Supplementary Materials

Brief Report

Attempting to Create a Pathway to 15-Deacetylcalonectrin with Limited Accumulation in Cultures of *Fusarium Tri3* Mutants: Insight into the Trichothecene Biosynthesis Machinery

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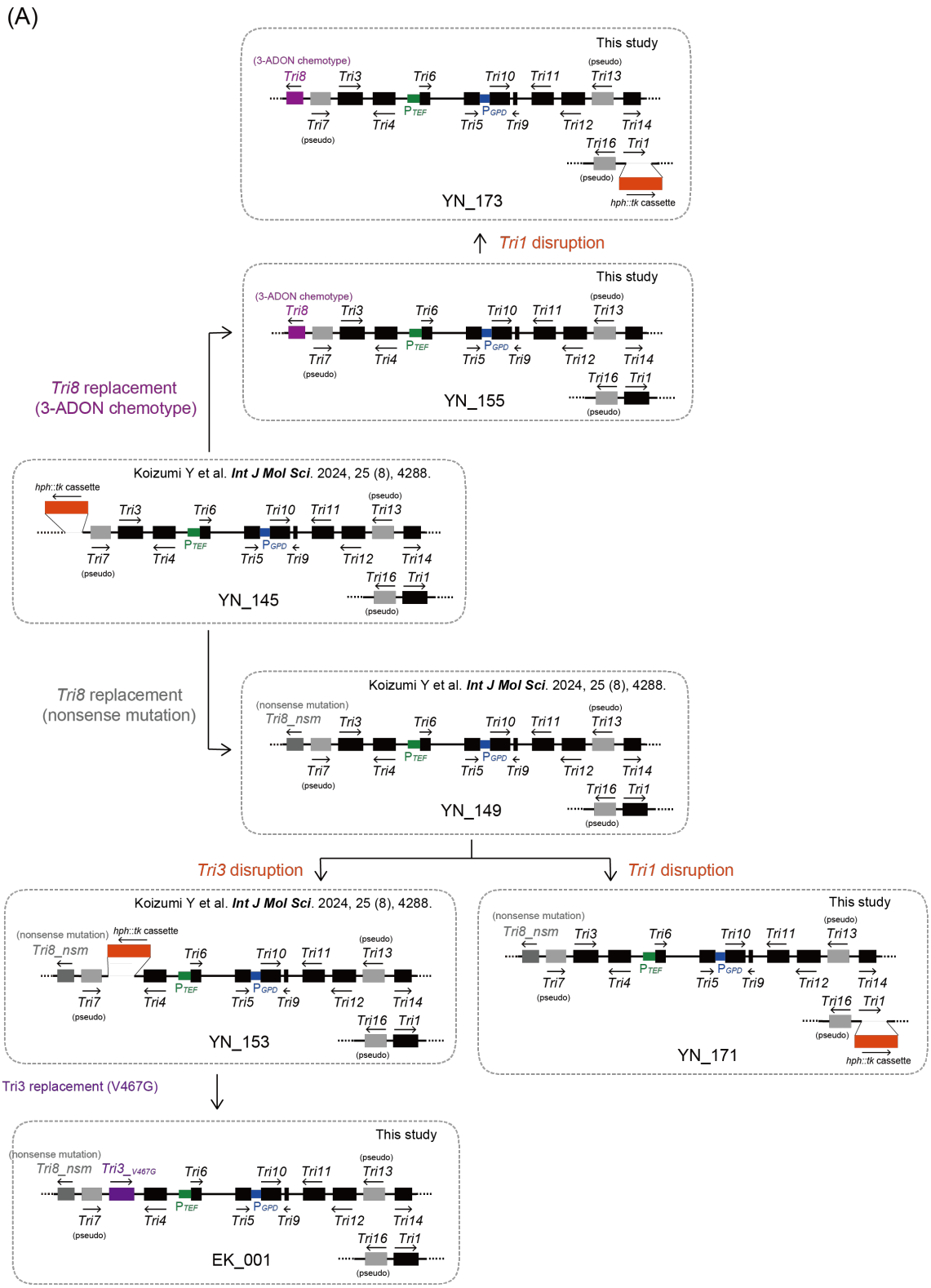
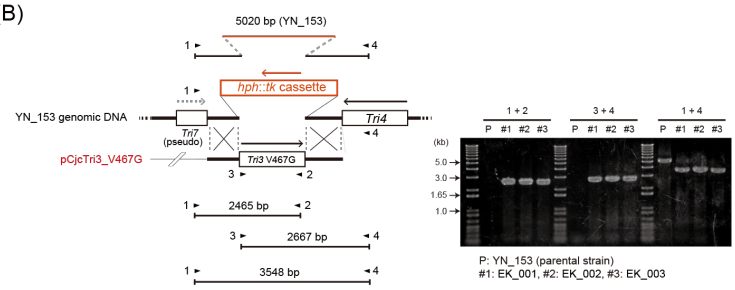
Supplementary Table S1. *Fusarium* strains and accession numbers of *EF-1α* and *RPB2* sequence used in molecular phylogenetic analysis.

Species	Strain[a]	GenBank accession nos.		References
		EF-1α	*RPB2*	
F. commune	MT-25	LC749603	LC749604	This study
F. beomiforme	RBG988	HQ667158	HQ646397	[1]
	RBG4549	HQ667157	HQ646396	[1]
F. commune	MRC 2564	MH582349	MH582180	[2]
	MRC 2566	MH582348	MH582181	[2]
	NRRL 28387	HM057338	JX171638	[2]
F. fujikuroi	MRC 2322	MH582343	MH582149	[2]
	MRC 2387	MH582340	MH582157	[2]
	MRC 2388	MH582341	MH582158	[2]
	MRC 2390	MH582342	MH582159	[2]
F. oxysporum	MRC 1694	MH582350	MH582094	[2]
	MRC 2066	MH582352	MH582126	[2]
	MRC 2199	MH582353	MH582140	[2]
	MRC 2325	MH582351	MH582152	[2]
	MRC 2536	MH582354	MH582173	[2]
F. proliferatum	MRC 2324	MH582344	MH582151	[2]
	MRC 2535	MH582346	MH582172	[2]
F. subglutinans	MRC 2627	MH582310	MH582187	[2]
	NRRL 22016	HM057336	JX171599	[3]
F. verticillioides	MRC 137	MH582315	MH582078	[2]
	NRRL 22172	MW402146	EF470122	[4]
				[5]

[a]RBG, Royal Botanic Gardens Trust, Sydney, New South Wales, Australia; MRC, South African Medical Research Council, Cape Town, South Africa; NRRL, The Agriculture Research Service Culture Collection, National Center for Agricultural Utilization Research, USDA/ARS, Peoria, IL, USA


(A)
This study
(3-ADON chemotype)
Tri8 Tri3 Tri6 Tri10 Tri11 Tri13
Tri7 (pseudo) Tri4 PTEF Tri5 PGPD Tri9 Tri12 Tri14
Tri16 Tri1
(pseudo)
YN_173
hph::tk cassette
Tri1 disruption
This study
(3-ADON chemotype)
YN_155
Tri8 replacement
(3-ADON chemotype)
Koizumi Y et al. Int J Mol Sci. 2024, 25 (8), 4288.
hph::tk cassette
YN_145
Tri8 replacement
(nonsense mutation)
Koizumi Y et al. Int J Mol Sci. 2024, 25 (8), 4288.
(nonsense mutation)
Tri8_nsm
YN_149
Tri3 disruption
Tri1 disruption
Koizumi Y et al. Int J Mol Sci. 2024, 25 (8), 4288.
(nonsense mutation) hph::tk cassette
Tri8_nsm
YN_153
This study
(nonsense mutation)
Tri8_nsm
YN_171
hph::tk cassette
Tri3 replacement (V467G)
This study
(nonsense mutation)
Tri8_nsm Tri3_V467G
EK_001

