLC-UV and UHPLC-QTOF-MS/MS

Crude extracts were fractionated on a C-18 Kaseisorb LC ODS 2000 column (4.6 mm x 150 mm) connected to an Agilent Technologies 1200 HPLC system and eluted with a CH_3OH:H_2O gradient from 5-95% for 17 min and 95% for 10 min, respectively, at a flow rate of 1 mL/min. The UV absorbance was detected at 254 and 280 nm. Metabolite analyses were also performed on a SCIEX X500R QTOF system with Turbo V™ source using the electrospray ionization (ESI) probe operated in positive ion mode. Separation was performed using an ExionLC™ AD system equipped with a 2.6 µm C-18 Phenomenex column (4.6 mm x 100 mm) with a 1 µL injection volume. Samples were eluted with a flow rate of 0.4 mL/min of CH_3OH:H_2O supplemented with 0.05% formic acid. The gradient used for separation was 5-95 % for 19 min and 95% for 10 min, respectively. A SWATH acquisition was also performed on a X500R QTOF System. The TOF MS (scan range: 80-500 Da) parameters were as follows: DP = 80 V, CE = 10 V and accumulation time = 0.25 sec. The TOF MS/MS (scan range 20-500 Da) parameters were DP = 80 V, CE = 30 V, CES = +/-5 V and accumulation time = 0.02 sec. The collision energy spread (CES) was applied to ensure that a comprehensive MS/MS pattern was collected. Data were analysed using the Explorer portion of the SCIEX OS acquisition software.

Isolation and structure determination of alternapyrone (1)

The acetone crude extract of wild type transformants was further isolated using a semi-preparative C-18 Kaseisorb LC ODS 2000 column (10.0 mm x 250 mm) to yield alternapyrone (**1**). The structure of alternapyrone was elucidated by NMR and HRMS. All NMR data (^{1}H, ^{13}C, COSY, HSQC

and HMBC) were collected on an AVANCE III HD 400 spectrometer using acetone-d_6 as solvent. Both ^{1}H and ^{13}C NMR chemical shifts are shown in Table S2. All HRMS experiments were performed on a Bruker micrOTOf-Q III instrument using atmospheric pressure chemical ionisation (APCI) in positive mode.

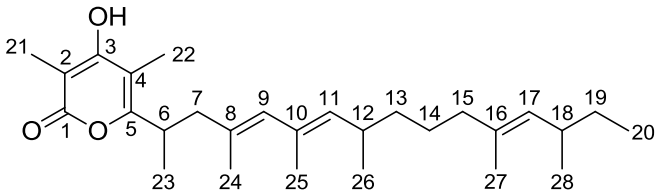
Fluorescence analysis

The expression of *altpks* and its CMeT domain mutants was monitored by fluorescence microscopy using a Leica Thunder Imager 3D microscope system with an excitation wavelength of 488 nm for EGFP.

Table S1 Primers used for amplification of *mtalt* genes and mutations of *mtaltA*. Underlined nucleotides of primers for constructing *mtaltA, mtaltB* and *mataltC* are regions of overlap with pEYA2eGFP, *adh* and *eno* expression cassettes, respectively. The mutated nucleotides in the primers used for site-directed mutagenesis are also underlined.

Primer	Sequence 5′-3′	Description
mtaltA-fwd	TAATGCCAACTTTGTACAAAAAAGCAGGCTCCATGGCCCAACATACAGCCCG	wild-type AltPKS
mtaltA-rev	GGTGAACAGCTCCTCGCCCTTGCTCACCATTTCGCCAGCCTTGGCAGCCT	
mtaltB-fwd	TTTCTTTCAACACAAGATCCCAAAGTCAAAATGGCCGCGGCCGAGAACAG	MtAltB
mtaltB-rev	CATTCTATGCGTTATGAACATGTTCCCTCTAGTGACTCTCATCTTCTACC	
mtaltC-fwd	CGACTGACCAATTCCGCAGCTCGTCAAAATGGACGTCCCCGGCCTTCTTG	MtAltC
mtaltC-rev	TTGGCTGGTAGACGTCATATAATCATACTTACAAGTCCCGCCTTGGGATC	
H1578A-fwd	GCCGCCAACGTCCTGGCCGCCACCAAGAGCATCG	H1578A AltPKS
H1578A-rev	CGATGCTCTTGGTGGCGGCCAGGACGTTGGCGGC	
H1578Q-fwd	GCCGCCAACGTCCTGCAGGCCACCAAGAGCATCG	H1578Q AltPKS
H1578Q-rev	CGATGCTCTTGGTGGCCTGCAGGACGTTGGCGGC	
E1604A-fwd	GCAAAATCGTGCTGAGCGCGGTGACCATCAAGCG	E1604A AltPKS
E1604A-rev	CGCTTGATGGTCACCGCGCTCAGCACGATTTTGC	

Table S2 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR chemical shifts in $(CD_3)_2CO$ and HRMS result for alternapyrone (**1**).



alternapyrone (1)

Position	δ_C (ppm)	δ_H (ppm), multiplicity (*J*)
1	165.2	-
2	98.3	-
3	165.0	-
4	106.8	-
5	161.4	-
6	34.0	3.22, m
7	45.9	2.33, dd (*J* = 13.0, 8.3 Hz) 2.19, dd (*J* = 13.0, 6.5 Hz)
8	133.2	-
9	132.1	5.58, s
10	131.8	-
11	136.6	4.91, br d (*J* = 11.1 Hz)
12	33.0	2.39-2.45, m
13	37.8	1.16-1.22, m 1.26-1.34, m
14	26.6	1.36-1.42, m
15	40.5	1.93-1.98, m
16	134.4	-
17	132.0	4.88, br d (*J* = 10.9 Hz)
18	34.7	2.23-2.27, m
19	31.3	1.12-1.22, m 1.27-1.38, m
20	12.3	0.82, t (*J* = 7.4 Hz)
21	9.1	1.91, s
22	10.0	1.96, s
23	18.2	1.17, d (*J* = 7.3 Hz)
24	18.0	1.74, d (*J* = 1.4 Hz)
25	17.3	1.62, d (*J* = 1.4 Hz)
26	21.5	0.92, d (*J* = 6.7 Hz)
27	16.2	1.57, d (*J* = 1.4 Hz)
28	21.4	0.89, d (*J* = 6.7 Hz)

