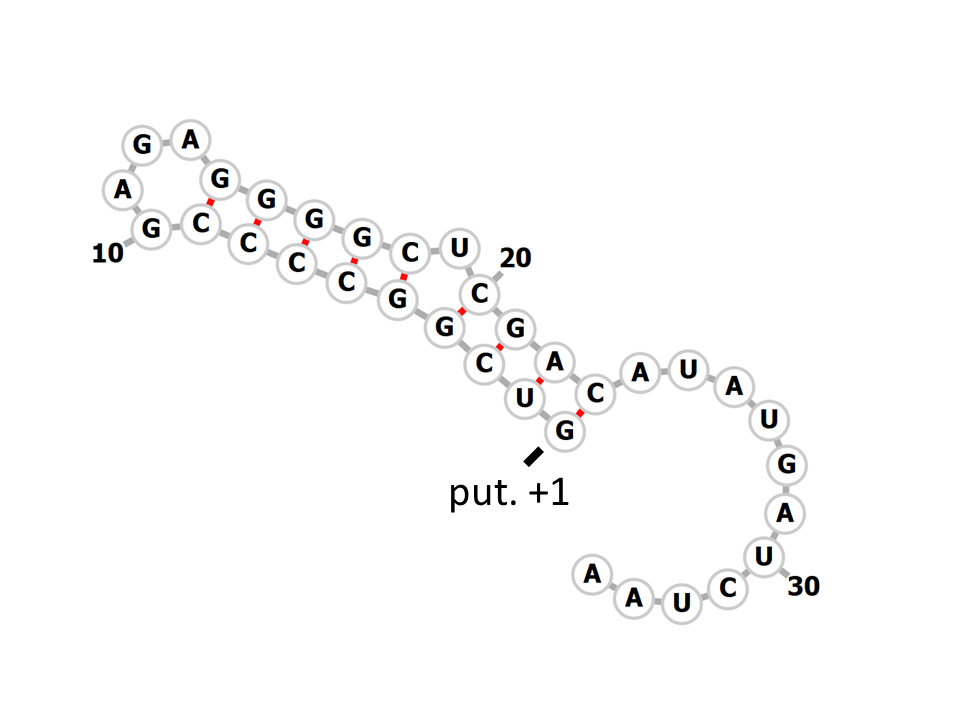
**SUPPLEMENTAL MATERIAL**

**Marker-free genome editing in *Ustilago trichophora* with the CRISPR-Cas technology**

Simon Huck, Josephine Bock, Joerg Girardello, Marc Gauert, Ümit Pul\*

A****

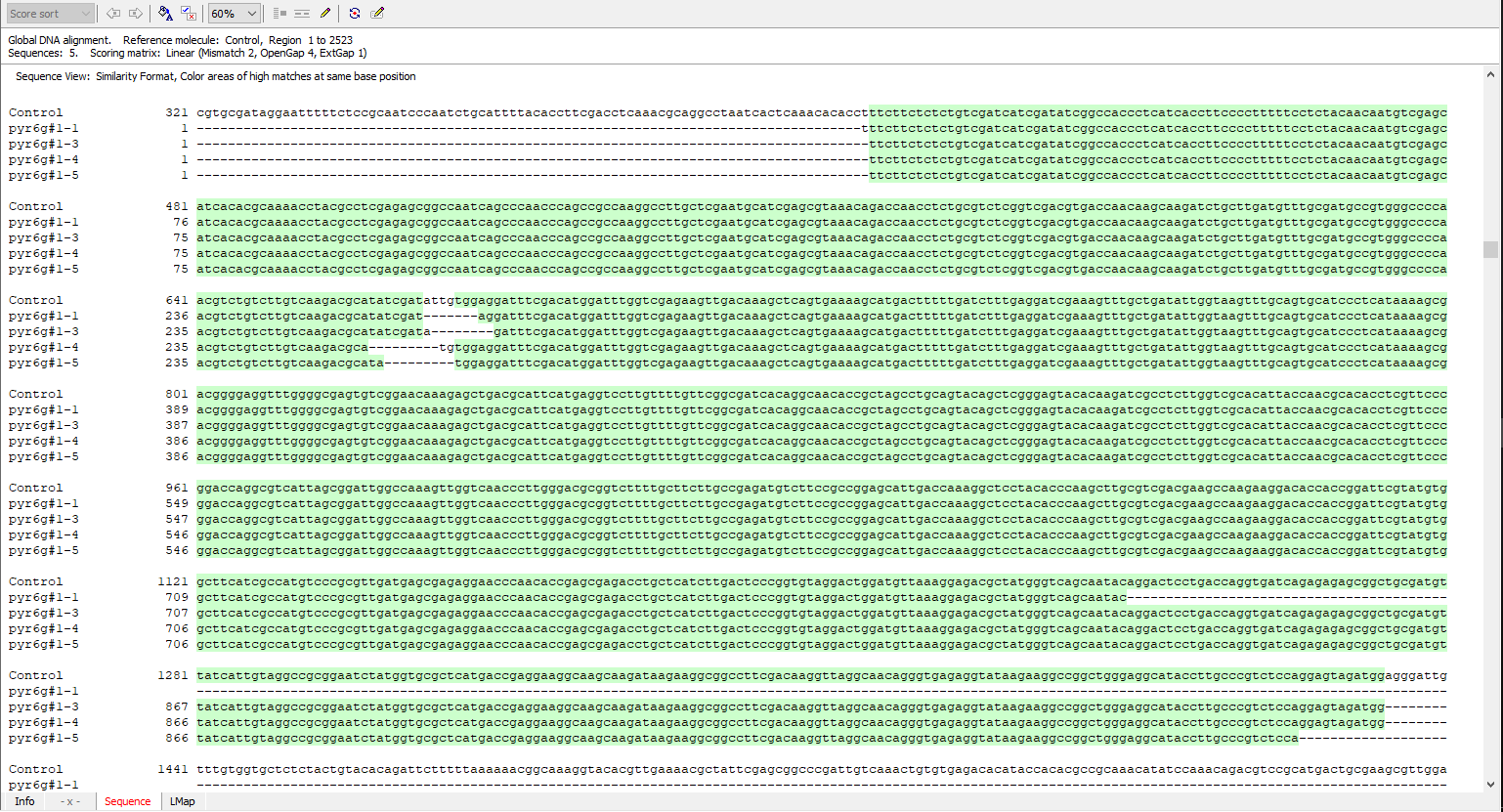
**B**

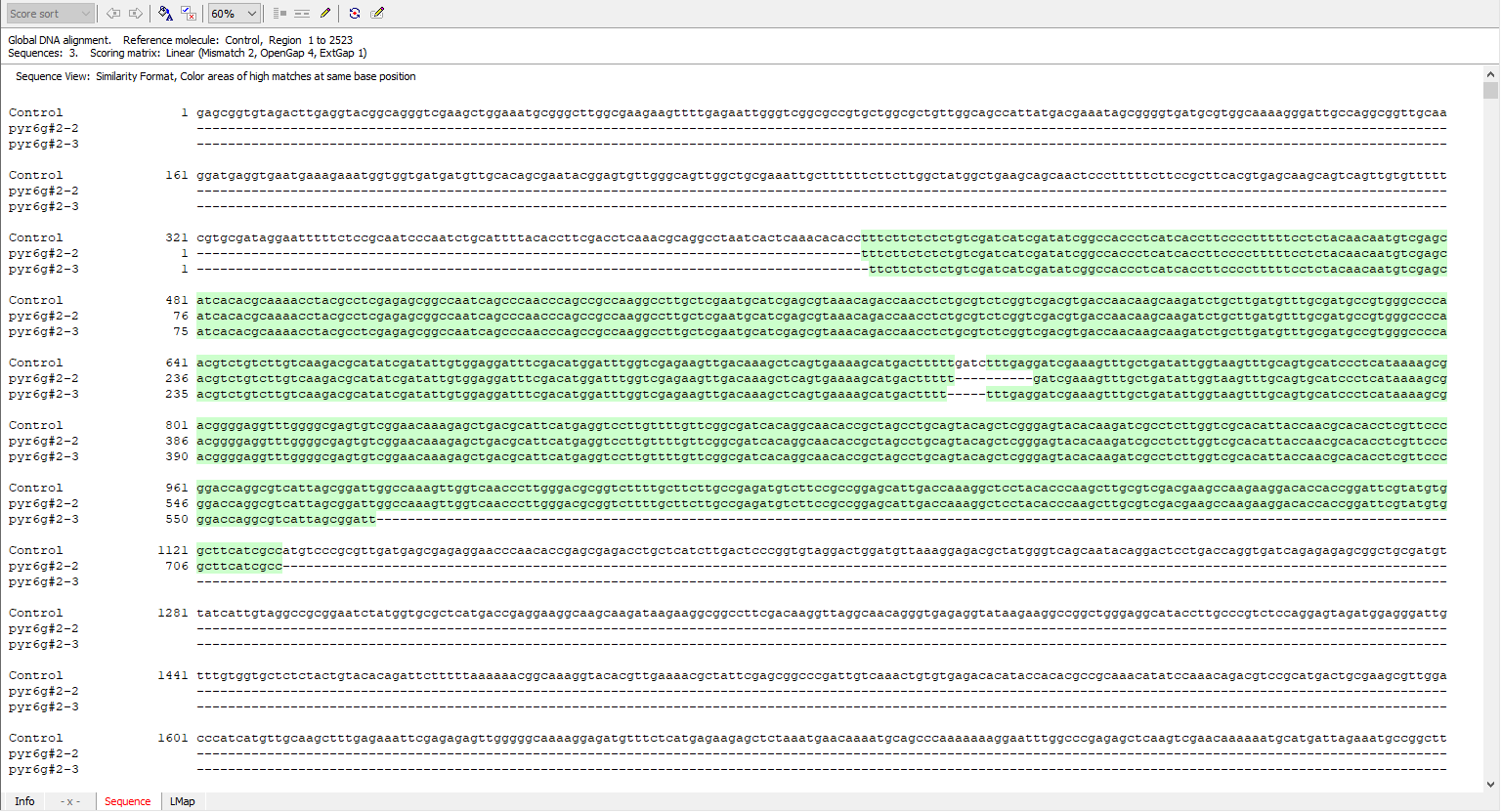
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**Supplemental Figure S1.** (A) Alignment of the *U. maydis* U6 region (Schuster et al., 2016) and the U6 region of *U. trichophora*. The U6 genes have a sequence similarity of 80 %, whereas the putative promoter regions exhibit 44 % similarity. (B) The predicted structure of the first 34 nucleotides of the putative *U. trichophora* U6 gene is shown. The putative transcriptional start site is indicated with +1. The prediction of the structure was performed using RNAfold tool (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi).