(B)



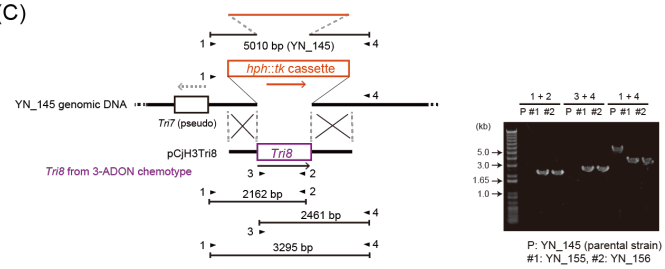
P: YN_153 (parental strain)
#1: EK_001, #2: EK_002, #3: EK_003

1 ► dTri3-Long_S: GAGCTTTGTGAACACCATTGGCGTCGACAC
2 ► GA_sw_Tri3_AS: AGAATAAACAGCAAATTACAGTTTGAATGCCAACATGAAC
3 ► GA_sw_Tri3_S: CCTACAACCATCAAGATGAGCGCTTCATCCTCCGCCTTGC
4 ► dTri3-Long_AS: GCGAGCTTACGACATTGAGCTTTATGACACC

↓

EK_001, EK_002, EK_003

(C)



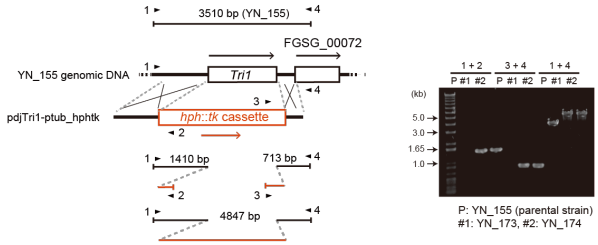
P: YN_145 (parental strain)
#1: YN_155, #2: YN_156

1 ► dTri8-Long_S: AGGACAATGACAGGAGAGGCATATCGCACC
2 ► F15-8sw-8_AS: GAAATGAAATCACAACTCTCGTGTTACGTCTCTC
3 ► F15-8sw-8_S: CACTCAGTATGGTTCTCGATCGTTTGTTGTTTCTTC
4 ► dTri8-Long_AS: GCTACATATACAGTAGGTATTTCTTCCTCCTGC

↓

YN_155, YN_156

(D)



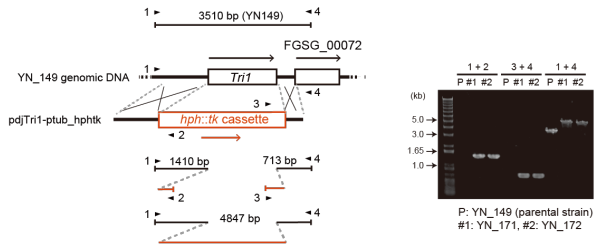
P: YN_155 (parental strain)
#1: YN_173, #2: YN_174

1 ► dTri1-Long_S: CCCGAGTAGACATCATGCCAACAGTTAGTTCTC
2 ► Specific_on_Ptub2: AACTGTGTAACTGCGTTATCGGTAGTGCGCTCC
3 ► Specific_on_HSVtk2: GCACGTCTTTATCCTGGATTACGACCAATCG
4 ► dTri1-Long_AS: CCGCCACATTCGCTAGTTTGTACTTAAGCGAG

↓

YN_173, YN_174

(E)



P: YN_149 (parental strain)
#1: YN_171, #2: YN_172

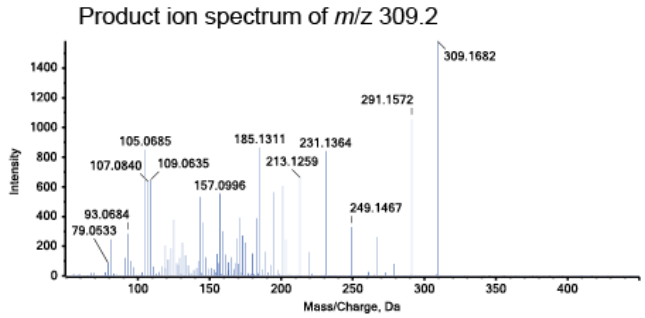
1 ► dTri1-Long_S: CCCGAGTAGACATCATGCCAACAGTTAGTTCTC
2 ► Specific_on_Ptub2: AACTGTGTAACTGCGTTATCGGTAGTGCGCTCC
3 ► Specific_on_HSVtk2: GCACGTCTTTATCCTGGATTACGACCAATCG
4 ► dTri1-Long_AS: CCGCCACATTCGCTAGTTTGTACTTAAGCGAG

