Supplementary Materials:

A Major Facilitator Superfamily Transporter Contributes to Ergot Alkaloid Accumulation but Not Secretion in *Aspergillus leporis*

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Table S1. Primers and PCR Protocol Information.

Primer pair	Primer sequences (5′ to 3′)[a]	Product (length)	Annealing temperature (°C), Extension time (s)
1	CATGCTTCTAATCCACCAAGTAC + GACAGCCGAAATAACGTACCATGGTGCGGAGTGCCTAC	*A. fumigatus easA* promoter with 22-nt overlap with *A. leporis easT* (812 bp)	63, 30
2	GTAGGCACTCCGCACCATGGTACGTTATTTCGGCTGTCC + TAGGAACAATGCATCTCAAG	*A. leporis easT* with 16-nt overlap with *easA* promoter from *A. fumigatus* (2050 bp)	60, 60
3	CATGCTTCTAATCCACCAAGTAC + TAGGAACAATGCATCTCAAG	*A. fumigatus easA* promoter fused to *A. leporis easT* (2824 bp)	60, 90
4	AGTCGGAGCTCCGCAGATTCTAGAAGTCCTG + GCTAGACTAGTTGTGTAGATTCGTCTGGTAC	*A. fumigatus gpdA* promoter (990 bp)	61, 30

5	GTCACCTGCAGGTCCGTCTCCATTGGCTCTTG + AGCTCCTGCAGGCTATTCCTTTGCCCTCGGAC	Hygromycin resistance gene including promoter and 3'UTR (1851 bp)	64, 60
6	GTCACCTGCAGGGTACCCGGGGATCTTTCGAC + AGCTCCTGCAGGTACATGCGTACACGCGTCTG	Phleomycin resistance gene including promoter and 3'UTR (2945 bp)	65, 90
7	GCTAGACTAGTATGGTGAGCAAGGGCGAG + GGTCAGTCGACCTACAACTTCGACTTGTACAGCTCGTCCATGC	mCherry gene with terminal nucleotides for -SKL amino acid sequence (742 bp)	53, 60
8	*ACAAGTTTGTACAAAAAAGCTGAACGAGAA*ATGGTACGTTATTTCGG CTG + *ACCACTTTGTACAAGAAAGCTGAACGAGAA*CCGTCAGCATGTCTTCG CTT	*A. leporis easT* without stop codon and 3'UTR and *attR1/2* overlaps (1793 bp)	61, 60
9	*ACAAGTTTGTACAAAAAAGCTGAACGAGAA*ATGGTACGTTATTTCGG CTG + *ACCACTTTGTACAAGAAAGCTGAACGAGAA*ATACAGATCCGGAGATG ATG	*A. leporis easT* with stop codon and 3'UTR and *attR1/2* overlaps (2070 bp)	60, 60

10	GATGGGCTGCAGGAATTCGATATCAAGCTTAATGGTGAGC + *ACCACTTTGTACAAGAAAGCTGAACGAGAA*CCGTCAGCATGTCTTCG CTT	Linear Af*easTCFP*-PhleoR plasmid from exponential megapriming PCR (12,348 bp)	72, 390
11	GCCGGTACCCAATTCGCCCTATAGTGAGTC + ACCACTTTGTACAAGAAAGCTGAACGAGAAATACAGATCCGGAGA TGATG	Linear Af*CFPeasT*-PhleoR plasmid from exponential megapriming PCR (12,385 bp)	72, 240
12	GTACGTTATTTCGGCTGTCC + GTTACTTGTACAGCTCGTCC	A portion of *A. leporis easT*-CFP fusion (2512 bp genomic DNA, 2266 bp cDNA)	61, 90
13	CACATGAAGCAGCACGACTT + CCCTTCTCTCTGGCTCGAG	A portion of CFP-*A. leporis easT* fusion for RT-PCR (1470 bp genomic DNA, 1224 bp)	64, 60
14	CAAGGTGCATCATCTGCCG + CCCTTCTCTCTGGCTCGAG	*A. leporis easT* locus before (306 bp) and after (~2300 bp) knockout	65, 90

[a] Underlines indicate unique restriction sites inserted to facilitate cloning of products: GAGCTC, *Sac*I; GTCGAC, *Sal*I; CCTGCAGG, *Sbf*I; ACTAGT, *Spe*I.