APCI-TOFMS (m/z): calculated for $C_{28}H_{45}O_3$ $[M+H]^+$ 429.3363, found 429.3374

Results

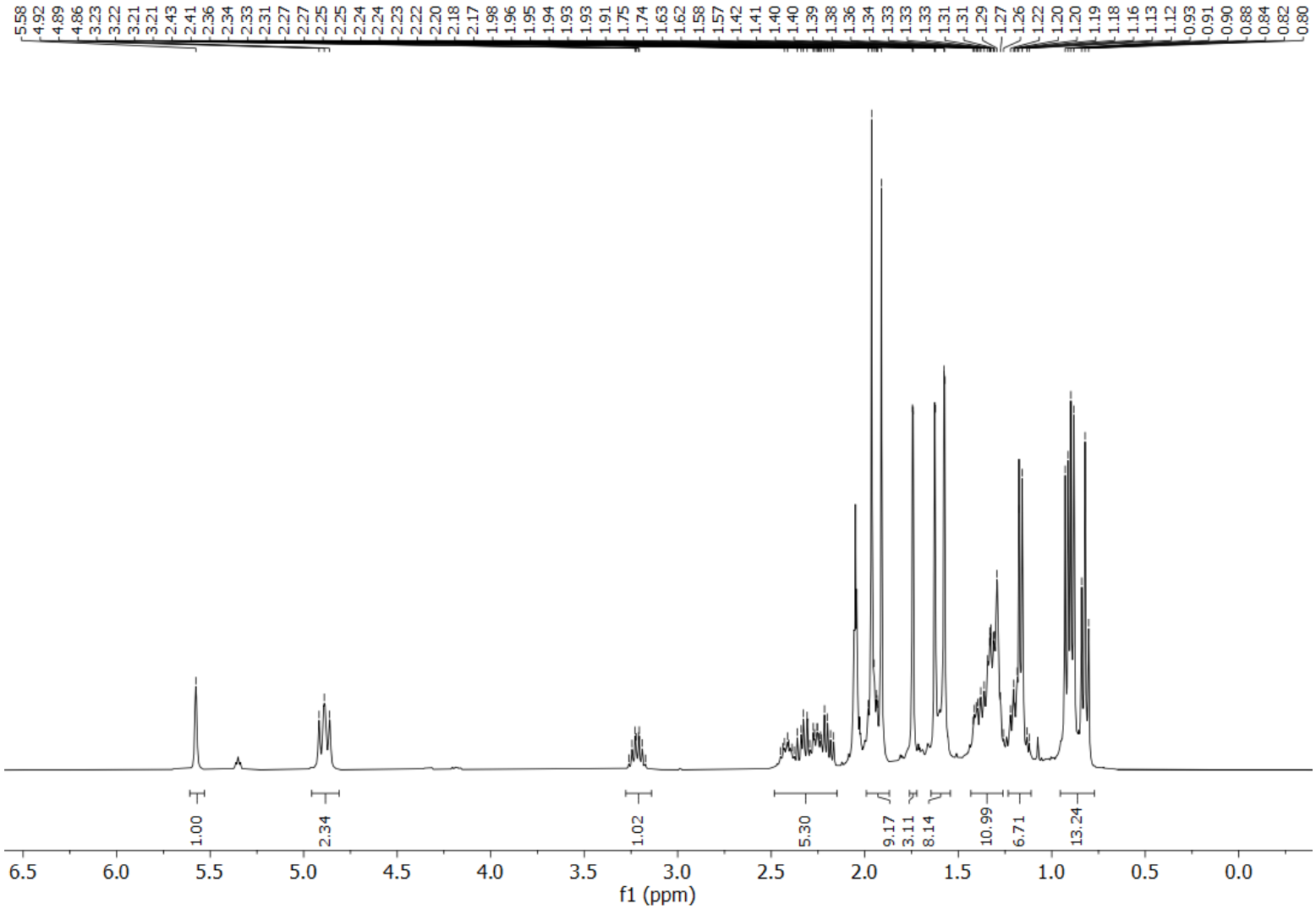


Fig. S1 ^{1}H (400 MHz) NMR spectrum of alternapyrone (**1**) in $(CD_3)_2CO$.