↓

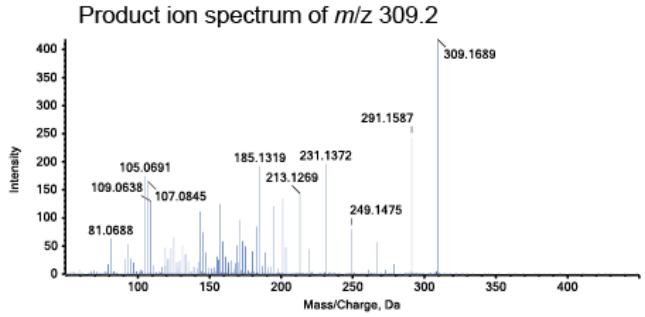
YN_171, YN_172

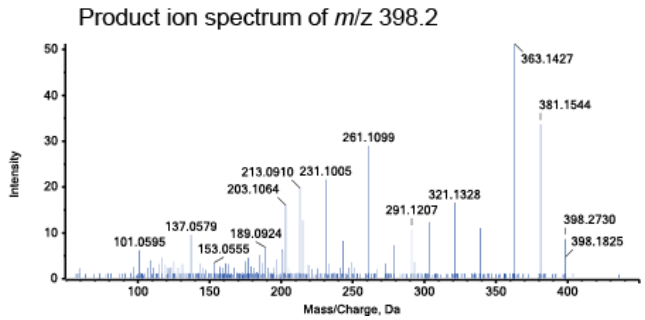
Supplementary Figure S1. Transgenic *F. graminearum* strains used in this study. Sequences and maps of transformation vectors are described in **Supplementary Figure S7**. (A) Pedigree of transformants with multiple genetic manipulations for production of trichothecene metabolites. Strain YN_145 (Δ*Tri6* / P_{TEF}*::Tri6*, ΔP_{Tri10} / P_{GPD}, Δ*Tri8* / P_{GPD}*::hph::tk*) is a *Tri8* disruptant derived from YN_120 (Δ*Tri6* / P_{TEF}*::Tri6*, ΔP_{Tri10} / P_{GPD}) [6]. Marker-free transformants YN_149 [6] and YN_155 (Δ*Tri6* / P_{TEF}*::Tri6*, ΔP_{Tri10} / P_{GPD}, Δ*Tri8* / *Tri8*$_{3\text{-}ADON\ chemotype}$) were obtained fromYN_145 by conditional negative selection against the *hph::tk* cassette with 2′-deoxy-5-fluorouridine (5-FdU). The gene disruptants, YN_171 (Δ*Tri6*/ P_{TEF}*::Tri6*, ΔP_{Tri10}/ P_{GPD}, Δ*Tri8*/ *Tri8_nsm*, Δ*Tri1*/ P_{TUB}*::hph::tk*) and YN_173 (Δ*Tri6*/ P_{TEF}*::Tri6*, ΔP_{Tri10}/ P_{GPD}, Δ*Tri8*/ *Tri8*$_{3\text{-}ADON\ chemotype}$, Δ*Tri1*/ P_{TUB}*::hph::tk*), were obtained from marker-free transformants by a one-step transformation process, where hygromycin B resistant transformants with double crossover homologous recombination were selected against a marker cassette containing *hph::tk*, a hygromycin B phosphotransferase gene (*hph*) translationally fused to a herpes simplex virus thymidine kinase gene (*tk*). A marker-free transformant EK_001, EK_002, and EK_003 (Δ*Tri6* / P_{TEF}*::Tri6*, ΔP_{Tri10} / P_{GPD}, Δ*Tri3* / *Tri3*$_{V467G}$) was obtained from YN_153 [6], *Tri3* disruptant derived from YN_149. (B) Generation of EK_001, EK_002, and EK_003. EK_001, EK_002, EK_003 were created from YN_153 by selection with 5-FdU following transformation with pCjcTri3_V467G (**Supplementary Figure S7**) (C) Generation of strain YN_155. YN155 was created from YN_145 by selection with 5-FdU following transformation with pCjH3Tri8 (**Supplementary Figure S7**). (D) Generation of strain YN_173. YN173 was created from YN_155 by selection with hygromycin B following transformation with pdjTri1-ptub_hphtk (**Supplementary Figure S7**). (E) Generation of strain YN_171. YN_171 was created from YN_149 by selection with hygromycin B following transformation with pdjTri1-ptub_hphtk.

Tri3 locus: *hph::tk*

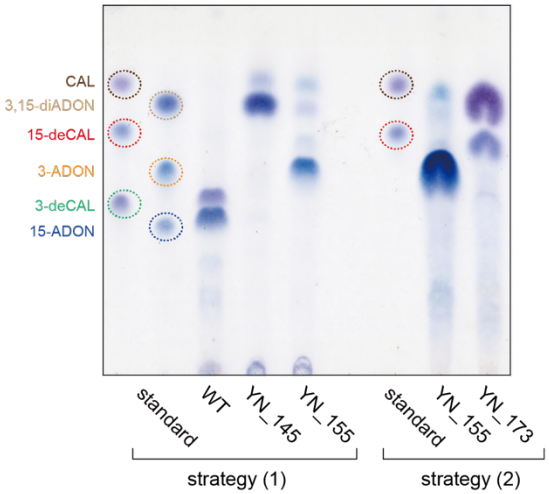


Tri3 locus: *Tri3_V467G*





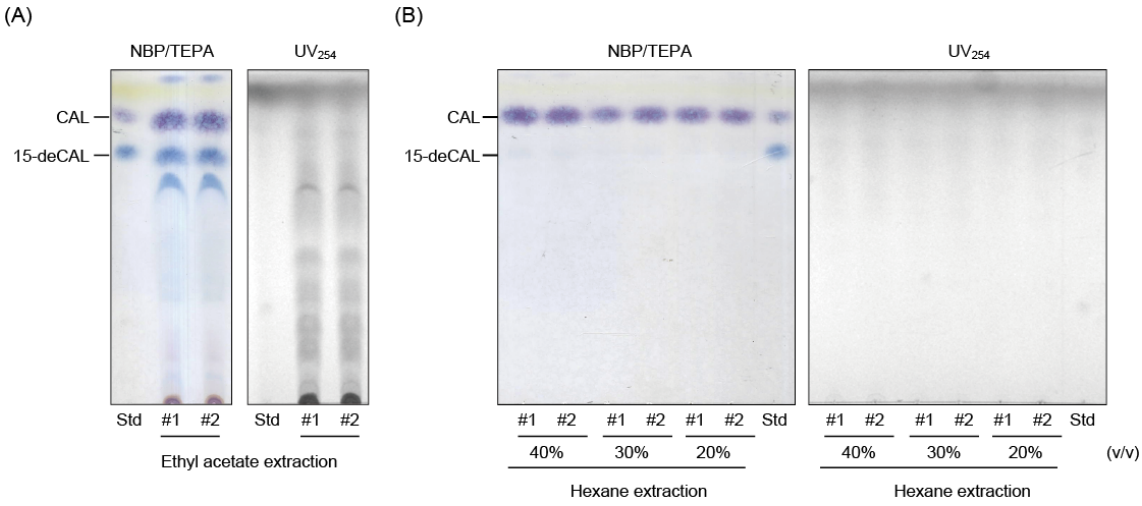
Supplementary Figure S2. LC-MS/MS analyses of the metabolites of the *Tri3* mutants (derived from a 3,15-diADON-overproducer described in **Figure 2**; native *Tri3* locus replaced with either the *hph::tk* maker or active-site mutant *Tri3_V467G*) cultured on liquid YS_60 medium. The MS/MS spectra of the LC-MS peaks of *m*/*z* 309.2 (*hph::tk* and *Tri3_V467G*) and *m*/*z* 398.2 (*Tri3_V467G*) detected in the positive ion mode are shown. The precursor ions of *m*/*z* 309.2 and *m*/*z* 398.2 correspond to 15-deCAL $[C_{17}H_{24}O_5 + H]^+$ (*m*/*z* 309.1696) and 3,15-diADON $[C_{19}H_{24}O_8 + NH_4]^+$ (*m*/*z* 398.1809), respectively. Although either mutant produced 15-deCAL, the production level was extremely low compared to that of 3,15-diADON in the parent YN_149 strain.



Transgenic strains derived from a trichothecene overproducer strain YN_120 (Δ*Tri6*/P_{TEF}::*Tri6*, ΔP_{Tri10}/P_{GPD})

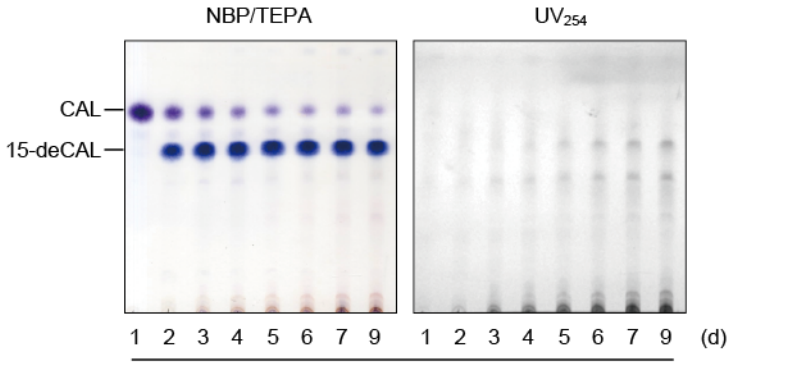
strain	*Tri8* locus	*Tri1* locus
YN_145	*hph::tk*	*Tri1* (native)
YN_155	*Tri8*$_{3\text{-ADON chemotype}}$	*Tri1* (native)
YN_173	*Tri8*$_{3\text{-ADON chemotype}}$	*hph::tk*