**A**

******

**B**

**C**

**Supplemental Figure S2.** (A) Analysis of *U. trichophora* clones transformed with either pMF-Cas9-PU6-Tri-pyr6g#1 or pMF-Cas9-PU6-Tri-pyr6g#2 on minimal media agar plates with or without 5-FOA/Cytidine. (B) and (C) Alignment of the sequences of selected clones targeted with either pMF-Cas9-PU6-Tri-pyr6g#1 (B) or pMF-Cas9-PU6-Tri-pyr6g#2 (C), respectively. The guide-sequence upstream of PAM is indicated with red box and the deleted nucleotides by hyphens.

**Supplemental Table S1**. List of potential off-target sites for pMF-Cas9-PU6-Tri-malAg#1 predicted with CCTop. The on-target site is colored in blue and the analyzed off-targets are indicated in green. The mismatches between the guide- and the off-targets are shown in red. None of the analyzed potential off-target sites were affected in two clones mutated with the pMF-Cas9-PU6-Tri-malAg#1 plasmid.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Target** | **Coordinates** | | **Strand** | **MM** | **Target Sequence** | **PAM** |
| on-target | 5841436 | 5841458 | + | 0 | CCATTTCCAAATCAAGACAC | TGG |
| off-target  (OT 1) | 16431224 | 16431246 | - | 6 | GGACCCCCATATCAAGACAC | AGG |
| off-target  (OT 2) | 12703630 | 12703652 | + | 7 | ACCCTGTGAAAACAAGACAC | GGG |
| off-target  (OT 3) | 5207479 | 5207501 | - | 6 | TCCTTTTTAAGACAAGACAC | CGG |
| off-target  (OT 4) | 13607213 | 13607235 | + | 7 | CCCCCTGTCAAACAAGACAC | AGG |
| off-target  (OT 5) | 9371022 | 9371044 | - | 7 | GCCCGGCCAAGTCCAGACAC | AGG |
| off-target  (OT 6) | 14485663 | 14485685 | + | 6 | CCCGTTTGAGATCATGACAC | TGG |
| off-target  (OT 7) | 15953542 | 15953564 | + | 7 | TGATCTTGAAATGTAGACAC | AGG |
| off-target  (OT 8) | 10492367 | 10492389 | - | 7 | GCGTCAGCAAATCCGGACAC | TGG |
| off-target  (OT 9) | 5299838 | 5299860 | - | 7 | GAAAAACAAAATCAAGAAAC | TGG |
| off-target  (OT 10) | 19449957 | 19449979 | + | 5 | GCATTTTGAGATCAAGAGAC | AGG |
| off-target  (OT 11) | 13319946 | 13319968 | + | 6 | CAAAGGCCAAAGCAAGAAAC | AGG |
| off-target  (OT 12) | 5376538 | 5376560 | + | 5 | CCACTCTCAAATCATGGCAC | AGG |
| off-target  (OT 13) | 14278150 | 14278172 | - | 7 | GAAAATGCAAAGCAAGAAAC | AGG |
| off-target  (OT 14) | 19856070 | 19856092 | - | 6 | CAAATCTCTAATCAAGACCC | TGG |
| off-target  (OT 15) | 2796319 | 2796341 | + | 6 | ACTTGTACAAAGCAAGACGC | AGG |
| off-target  (OT 16) | 992576 | 992598 | - | 7 | GGAAATTCAACTCAAGACTC | TGG |
| off-target  (OT 17) | 19069768 | 19069790 | + | 7 | CAGCAACCAAAACAAGACGC | CGG |
| off-target  (OT 18) | 20197363 | 20197385 | + | 7 | ACCAGTTCAAATCAAACCAC | AGG |
| off-target  (OT 19) | 9298167 | 9298189 | + | 6 | GCAAGCCCAGATCAAGACAT | TGG |
| off-target  (OT 20) | 1896360 | 1896382 | + | 6 | CAATCGCAAAATCCAGACGC | AGG |
| off-target  (OT 21) | 14823503 | 14823525 | + | 6 | TCATGCACAAGTCAAGACAT | AGG |
| off-target  (OT 22) | 20657448 | 20657470 | - | 6 | GCAACTGCAAATCAAGTCCC | AGG |
| off-target  (OT 23) | 3399233 | 3399255 | + | 7 | CAGCGTTCAAATCAAGTCTC | TGG |
| off-target  (OT 24) | 4352144 | 4352166 | + | 7 | GAAAATTCAAATCAAAACAA | AGG |
| off-target  (OT 25) | 1603295 | 1603317 | - | 7 | TTCTTACGAAATCAAGAGAT | TGG |

**Supplemental Table S2**. Deletion of DNA regions upstream and downstream of the malA#g1 targeted region. The wild-type malA sequence (malA) and the sequences of PCR fragments obtained from U. trichophora clones transformed with pMF-Cas9-PU6-Tri-malAg#1 are shown (S1 - S5). The malA#g1 target sequence is underlined and the deleted nucleotides are indicated by hyphens.