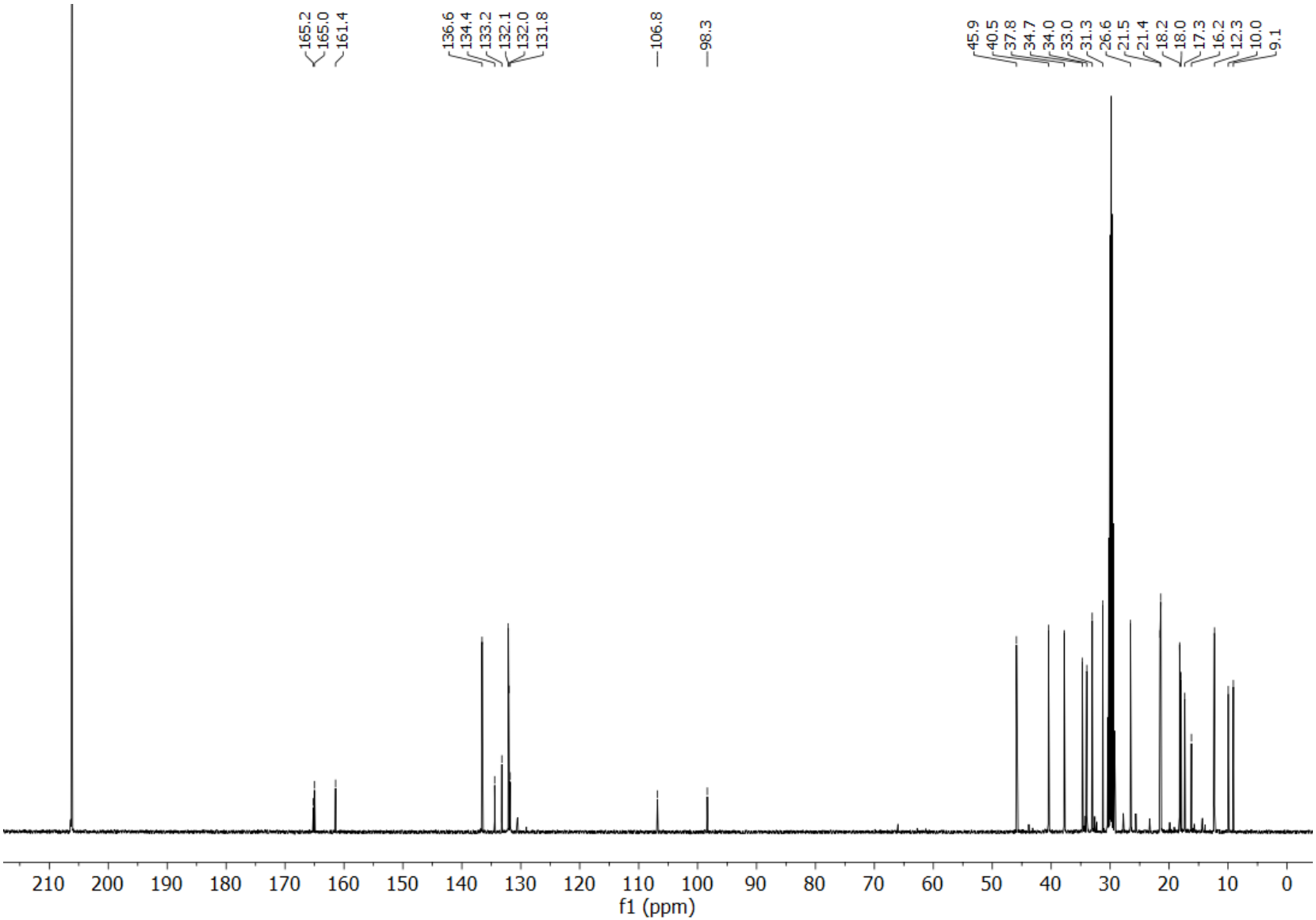


Fig. S2 ^{13}C (100 MHz) NMR spectrum of alternapyrone (**1**) in $(CD_3)_2CO$.

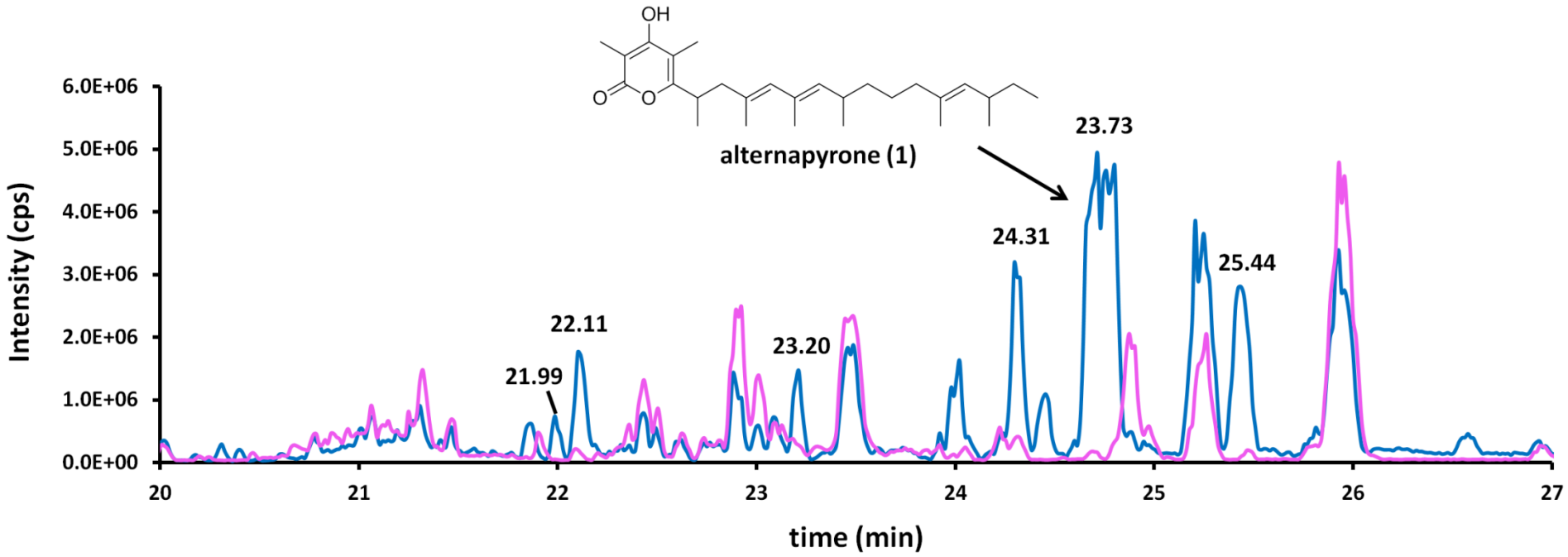


Fig. S3 Comparison of base peak chromatograms of the acetone crude extracts of a transformant expressing AltPKS (blue) with a control (a transformant without *mtaltA*) (pink). Peaks labelled with retention time belong to compounds with the MS/MS fragmentation pattern related to alternapyrone.