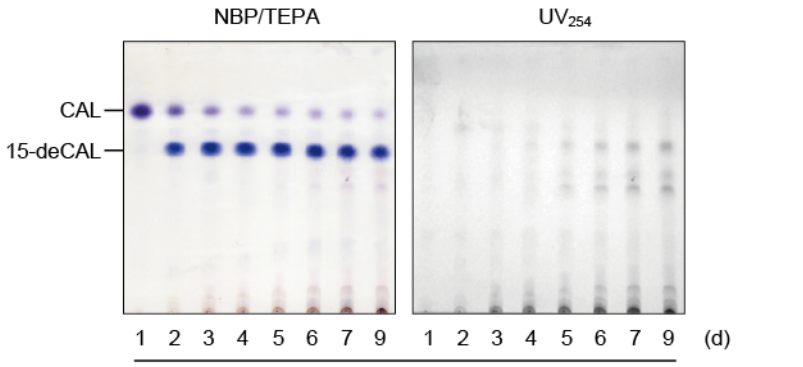
Supplementary Figure S3. TLC of culture metabolites of the transgenic *F. graminearum* strains. The three transgenic strains used in this experiment are derived from a 15-ADON overproducer strain YN_120 [6]. A synthetic methionine medium [7] and YG medium were used for culturing of the strain for strategy (1) and strategy (2), respectively. Ethyl acetate extract from 0.5 mL of the culture was loaded on each lane. After developing the TLC plate（Kieselgel F_{254}, Merck）with solvent mixture of ethyl acetate/toluene (3:1), trichothecenes were visualized with 4-(*p*-nitrobenzyl)pyridine (NBP)/tetraethylene pentamine (TEPA) [8].



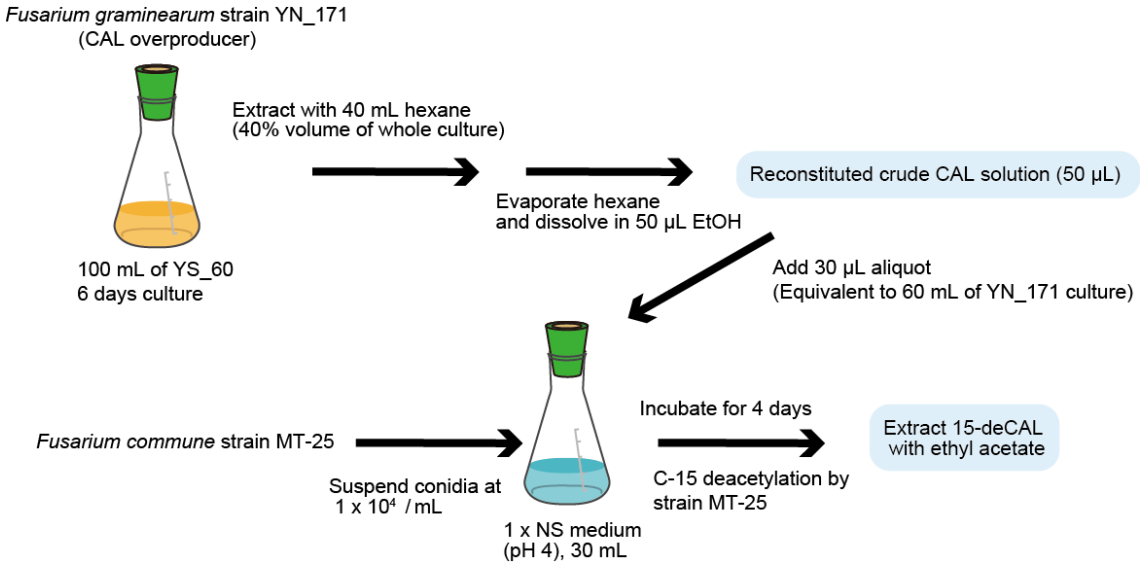
Supplementary Figure S4. Evaluation of the efficiency of the CAL extraction method. Each lane of TLC contains the metabolites extracted from 250 µL of the culture. To check the presence of fungal metabolites other than trichothecenes, the TLC plate was placed under UV light at 254 nm. The experiments were carried out in duplicate (#1 and #2). (A) A failure to directly use spent medium of the CAL-overproducer strain YN_171 (**Supplementary Figure S1**) for bioconversion of CAL to 15-deCAL. The conidia of YN_171 were inoculated onto YG medium at a final density of 1.0×10^4/mL and incubated for 16 h. One milliliter of the YG pre-culture thus prepared was transferred to 100 mL of YS_60 medium. After 6 days of incubation with gyratory shaking at 135 rpm and 25 ^{o}C, the CAL-containing spent medium was collected by filtration through a sterilized tea strainer (mesh size; 20 µm). The CAL-containing spent medium was aseptically supplemented with the nutrients of the synthetic 1 × NS medium (pH 4) and used for the feeding of strain MT-25. After 16 days of incubation, the whole culture was extracted with equal volume of ethyl acetate and analyzed by TLC. The TLC demonstrated the presence of CAL, its transformed product, and other fungal metabolites in the culture of strain MT-25; however, a significant amount of CAL still remained in the medium after 16 days of incubation. Besides, the presence of numerous contaminating metabolites, as demonstrated by the UV absorption, hampered efficient purification of the trichothecene metabolites. (B) Optimization of the CAL extraction method. By using the above spent medium containing CAL, its transformed product 15-deCAL, and other fungal metabolites, conditions for selective and efficient CAL extraction were investigated. When the culture was extracted with varying

amounts of hexane, the contaminating metabolites remained in the liquid phase; only CAL was detected from the solvent phase by the TLC analysis. The amounts of hexane used for the extraction are shown as percentage relative to the volume of the whole culture. By comparing the intensity of the CAL spots on a TLC plate, 40% the total culture volume of hexane proved to be sufficient in terms of quantity and quality for use in the subsequent feeding to *F. commune* MT-25 without further purification.