>malA\_wildtype

GGCAGTGACGGCAAGCAAAATTTCTTGAAGAGACGCACACACGCAAAGCGTCGTTCTCACAGCGACGTTCAC

CGGTCCGGTGATCCGTTCATTCGTCCATCACTTCACCCAACAGGATCGGTCAATTGATTGGCCTCGTGGTCG

TGTGGGCGTCTCTGCCTCGTCGACACTGCACTTTCAAGTTCCCGGACTTCTCCGGCGGAGACGTACGGAAGC

ACAAGTGCGGAGACGGAGCATCGACGGGGCCAGTGATTGGATCTTTCCTGTCGAACGAGCCCCGCTTGTCTG

CTTGTATGCAGCTGGACGCTTGCCGTGCATGAAGTGGGTCGCGCAGCGAGCGAGCAAGCGAAAAACTTCAAA

GCGGCCGAGCCTCCGGTTGCTCGGCAACGAAAGGGGTTTTTTTTTGTGGGGATGGATGAGCCAGTCGAATCC

GTGGTCAGCGAGTGACGGACAGCTGCCTCGCTGCGTGTTCCATTATTTTGCTCGATTCGCCGGTCATTTCTT

TGCGGCCTTCAACCCTCTCCGTCGAATGCTCGTGTACCTGGTGCCCTTCAGTTTCGTCTTTTCTCCATGCCA

GTCGGAAGGCGAATTGAGCAAGGCATCGGAACTTCCGTTGTATTGCCGATTCGGTTTTCTCGTTCAACTTTG

CACCTTTCCACATGACCAGGCTCAAGAGAAGTACTTGACCTAGCTCTTTTGCCGCTGCTCAAAGTCTTGCGG

TGGGGATGGCAATATTTTGCTGTAGGCAACGAGTCATCTCGCTCAACAGCGAGCATGCATGCTCGTCAGCTT

GAAAGACCACCCCATTGGTCGGTAATCCATTGTGCATCATGCCGTTCGACCGTGCCAAAGTGGGGAATGCAA

ATTTGCTGCCGCTGCTGCTTGTTGGACTTCGAAGAGACTCTCTTTTGGTTGGTTCTGCGACACCATTTTCCC

CAATTTTCATTTCTTCTCCAAAAAACAAAAAACAGAAAAAGAAAAAGAAAAAAAAGATCCAATTTAGCTCGC

GATTTTACCCGAATTGCATTGACTTGGCTGTCGAGTCAGGTTTCGCCTTTTGGTCACGACCTTGCCCGAAGC

TCTATCTCGTGACGCGTGTGGCTTGATGGCAGCTGCCTCCGAGTCACTTTCCGTCTTCTTCCCCATTTCACG

CTTCGCTCTTTGTGAAAGACCCTGACTTTTGCTCTTCACACGGGCCAATCTTCTCAAATTGATCCGGCGGCC

ATACCTGCTTCCTTGATGTTTCCGAAAGCGTTCTTCCTTCCCCACTCCATCTTCCGTCTTCTTGTATCAGGT

GCCGTTGAGATCTCTTGCGGACATTATCGAACGCAGTAGACGTCGCCGGTTTCTACAATTCGCAGACCGACC

GACGTACGCGCCGGCTTTCTCCACAAATTTCTTGCCGCTGCAGCACTTTTTCTCCGCCAAAGAATAACTGCT

TGCCGAGTCACTTCAGAGAAGAAGCCGTTCCTCACTTTCAGCTCTTCATTGTAGCGAAGCACAGCAAAGCAC

AGCATGCTTCGTTCATCACATCGGTCCATAGGCAGTTGCCTTCGTTGTGCATCAGAAGAATGGTATCAATCC

ACAGGGATGGTAAATGCCATTGCTGGGCCATCTGTTGCTAGCCAAGCATCTCCTGCATCCCCCTTCCACAGT

AGCACGAGACAGCGCTCATCACGCAAGACTTCCGCCGCATCGTTCTCCACATCGTCCACAAGATCAAACCAG

CTCGCTGACCATCGCATTATCTCCGAT**CCATTTCCAAATCAAGACAC**TGGCTTTTCATATGAGAAGCGTGAC

CAGCTCGGCCTCCGCGGCCTCCTCCCTCCTGCCAAGCAGAGCCTCAACACACAAGTCCTGCGCGTCCTGCAC

CAGCTCCGCAGCAAGTCGACCCCTCTCGAAAAGCACGTCATGCTCGCTTCGCTTAGACAGACCAACACTCGT

CTTTATTACGCCACAATCCTTGCCAACAAGGAAGAGATTCTTCCTCTTATCTACACTCCTACCGTCGGCGAG

GCATGCCAGAAGTTCTCCCACATCTACCGAAGGCCCGAGGGCCTCTCTATCTCGCTCGAGGACAAGGGCAAG

ATCGCCAGTATTGTTGAGAACTGGCCTGTCCCCGCCGGCTCGCCACGAATCGCTGTCATCACCGACGGCTCG

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CTGGAGGACCCCCTCTACCTGGGTCTCCGACGCGAGCGTGCAGGCACCGAGGAGTACATCGAGTTCATGGAC

GAGGTTATGGATGCTCTTCACTCCAGATACCCCAACCTCATCATCCAGTTTGAGGACTTTACCTCGGAGAAT

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CGCATCCTCATGGCCGGTGCCGGTTCGGCTGCTGTCGGTGTCGGCAAGCAGCTCATGTCGTTCTTCACCCGC

CAAGGTCTTTCCGAGGACGAGGCTCGCGAGCGCATCTACATCACCGACTCTAAGGGTCTGGTCACCAAGGAC

CGTGGAGACAAGCTGCAGGAGCACAAGGTCTTTTTCGCCCGCGACGACAACAATGGCAAGCAGATCAAGGAT

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GGAATCGCTTTCGCGTGCTATAGTCAAGCCGGAATACCCAAGACGGAACACCAGAAAGGTCTTGCGGATGAG