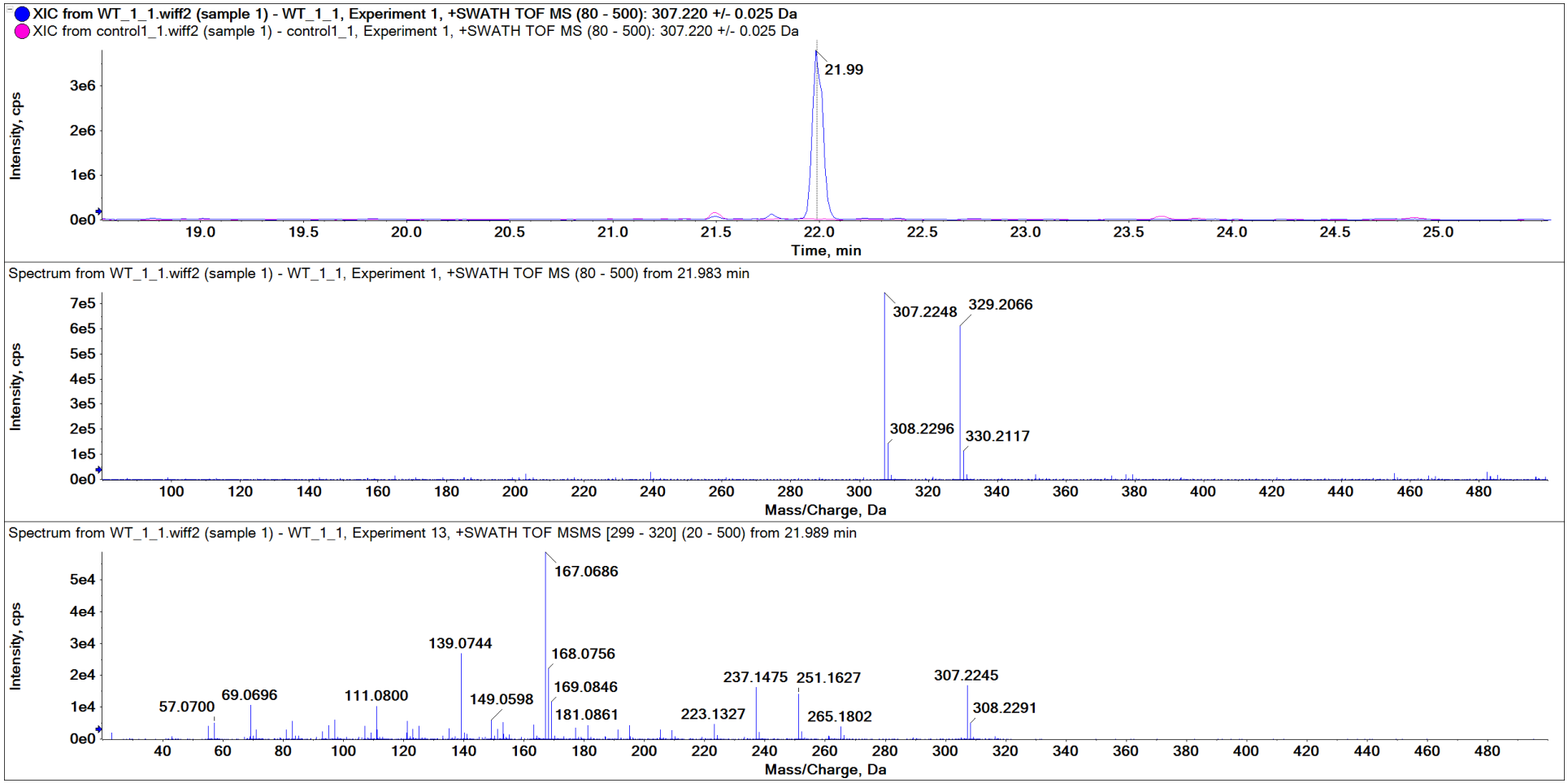


Fig. S4 Comparison of extracted ion chromatograms (top) with a *m/z* range of 307.220 ± 0.025 Da of the acetone crude extract of a transformant expressing AltPKS (blue) with a control transformant (pink). MS and MS/MS spectra of a product eluted at 21.99 min are also shown in the middle and bottom panels. UHPLC-ESI-QTOF-MS: calculated for $C_{19}H_{31}O_3$ $[M+H]^+$ 307.2268, found 307.2248 and $C_{19}H_{30}O_3Na$ $[M+Na]^+$ 329.2087, found 329.2066.