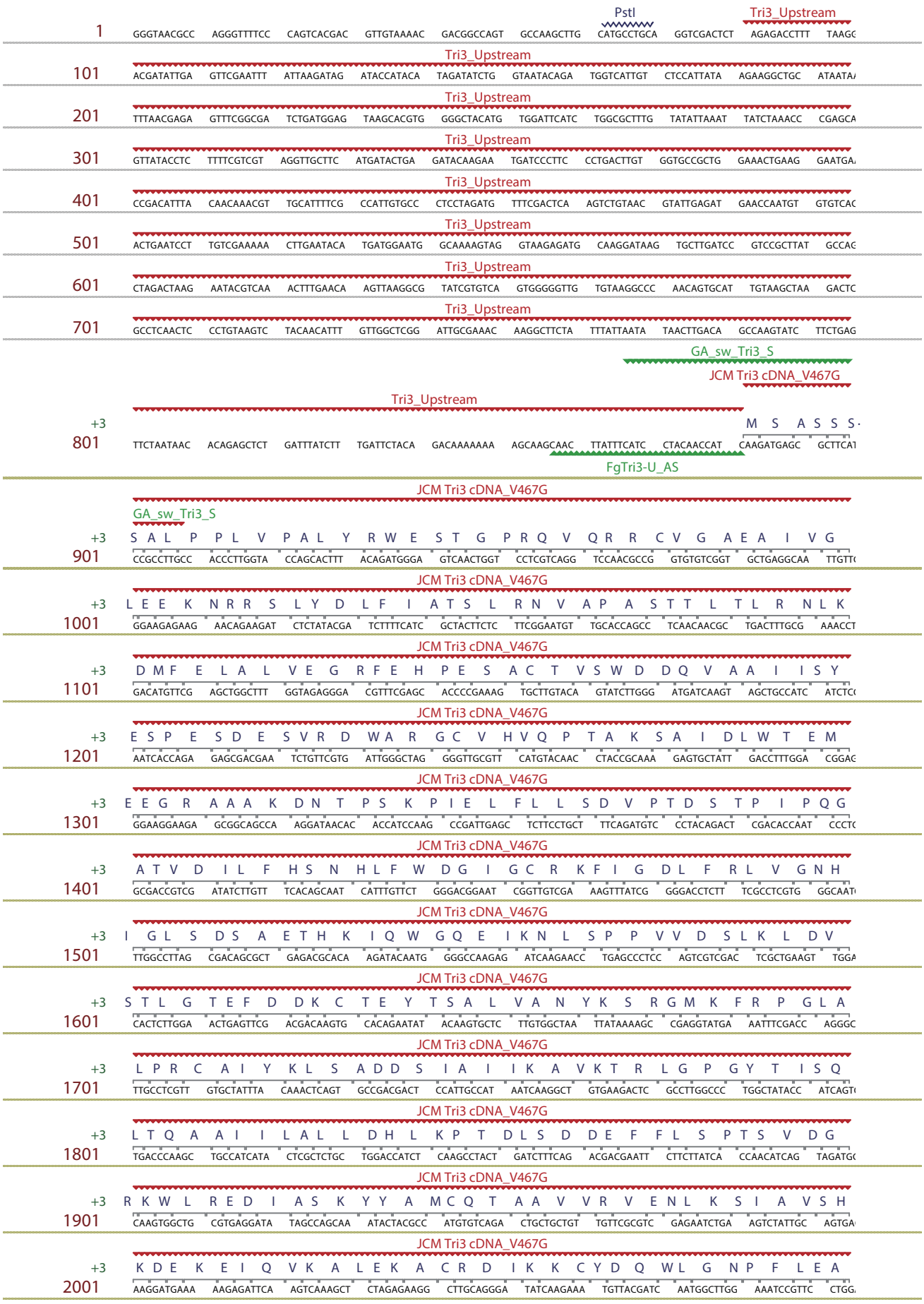




Supplementary Figure S5. Optimization of the amount of CAL and the period of incubation for bioconversion of CAL to 15-deCAL by MT-25. The CAL-overproducer YN_171 was grown on 100 mL of YS_60 medium and incubated for 6 days with gyratory shaking at 135 rpm and 25 ºC. The whole culture was extracted with 40 mL of hexane, the solvent was concentrated in a hume hood, and the dried materials were dissolved in 50 µL of ethanol. Three-fifths (60 mL; upper panel) and two-fifths (40 mL; lower panel) the volumes of the CAL solutions were then added to 30 mL of fresh conidia of strain MT-25 (1.0×10^4/mL) dispersed in 1 × NS medium, pH 4. In adding the crude CAL extract to the MT-25 culture, the volume of ethanol was adjusted to 30 µL. Each culture was sampled on the day indicated, extracted with equal volume of ethyl acetate, and analyzed by TLC. Each lane contains metabolites extracted from 500 µL of the MT-25 culture.



Supplementary Figure S6. An overview of the 15-deCAL preparation method established in this study. The extracted 15-deCAL can simply be purified in one step by preparative TLC or reversed phase column chromatography.