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CCAATGGCCTGGCAGGTGCTGGGATGTTGGGATCAACGGATCGATCCGTCCGTACGCTTCTGAAACCCCATC

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ATGCGCCAGACATGTGCTTCTTCCTTCGCCTGCGAACTAGGTCTGAGAAGGATCGTGAGCGGGTCGGCTCGC

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ATACCTGCTTCCTTGATGTTTCCGAAAGCG------------------------------------------

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>S4\_deletion\_of\_1165bp

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GCCGTTGAGATCTCTTGCGGACATTATCGAACGCAGTAGACGTCGCCGGTTTCTACAATTCGCAGACCGACC

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-CCTTTCCCGGGAATGGCCCTTGGCCGGNNNNTCGGAAGGNNGGTCGGGTCCCANNGAAAATAAC--CCAAA

TCCGC-NTGGCCCTTTCNNNTNNNC---ACAAAAAGAAACGCCCCAAGGAAN--NNGTNCCCCACC---GGA

AAAATTCTGGAAATC

**Supplemental Table S3.** Oligonucleotides used in this study.

|  |  |  |  |
| --- | --- | --- | --- |
| **No in this study** | **Name** | **Sequence (5’-3’)**  **Purpose** | **Experiment** |
| 1 | cas9-phsp-ovl-fwd | TTGCCAGATCCCGGGGGGCCATGGGCAAGCCCATCCCTAAC | construction of pMF-Cas9-U6tri-scaff, amplification of Cas9 including SV40 NLS |
| 2 | ofp-tnos-ovl-rev | TCTGCAGCCGGGCGGCCGCTCTAAGCTTCGAACTTGGGAAGATCAC | construction of pMF-Cas9-U6tri-scaff, amplification of Cas9 including SV40 NLS |
| 3 | tnos-ofp-ovl-fwd | TTCCCAAGTTCGAAGCTTAGAGCGGCCGCCCGGCTGCAGATCGTTCAAAC | construction of pMF-Cas9-U6tri-scaff, amplification of pMF1-hs backbone without hyg |
| 4 | phsp-cas9-ovl-rev | TTAGGGATGGGCTTGCCCATGGCCCCCCGGGATCTGGCAAC | construction of pMF-Cas9-U6tri-scaff, amplification of pMF1-hs backbone without hyg |
| 5 | u6tri-pmf-ovl-fwd | ATCGCCCTTCCCAACAGTTGTTGCGGATGTGAAAGTGGTTGATG | construction of pMF-Cas9-U6tri-scaff, amplification of U6 promoter of U. trichophora |
| 6 | u6tri-pmf-ovl-rev | CCCATTCGCCATTCAGGCTGGTCTTCGAGAAGACGCGTAAATCAAAGTGCGAGATG | construction of pMF-Cas9-U6tri-scaff, amplification of U6 promoter of U. trichophora |
| 7 | pmf-u6tri-ovl-fwd | TTACGCGTCTTCTCGAAGACCAGCCTGAATGGCGAATGGG | construction of pMF-Cas9-U6tri-scaff, amplification of pMF-Cas9 backbone |
| 8 | pmf-u6tri-ovl-rev | AACCACTTTCACATCCGCAACAACTGTTGGGAAGGGCGATCGG | construction of pMF-Cas9-U6tri-scaff, amplification of pMF-Cas9 backbone |
| 9 | spacer-tri-1A | TTACGCCATTTCCAAATCAAGACAC | spacer cloning, malAg#1 in PU6-Tri vector |
| 10 | spacer-tri-1B | AAACGTGTCTTGATTTGGAAATGGC | spacer cloning, malAg#1 in PU6-Tri vector |
| 11 | spacer-tri-2A | TTACGCGTGACCAGCTCGGCCTCCG | spacer cloning, malAg#2 in PU6-Tri vector |
| 12 | spacer-tri-2B | AAACCGGAGGCCGAGCTGGTCACGC | spacer cloning, malAg#2 in PU6-Tri vector |
| 13 | spacer-may-1A | CAACGCCATTTCCAAATCAAGACAC | spacer cloning, malAg#1 in PU6-May vector |
| 14 | spacer-may-1B | AAACGTGTCTTGATTTGGAAATGGC | spacer cloning, malAg#1 in PU6-May vector |
| 15 | U6may-2-fwd | TACTCAGAACTGCCGGAACG | sequencing, check for correct spacer insertion in PU6-May vector |
| 16 | U6tri-2-fwd | CGGTGGCCGTCATCTTTGAG | sequencing, check for correct spacer insertion in PU6-Tri vector |
| 17 | malA-rev | ATGCGTATGATGACCGAGTG | PCR analysis of malA target locus  PCR plasmid curing |
| 18 | malA-lb-seq | CCCGAATTGCATTGACTTGG | PCR analysis of malA target locus, respective sequencing and plasmid curing |
| 19 | 493\_Cas9\_seq6 | GATCGCTAAGAGCGAACAGG | PCR plasmid curing |