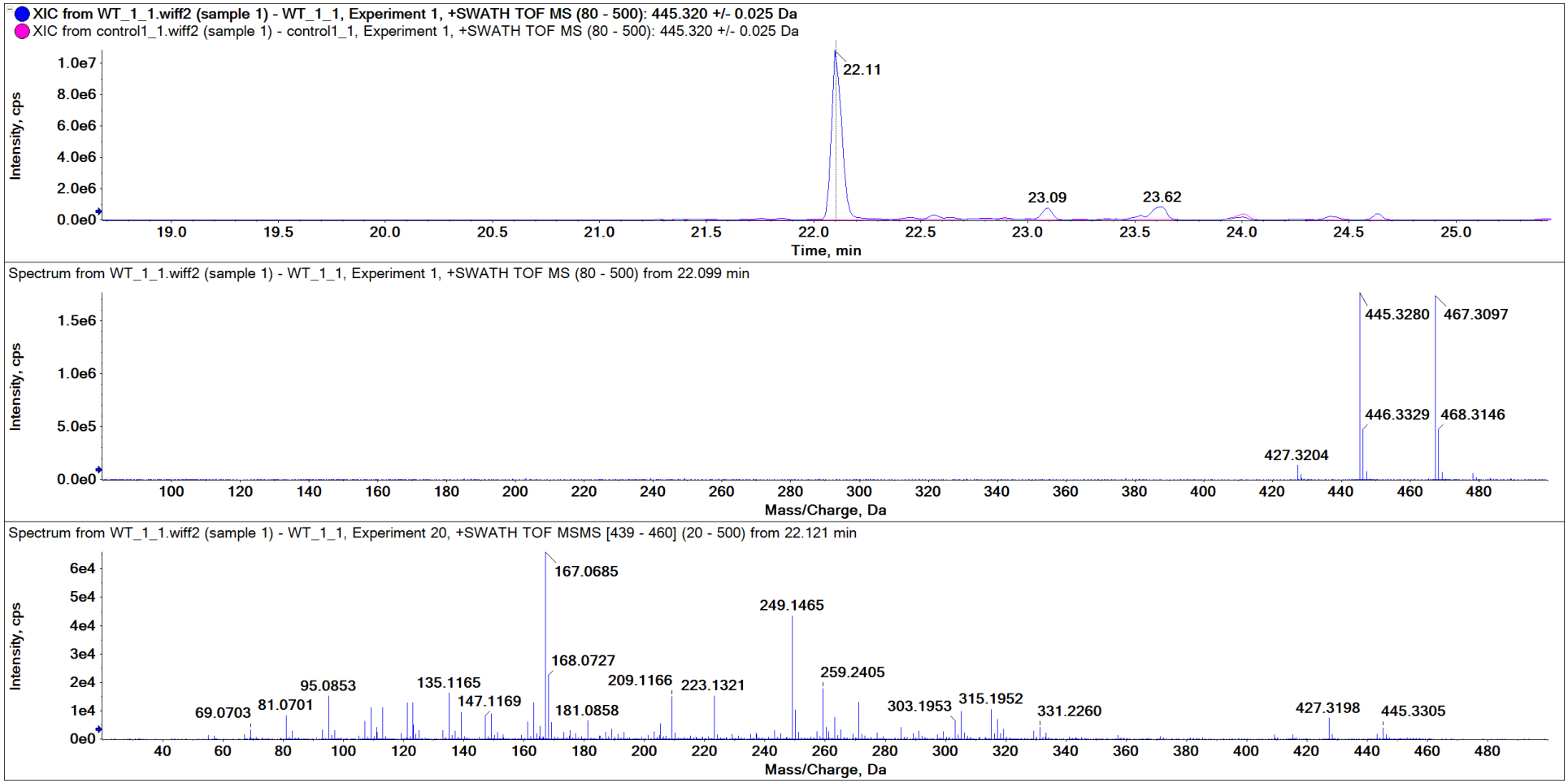


Fig. S5 Comparison of extracted ion chromatograms (top) with a *m/z* range of 445.320 ± 0.025 Da of the acetone crude extract of a transformant expressing AltPKS (blue) with a control transformant (pink). MS and MS/MS spectra of a product eluted at 22.11 min are also shown in the middle and bottom panels. UHPLC-ESI-QTOF-MS: calculated for $C_{28}H_{45}O_4$ $[M+H]^+$ 445.3312, found 445.3280 and $C_{28}H_{44}O_4Na$ $[M+Na]^+$ 467.3132, found 467.3097.

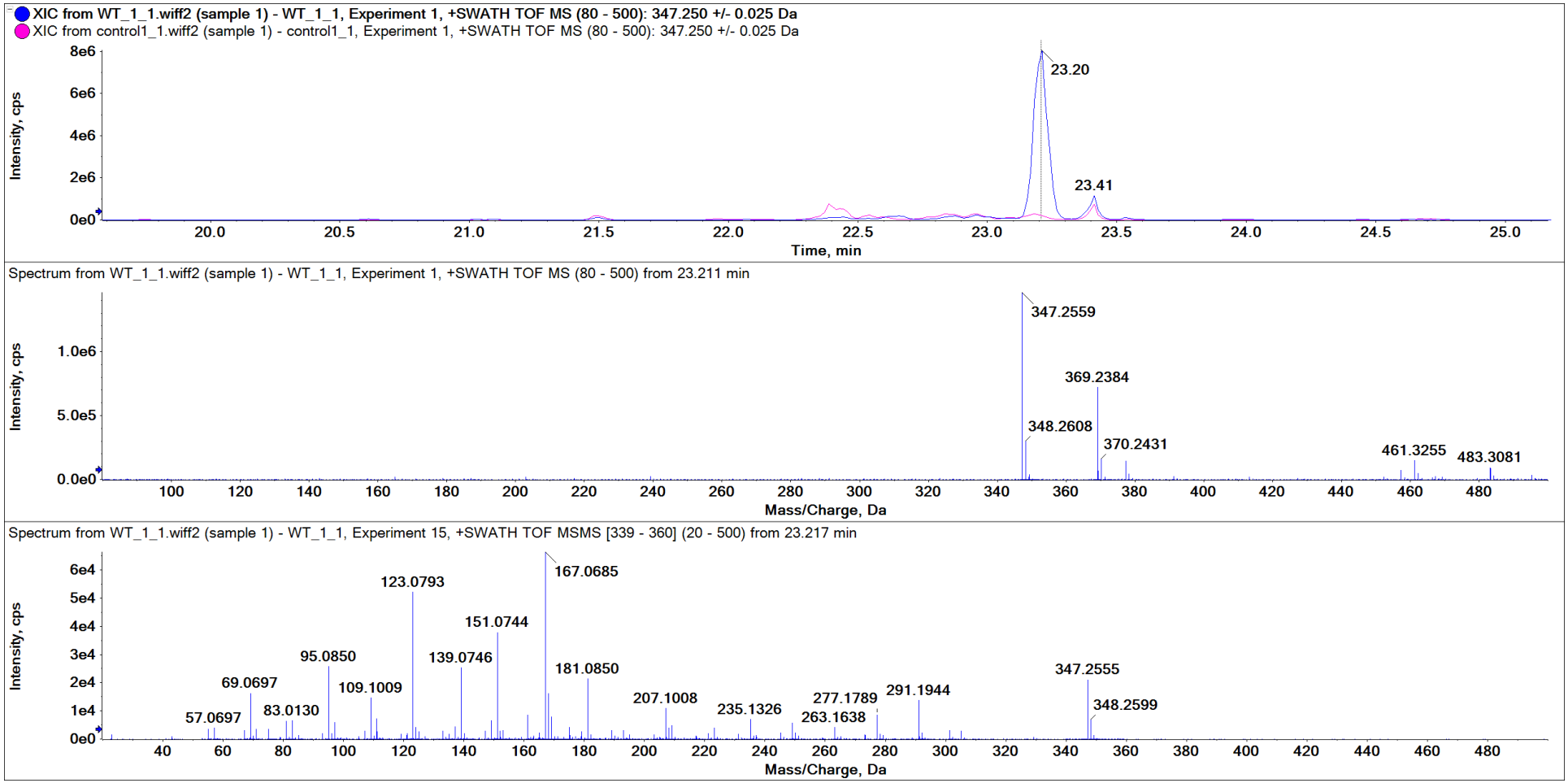


Fig. S6 Comparison of extracted ion chromatograms (top) with a *m/z* range of 347.250 ± 0.025 Da of the acetone crude extract of a transformant expressing AltPKS (blue) with a control transformant (pink). MS and MS/MS spectra of a product eluted at 23.20 min are also shown in the middle and bottom panels. UHPLC-ESI-QTOF-MS: calculated for $C_{22}H_{35}O_3$ $[M+H]^+$ 347.2581, found 347.2559 and $C_{22}H_{34}O_3Na$ $[M+Na]^+$ 369.2400, found 369.2384.