PstI
Tri3_Upstream
1
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101
ACGATATTGA GTTCGAATTT ATTAAGATAG ATACCATACA TAGATATCTG GTAATACAGA TGGTCATTGT CTCCATTATA AGAAGGCTGC ATAATA
201
TTTAACGAGA GTTTCGGCGA TCTGATGGAG TAAGCACGTG GGGCTACATG TGGATTCATC TGGCGCTTTG TATATTAAAT TATCTAAACC CGAGCA
301
GTTATACCTC TTTTCGTCGT AGGTTGCTTC ATGATACTGA GATACAAGAA TGATCCCTTC CCTGACTTGT GGTGCCGCTG GAAACTGAAG GAATGA
401
CCGACATTTA CAACAAACGT TGCATTTTCG CCATTGTGCC CTCCTAGATG TTTCGACTCA AGTCTGTAAC GTATTGAGAT GAACCAATGT GTGTCAC
501
ACTGAATCCT TGTCGAAAAA CTTGAATACA TGATGGAATG GCAAAAGTAG GTAAGAGATG CAAGGATAAG TGCTTGATCC GTCCGCTTAT GCCAC
601
CTAGACTAAG AATACGTCAA ACTTTGAACA AGTTAAGGCG TATCGTGTCA GTGGGGGTTG TGTAAGGCCC AACAGTGCAT TGTAAGCTAA GACTC
701
GCCTCAACTC CCTGTAAGTC TACAACATTT GTTGGCTCGG ATTGCGAAAC AAGGCTTCTA TTTATTAATA TAACTTGACA GCCAAGTATC TTCTGAG
GA_sw_Tri3_S
JCM Tri3 cDNA_V467G
+3
M S A S S S
801
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FgTri3-U_AS
GA_sw_Tri3_S
+3
S A L P P L V P A L Y R W E S T G P R Q V Q R R C V G A E A I V G
901
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L E E K N R R S L Y D L F I A T S L R N V A P A S T T L T L R N L K
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+3
D M F E L A L V E G R F E H P E S A C T V S W D D Q V A A I I S Y
1101
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+3
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1201
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E E G R A A A K D N T P S K P I E L F L L S D V P T D S T P I P Q G
1301
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1401
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+3
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1701
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L T Q A A I I L A L L D H L K P T D L S D D E F F L S P T S V D G
1801
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R K W L R E D I A S K Y Y A M C Q T A A V V R V E N L K S I A V S H
1901
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K D E K E I Q V K A L E K A C R D I K K C Y D Q W L G N P F L E A
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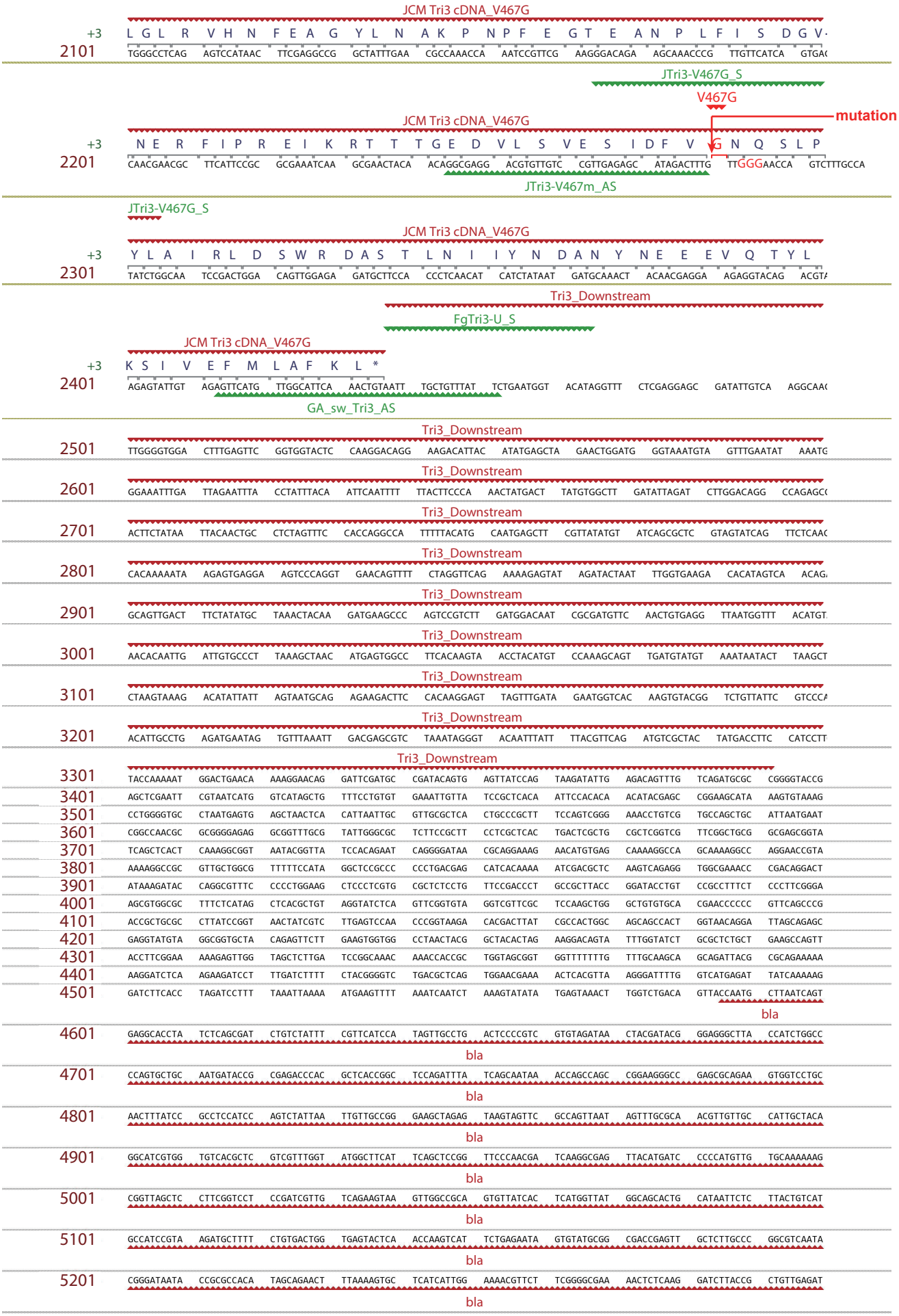
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V467G
mutation
JCM Tri3 cDNA_V467G
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Tri3_Downstream
FgTri3-U_S
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Tri3_Downstream
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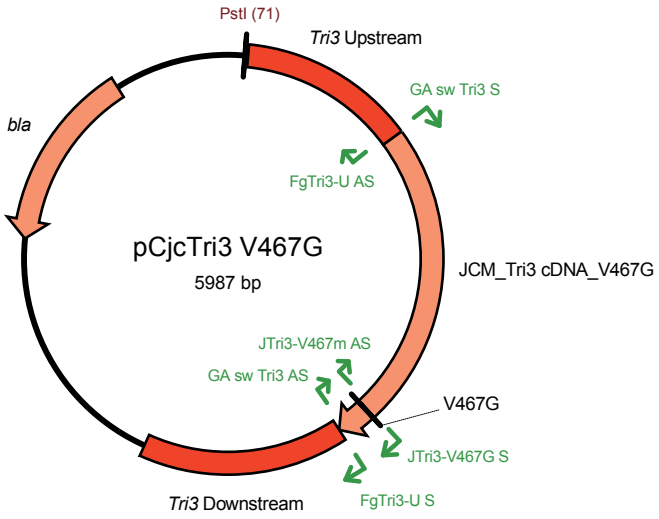

dTri8-U_S

PstI Tri8 upstream region

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dTri8-U_S

Tri8 upstream region

101 TACCTATCGC ATTGTCGCTG GTGCTGGAAA AGATTCCGTC CGACTTTCGG TCCGATTAGA ACTATTGCGA AGGCCGTGAA AATGAACCGC TTGAT

Tri8 upstream region

201 AAGACTAGAG GTCCAGACAA GTAAGTGAAA GAGCAATACA AGACAGATGA AGCCTTACTT TTCTCAGGCC TCGCTGGTGG ACCTAGTAAA AAG

Tri8 upstream region

301 TCGAAACGAA CCAGCTGTGA TGTGTAGAAT GGAGGGCCTG ATGAAAGGGC CAGGGGCCTG ACATGGACAT GGGGACAATA AATCGGCTGT TC

Tri8 upstream region

401 CGTAACTACA CTTAAAGAAC CCAGGACTCA ATCTAGAATT CTTTGATAAA GCGAGCTTCA CTTTACTATG TATAAAGTAA AGTTTGTAGT GTATAC


Tri8 upstream region
501
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Tri8 upstream region
601
CATTTCACTC TAGCGCATTG AGGATTGATG TACTCTGTAC GTGCTGAGGT CTTGGACCCC TTCACCCCTG GCACAACAAC ATCAAGGTTC AAACA
F15-8sw-8_S
Tri8_3-ADON chemotype
Tri8 upstream region
+3
M V L D
701
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F15-8sw-U_AS
Tri8_3-ADON chemotype
F15-8sw-8_S
+3
R L L F L L S L W L G F V G A T Q A A L S E P I P P S K D P W Y T A·
801
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Tri8_3-ADON chemotype
+3
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+3
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+3
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+3
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+3
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Tri8_3-ADON chemotype
+3
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1901
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F15-8sw-D_S
Tri8_3-ADON chemotype
Tri8 downstream region
+3
Q R D T K G C V I K D V T R D V T G D V T R D V T R D V T R E L *
2001
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F15-8sw-8_AS
F15-8sw-D_S
Tri8 downstream region
2101
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F15-8sw-8_AS