| 20 | 491\_Cas9\_seq4 | GGAAGCTCATCAACGGCATTA | PCR plasmid curing |
| 21 | 489\_Cas9\_seq2 | GGCGACCAGTACGCTGACCTG | PCR plasmid curing |
| 22 | 497\_Cas9\_seq\_1A | GCTGGGCCGTTATCACAGAC | PCR plasmid curing |
| 23 | Cas9-seq1-rev | CGGTGCCATCCATCTTCTCC | PCR plasmid curing |
| 24 | Cas9-seq2-rev | CGGTGTATCTTCGTCTCTTC | PCR plasmid curing |
| 25 | Cas9-seq3-rev | ACCACGGCGTTCAGGTATGC | PCR plasmid curing |
| 26 | Cas9-seq4-rev | AGGCCGGTAATGGACTGGTG | PCR plasmid curing |
| 27 | inHygR\_rev | CGCTGTTATGCGGCCATTG | PCR plasmid curing |
| 28 | Phsp70-2-fwd | ACCCTTCCGTTGCCAGATCC | PCR plasmid curing |
| 29 | malA-sp1-EcoRI\_A | AGCTCGCTGACCATCGCATTATCTCCGATCCATTTCCAAATCAAGAGAATTCCACTGGCTTTTCATATGAGAAGCGTGACCAGCTCGGCCTCCG | EcoRI homology directed repair template |
| 30 | malA-sp1-EcoRI\_B | CGGAGGCCGAGCTGGTCACGCTTCTCATATGAAAAGCCAGTGGAATTCTCTTGATTTGGAAATGGATCGGAGATAATGCGATGGTCAGCGAGCT | EcoRI homology directed repair template |
| 31 | Spacer-pyr6-1A | TTACGCAAGACGCATATCGATATTG | spacer cloning, pyr6g#1 in PU6-Tri vector |
| 32 | Spacer-pyr6-1B | AAACCAATATCGATATGCGTCTTGC | spacer cloning, pyr6g#1 in PU6-Tri vector |
| 33 | Spacer-pyr6-2A | TTACGCATGACTTTTTGATCTTTG | spacer cloning, pyr6g#2 in PU6-Tri vector |
| 34 | Spacer-pyr6-2B | AAACCAAAGATCAAAAAGTCATGC | spacer cloning, pyr6g#2 in PU6-Tri vector |
| 35 | OT-M1-fwd | CGTTGGAAATGTGCGGCATC | PCR, Off-Target #1 analysis |
| 36 | OT-M1-rev | GCAGCGCCTCCAATTTGGTG | PCR, Off-Target #1 analysis |
| 37 | OT-M2-fwd | CTATGTGAGATGGCTGGAAG | PCR, Off-Target #2 analysis |
| 38 | OT-M2-rev | AGTGTTGCCCTTGCTGTTGG | PCR, Off-Target #2 analysis |
| 39 | OT-M3-fwd | TCATCGCGACACTGGTCTGG | PCR, Off-Target #3 analysis |
| 40 | OT-M3-rev | CAGGAGATGAGCAGCAATAC | PCR, Off-Target #3 analysis |
| 41 | OT-M4-fwd | ATCTCTGCACGCGCCAACTC | PCR, Off-Target #4a analysis |
| 42 | OT-M4-rev | TCTCGAATACGGCGTATTGG | PCR, Off-Target #4a analysis |
| 43 | OT-M5-fwd | TTCCGGTCTGACGCGAAATC | PCR, Off-Target #5 analysis |
| 44 | OT-M5-rev | CGGGTTGCAATTTACTAGGC | PCR, Off-Target #5 analysis |
| 45 | OT-M6-fwd | GCATGCTCATATGCGTCTTG | PCR, Off-Target #6 analysis |
| 46 | OT-M6-rev | ACGGTACGAGCTACGGTACG | PCR, Off-Target #6 analysis |
| 47 | OT-M7-fwd | ATGCATGAATCGGGCTTCAG | PCR, Off-Target #7 analysis |
| 48 | OT-M7-rev | CACGGCCTATCAACAGAATC | PCR, Off-Target #7 analysis |
| 49 | OT-M8-fwd | TAGGGAGTTTGGCAAGTTGG | PCR, Off-Target #8 analysis |
| 50 | OT-M8-rev | AAGAAGGGCCGGCGAAGAAG | PCR, Off-Target #8 analysis |
| 51 | OT-M9-fwd | TGCAGATTCAGCCCTGCTTC | PCR, Off-Target #9a analysis |
| 52 | OT-M9-rev | CCCGAACAATCAGAATCTCC | PCR, Off-Target #9a analysis |
| 53 | OT-M10-fwd | GGTATGCAGAGACGATGATG | PCR, Off-Target #10 analysis |
| 54 | OT-M 10-rev | GAGCGTGTCCGGCAAGTATC | PCR, Off-Target #10 analysis |
| 55 | OT-M4a-fwd | AAACTTCGGCGAGGGTGCTG | PCR, Off-Target #4b analysis |
| 56 | OT-M4a-rev | TGAGCCCTGAAGTCGGTATG | PCR, Off-Target #4b analysis |
| 57 | OT-M9a-fwd | CCTCGAAGCATTCCTCAAAG | PCR, Off-Target #9b analysis |
| 58 | OT-M9a-rev | GCCACCTCATTCCAACATCC | PCR, Off-Target #9b analysis |
| 59 | pyr6\_lb\_fwd | CTGAGGATGTCTTGCGAGTACGAGAG | PCR |
| 60 | pyr6\_rb\_rev | TCAACCCGAGCAATCGACTCTTCTCC | PCR |
| 61 | py6\_fwd | CGGCGTGTGGTATGTGTCTC | Sequencing |
| 62 | py6\_rev | TACACCTTCGACCTCAAACG | Sequencing |