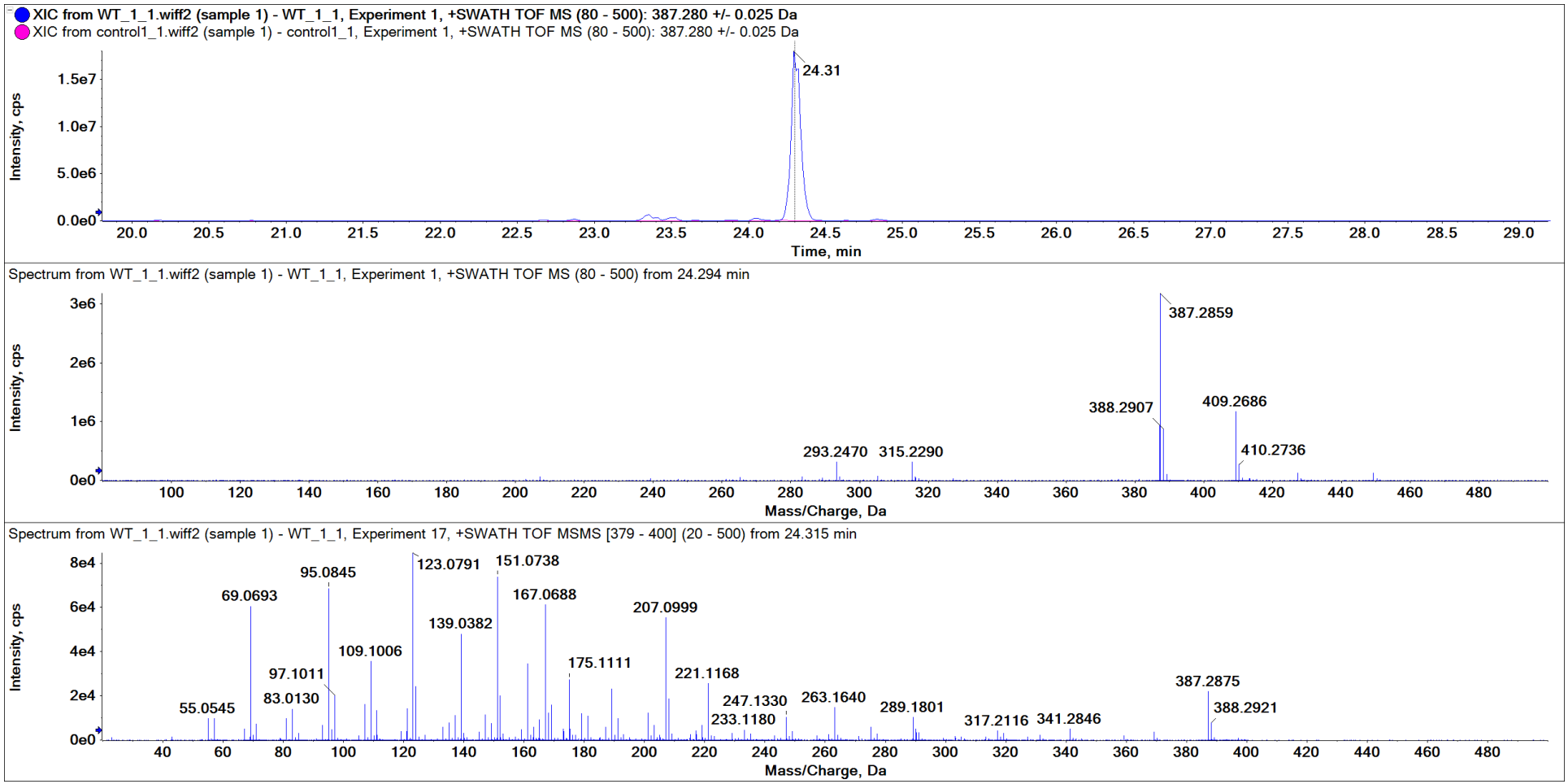


Fig. S7 Comparison of extracted ion chromatograms (top) with a *m/z* range of 387.280 ± 0.025 Da of the acetone crude extract of a transformant expressing AltPKS (blue) with a control transformant (pink). MS and MS/MS spectra of a product eluted at 24.31 min are also shown in the middle and bottom panels. UHPLC-ESI-QTOF-MS: calculated for $C_{25}H_{39}O_3$ $[M+H]^+$ 387.2894, found 387.2859 and $C_{25}H_{38}O_3Na$ $[M+Na]^+$ 409.2713, found 409.2686.