Tri8 downstream region

2201 TAGTGGTCTT ATACTGTGTC TTATAAAATA TTTATTGATC CTTTTCTATG AGTAACTTTA TCTGTCTCCG GCGATGTAGC TGCATCTGTA AGATTCAA

Tri8 downstream region

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Tri8 downstream region

2401 GATCAGCTCC CATTTCCCTT CAAGTTCCCT CCATTGTGGT CAGAAGACAA GGGAAGCATG AGGGTGGACT CATTTGAAGC ACGATAATTA CACT

Tri8 downstream region

2501 AAACACAGGT TTAGCTTGAA CTGAAAAACC ACTCATCACA TTCACAGTAG CACCACTGGA TAGATGTTGG GTTCAAGTAA GCCTCATGTT GCTCT

Tri8 downstream region

2601 CGCTACTTAC CCCTTGCCGC AACAATATGC ATCTTTTACA CTTCATGCAC AAACAAGGGC ATGGTATCGT TGAGGCAATA TCAGACATAT AAATT

Tri8 downstream region

2701 TAATATTGTC GGATACTGCG TGGAGGAAGG CGACTTCGTC TTTATGGACG TCAAGCCCTC CTGTAAGCCT CTTTTCAAGG TCCCTCAGGC TCGCA

Tri8 downstream region

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Tri8 downstream region

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Tri8 downstream region

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Tri8 downstream region

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dTri8-D_AS

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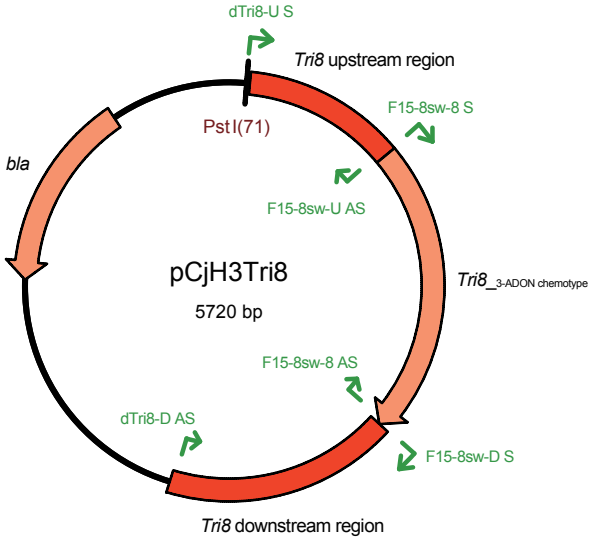

dTri1-U_S

HindIII

Tri1 upstream region

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dTri1-U_S

Tri1 upstream region

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Tri1 upstream region

201 ACCAACATTG AAACACCAAA GTCAATTACA GGGCCTCTGC AGATACCCTA TAGAGAGGTT GGAGGCAGTA AATAAATAGA TGGAATGTTG AAG

Tri1 upstream region

301 AGATTACGGA TAATACATGT ATGGTCTCGT AGTATAATTG ATGAGAATTT AATAAATATA TTTCCTTGTT AAGAATTTAA TTATTTTGCT GTCCAAG

Tri1 upstream region

401 ACTTCATGAC AGCTTTTGCC ATTATGACTG CTTACAGTAA TCCAGAGCCT TTGCCTTACT CGGCAGAAAC TTGTGCATTG TTTCACAAAG CTAGAC

Tri1 upstream region

501 AGTAGTTGCC GATAGTTAGA CCTTTACAGT CCTCAAGACA AACAAACAGA CACCATTTGT AGTTTCAAGG AGTGGTCGAT GGTGCCCTTT TGTCT

Tri1 upstream region

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Tri1 upstream region

701 ACCTAGCAGG CTGGAAGTTA CGAATCTAGA CTAGCATGTG AAGATAACGA TACCAAAGTC AATTCTGTTT GGTTACAAGT TTTGGATCTT GTGCA

Tri1 upstream region

801 GCCTGATAGT TAGTACCCTA CCAATCAATA TGAAGATCAA TGCAACAAAG TCAGTATTAT TCGGTAGGTC GTTTTGTTCG TTAGTGGGGG TTGGC

Tri1 upstream region

901 TCTACCAATT GAGGCTGATA CTGTTCAGTG TGGTTCGTTT TGTTAGCTGG CCTTATACTT AGGACCCTAC CGATGTGGAT CTGCAGAAAA TAGAGT

Tri1 upstream region

1001 GACAACACTC AATGAAACGG TCTAACCGTG AGGCAAGCCA TGTCACGGGC CCTGTAGGTA TATGTTATAA ATGTAGGATA CCCTCTACAA CTCT

pTub_S1

Tri1 upstream region

TUB promoter

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dTri1-U_AS

TUB promoter

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TUB promoter

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TUB promoter

1401 TATTGGGCCT GCCCGACAAG CTGCAACAGA TAACCCCAAC TGTCAGCACG GCATTTTACG AGTAGGTCCT ACCTGCACAT ATCCAAATGA TGAA

TUB promoter

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TUB promoter

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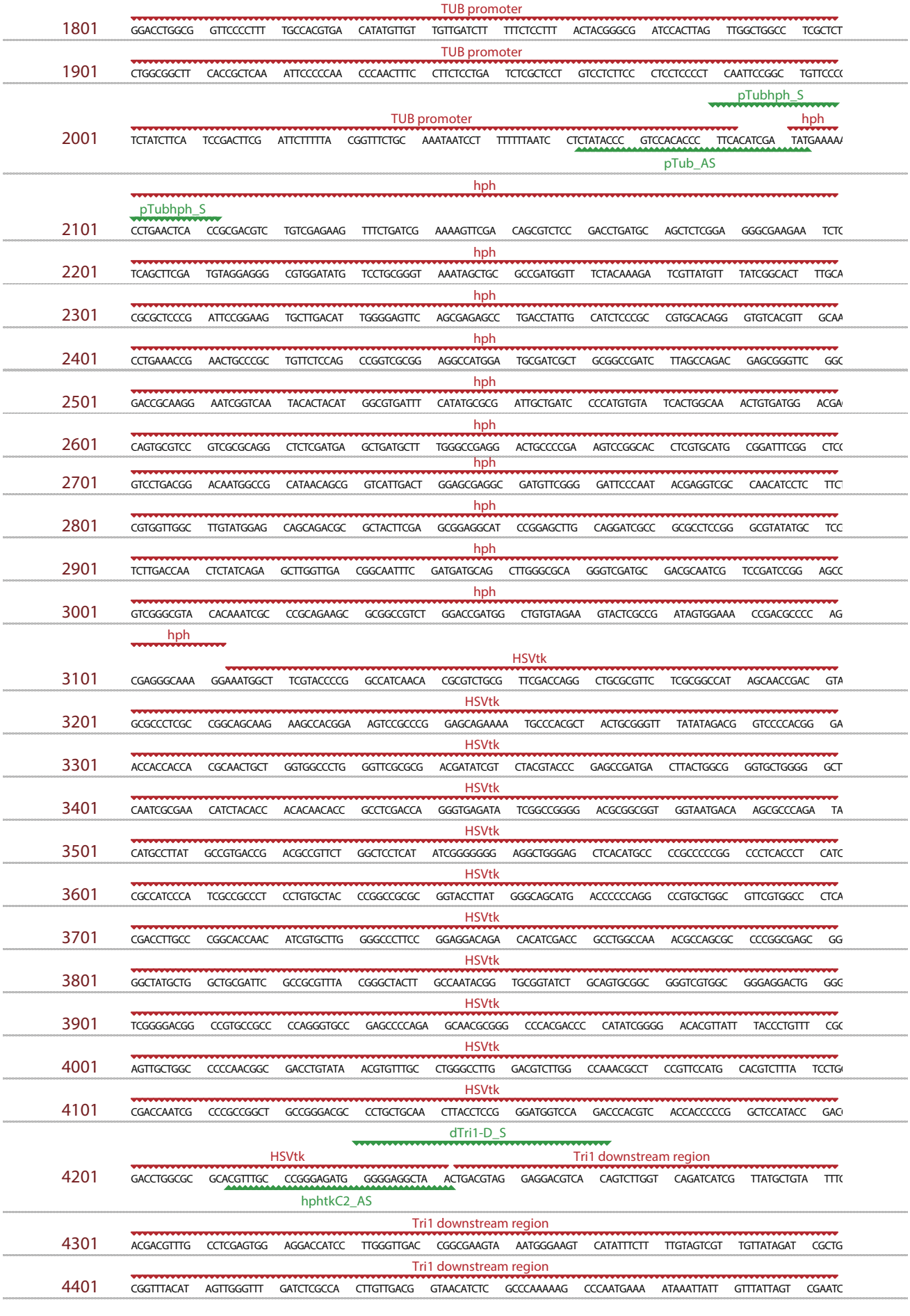
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pTubhph_S
TUB promoter
hph
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HSVtk
3301 ACCACCACCA CGCAACTGCT GGTGGCCCTG GGTTCGCGCG ACGATATCGT CTACGTACCC GAGCCGATGA CTTACTGGCG GGTGCTGGGG GCT
HSVtk
3401 CAATCGCGAA CATCTACACC ACACAACACC GCCTCGACCA GGGTGAGATA TCGGCCGGGG ACGCGGCGGT GGTAATGACA AGCGCCCAGA TA
HSVtk
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HSVtk
3601 CGCCATCCCA TCGCCGCCCT CCTGTGCTAC CCGGCCGCGC GGTACCTTAT GGGCAGCATG ACCCCCCAGG CCGTGCTGGC GTTCGTGGCC CTCA
HSVtk
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HSVtk
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HSVtk
3901 TCGGGGACGG CCGTGCCGCC CCAGGGTGCC GAGCCCCAGA GCAACGCGGG CCCACGACCC CATATCGGGG ACACGTTATT TACCCTGTTT CGC
HSVtk
4001 AGTTGCTGGC CCCCAACGGC GACCTGTATA ACGTGTTTGC CTGGGCCTTG GACGTCTTGG CCAAACGCCT CCGTTCCATG CACGTCTTTA TCCTG
HSVtk
4101 CGACCAATCG CCCGCCGGCT GCCGGGACGC CCTGCTGCAA CTTACCTCCG GGATGGTCCA GACCCACGTC ACCACCCCCG GCTCCATACC GAC
dTri1-D_S
HSVtk
Tri1 downstream region
4201 GACCTGGCGC GCACGTTTGC CCGGGAGATG GGGGAGGCTA ACTGACGTAG GAGGACGTCA CAGTCTTGGT CAGATCATCG TTATGCTGTA TTT
hphtkC2_AS
Tri1 downstream region
4301 ACGACGTTTG CCTCGAGTGG AGGACCATCC TTGGGTTGAC CGGCGAAGTA AATGGGAAGT CATATTTCTT TTGTAGTCGT TGTTATAGAT CGCTG
Tri1 downstream region
4401 CGGTTTACAT AGTTGGGTTT GATCTCGCCA CTTGTTGACG GTAACATCTC GCCCAAAAAG CCCAATGAAA ATAAATTATT GTTTATTAGT CGAATC