**Supplemental Table S4.** Sequences of Cas9-expression cassette and sgRNA transcription unit.

>Phsp70\_hCas9-2A-OFP\_Tnos

GAACGTGGTAACTACCAGCGAGTTCTGCAAACTTCAAAAAAAAAATCTGGGCACGATGAA

AGTTGAGCTAACGCTGACGCTCACAAATGGCGTGGCTAAAGGAAGCGAGACAATCGGAAA

ATTGTTCTCTCGGGCACCACAAAGCTGTTGTTAGTCGCTGAAGAACAATTCCAACTGATT

CCGCCGCCTTCCTATTGCGTCAGCCTTGTACCTAAGCTGCCGAGTAACGTCACTCAACCT

CTCTTTTCAGACTGCTTTGCTCCGCGAATACTTTTCTTCTATGCGCTCAAGAAAATGACA

CAGCACACCAAGCTCTGCAAACTTTCTTCGCTAATCTGACGCGAAATGTGAGCCATTTCT

TCTCGCCTGCAATGGCAATGCGTCTGTGCGGCGATGAGAATCACGATGCGGAATGGGTGG

CTGGAAGTTCATAGAGATGCTGAGTTGTTGGAGCGACATGGTACATAAGCATGAGTCTGT

CCTGATTTCCACCCTCCCGTCTTTCATCAACTTTCTCGTCTGACCCTTCCGTTGCCAGAT

CCCGGGGGGCCATGGGCAAGCCCATCCCTAACCCCCTGTTGGGGCTGGACAGCACCGCTC

CCAAAAAGAAAAGGAAGGTGGGCATTCACGGCGTGCCTGCGGCCGACAAAAAGTACAGCA

TCGGCCTTGATATCGGCACCAATAGCGTGGGCTGGGCCGTTATCACAGACGAATACAAGG

TACCCAGCAAGAAGTTCAAGGTGCTGGGGAATACAGACAGGCACTCTATCAAGAAAAACC

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GCAACGAGATGGCCAAGGTGGACGACAGCTTCTTCCACAGGCTGGAGGAGAGCTTCCTTG

TCGAGGAGGATAAGAAGCACGAACGACACCCCATCTTCGGCAACATAGTCGACGAGGTCG

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ATAAAGCCGACTTGCGACTTATCTACTTGGCTCTGGCGCACATGATTAAGTTCAGGGGCC

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CATGA

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