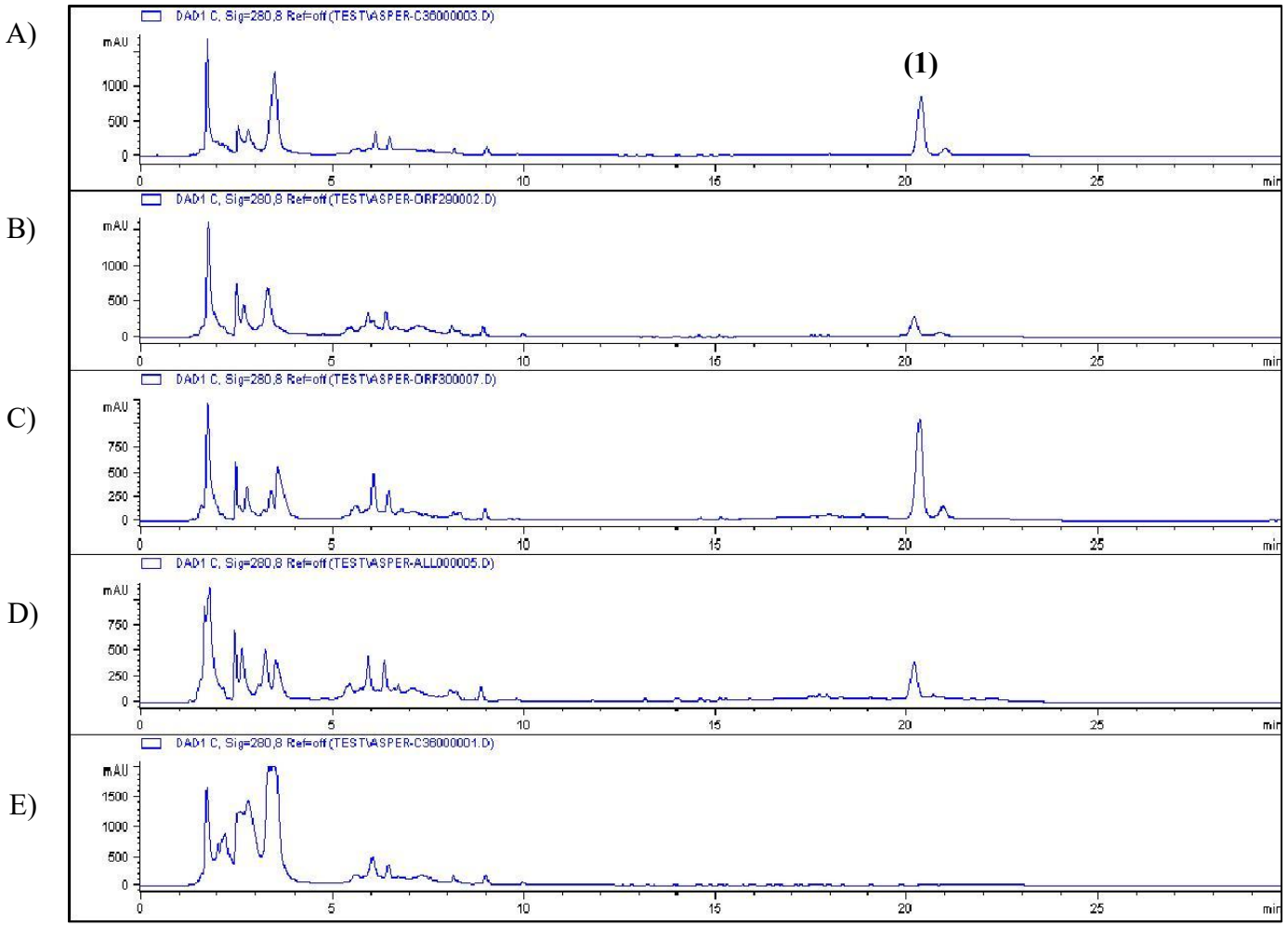


Fig. S8 HPLC chromatograms detected with UV at 280 nm of the acetone crude extract of transformants expressing A) MtAltA, B) MtAltAB, C) MtAltAC, D) MtAltABC and E) control (a transformant without *mtalt* genes). Alternapyrone (**1**) could be observed in all transformants except the control.