Tri1 downstream region

4501 TCTGTATATG AATCTTTTTT CAACATGGGA ATATCGAACT CAGAATTCAA ATGCAAGCAT TCGCACCTTG TTTGCATTGG CAGCACGTTG AATCAT

Tri1 downstream region

4601 AGTTCGAATT ACCCAGTTGA TGCAACAGTG AAAGAAAACT TGGTTGTCGA ATAAAAATAT ACTTGGGGGT ACCGAGCTCG AATTCGTAAT CATG

dTri1-D_AS

4701 GCTGTTTCCT GTGTGAAATT GTTATCCGCT CACAATTCCA CACAACATAC GAGCCGGAAG CATAAAGTGT AAAGCCTGGG GTGCCTAATG AGTGAGCTAA

4801 CTCACATTAA TTGCGTTGCG CTCACTGCCC GCTTTCCAGT CGGGAAACCT GTCGTGCCAG CTGCATTAAT GAATCGGCCA ACGCGCGGGG AGAGGCGGTT

4901 TGCGTATTGG GCGCTCTTCC GCTTCCTCGC TCACTGACTC GCTGCGCTCG GTCGTTCGGC TGCGGCGAGC GGTATCAGCT CACTCAAAGG CGGTAATACG

5001 GTTATCCACA GAATCAGGGG ATAACGCAGG AAAGAACATG TGAGCAAAAG GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG CCGCGTTGCT GGCGTTTTTC

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5401 CGTCTTGAGT CCAACCCGGT AAGACACGAC TTATCGCCAC TGGCAGCAGC CACTGGTAAC AGGATTAGCA GAGCGAGGTA TGTAGGCGGT GCTACAGAGT

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bla

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6001 CCACGCTCAC CGGCTCCAGA TTTATCAGCA ATAAACCAGC CAGCCGGAAG GGCCGAGCGC AGAAGTGGTC CTGCAACTTT ATCCGCCTCC ATCCAGTCTA

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bla

6201 TGGTATGGCT TCATTCAGCT CCGGTTCCCA ACGATCAAGG CGAGTTACAT GATCCCCCAT GTTGTGCAAA AAAGCGGTTA GCTCCTTCGG TCCTCCGATC

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bla

6401 CTGGTGAGTA CTCAACCAAG TCATTCTGAG AATAGTGTAT GCGGCGACCG AGTTGCTCTT GCCCGGCGTC AATACGGGAT AATACCGCGC CACATAGCAG

bla

6501 AACTTTAAAA GTGCTCATCA TTGGAAAACG TTCTTCGGGG CGAAAACTCT CAAGGATCTT ACCGCTGTTG AGATCCAGTT CGATGTAACC CACTCGTGCA

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7001 GGAGCAGACA AGCCCGTCAG GGCGCGTCAG CGGGTGTTGG CGGGTGTCGG GGCTGGCTTA ACTATGCGGC ATCAGAGCAG ATTGTACTGA GAGTGCACCA

7101 TATGCGGTGT GAAATACCGC ACAGATGCGT AAGGAGAAAA TACCGCATCA GGCGCCATTC GCCATTCAGG CTGCGCAACT GTTGGGAAGG GCGATCGGTG

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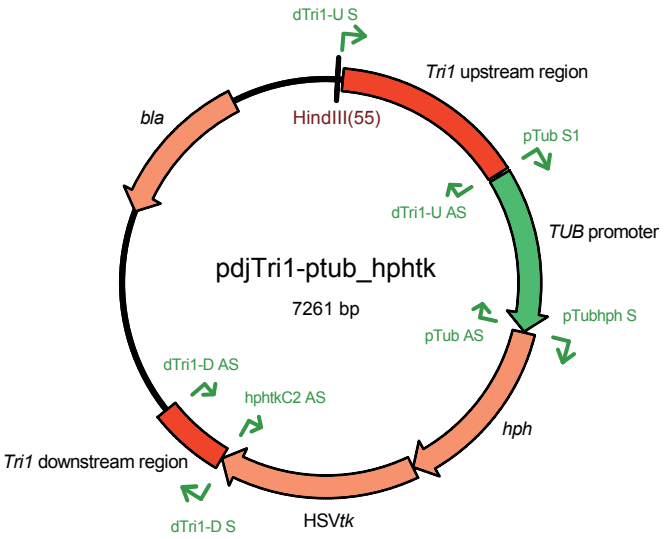

Supplementary Figure S7. Sequences and maps of replacement and disruption vectors. Gene replacement vectors pCjcTri3_V467G and pCjH3Tri8 were used for transformation of transgenic strains YN_153 (*Tri3* locus: *hph::tk*) and YN_145 (*Tri8* locus: *hph::tk*), respectively, to generate marker-free strains EK_001 and YN_155 (**Supplementary Figure S1**). Gene disruption vector pdjTri1-ptub_hphtk was used for transformation of YN_155 to generate YN_173 (*Tri1* locus: *hph::tk*) (**Supplementary Figure S1**).

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