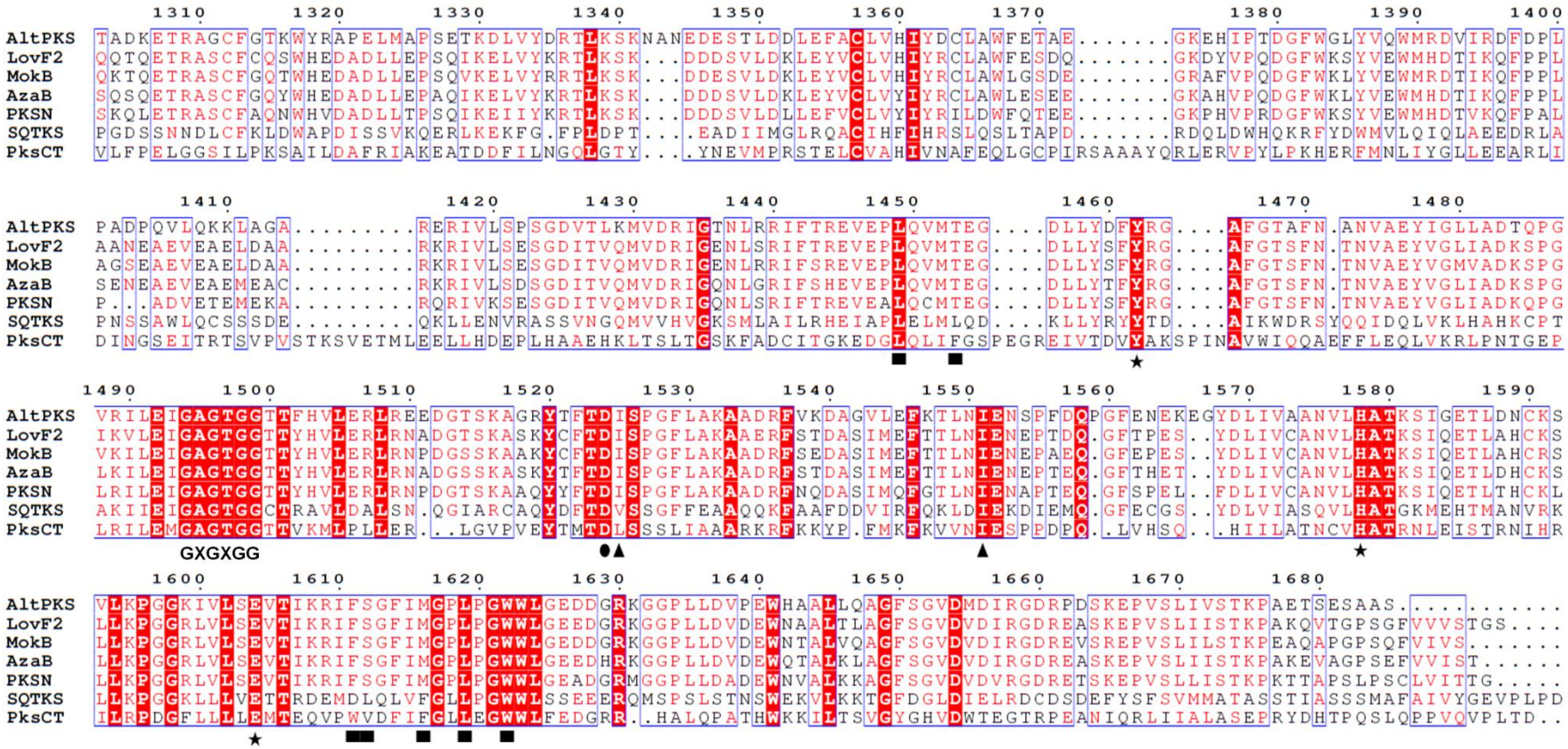


Fig. S9 Amino acid sequence alignment of the CMeT domain of reducing (AltPKS (ON131098), LovF2 (OLN91972), MokB (TIC96982), AzaB (KAF3802434), PKSN (Q5KTM9) and SQTKS (Q86ZD9)) PKSs compared with that of the known *C*-MeT domain structure of nonreducing PKS, PksCT (Q65Z23). GenPept accession numbers are given in brackets. The catalytic residues and residues forming a hydrophobic patch around substrate binding tunnel are marked with stars and squares, respectively. For the *S*-adenosylhomocysteine (SAH) binding pocket, the GXGXGG motif forming hydrogen bonds with the homocysteine moiety are conserved among all PKSs. The residues binding with ribose and forming a hydrophobic pocket for adenine moiety are marked with a circle and triangles, respectively

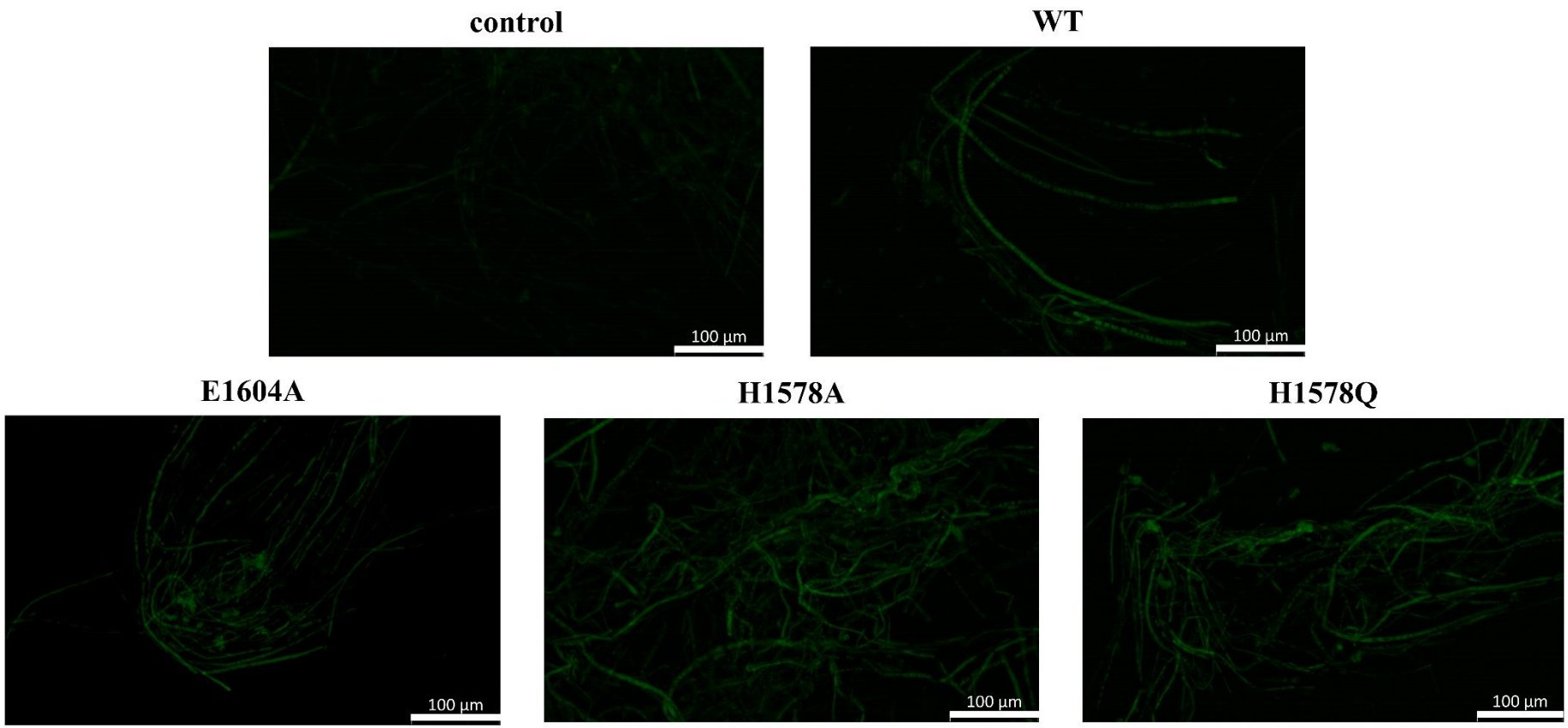


Fig. S10 Green fluorescence imaging of *A. oryzae* NSAR1 transformants expressing wild type AltPKS and its *C*-MeT domain mutants.

References

1. R. D. Gietz and R. A. Woods, *Methods Enzymol.*, 2002, **350**, 87-96.
2. T. A. Hall, *Nucleic Acids Symp. Ser.*, 1999, **41**, 95-98.
3. X. Robert and P. Gouet, *Nucleic Acids Res.*, 2014, **42**, W320-324.
4. M. N. Heneghan, A. A. Yakasai, L. M. Halo, Z. Song, A. M. Bailey, T. J. Simpson, R. J. Cox and C. M. Lazarus, *Chembiochem*, 2010, **11**, 1508-1512.