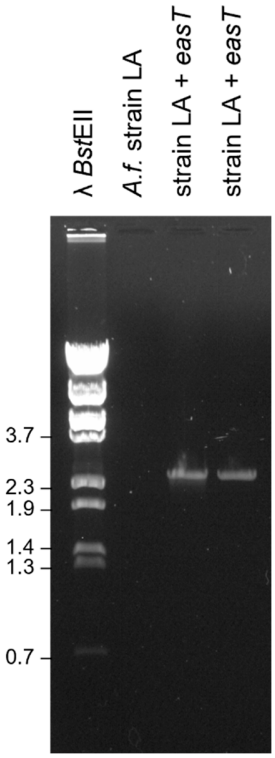

Figure S1. Detection of the *easT*-expression construct in transformants of *A. fumigatus* strain LA. PCR products (or lack thereof, in the case of the recipient strain) were obtained in reactions with primer combination 3 (Table S1). Sizes (in kb) of relevant fragments of *Bst*EII-digested bacteriophage lambda are indicated to the left of the gel.

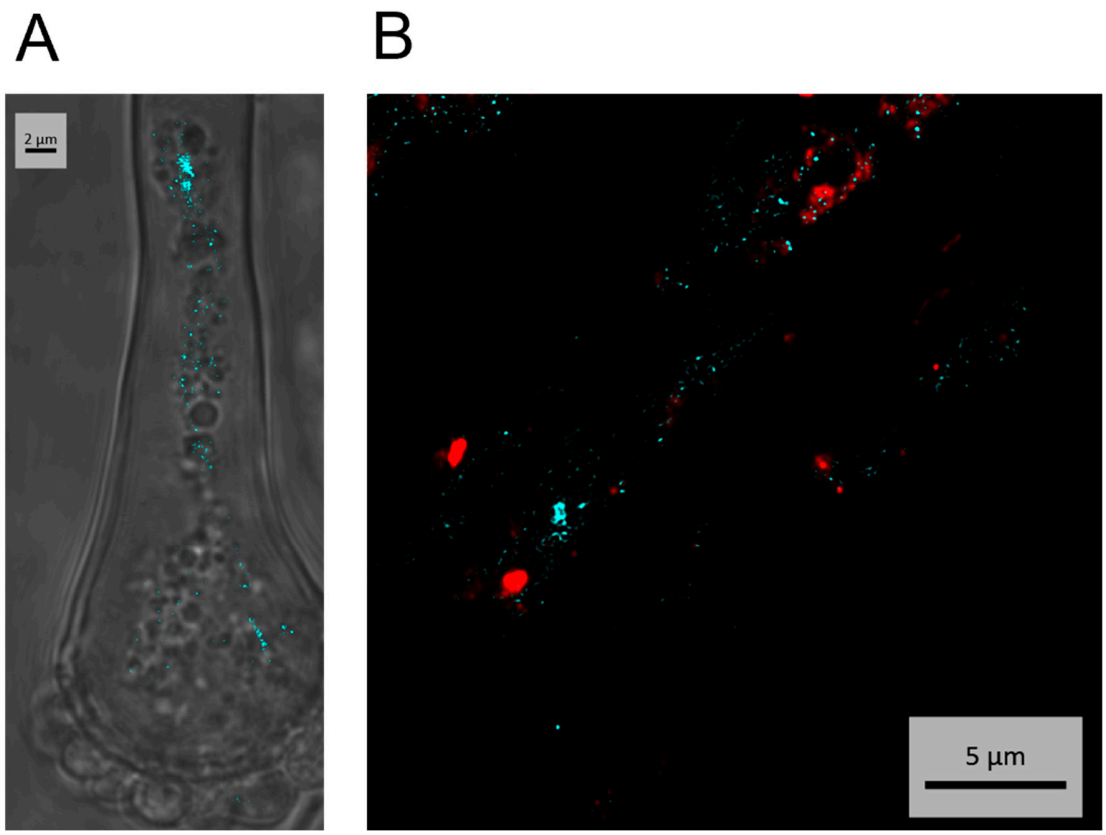

Figure S2. Localization of EasT-CFP and mCherry-SKL fusion proteins in transformants of *A. fumigatus* strain LA. (**A**) Localization of EasT-CFP and in a conidiophore visualized with overlaid fluorescence and differential interference contrast. (**B**) Localization of mCherry-SKL fusions (red) and EasT-CFP (blue) in hyphae of *A. fumigatus* strain LA.

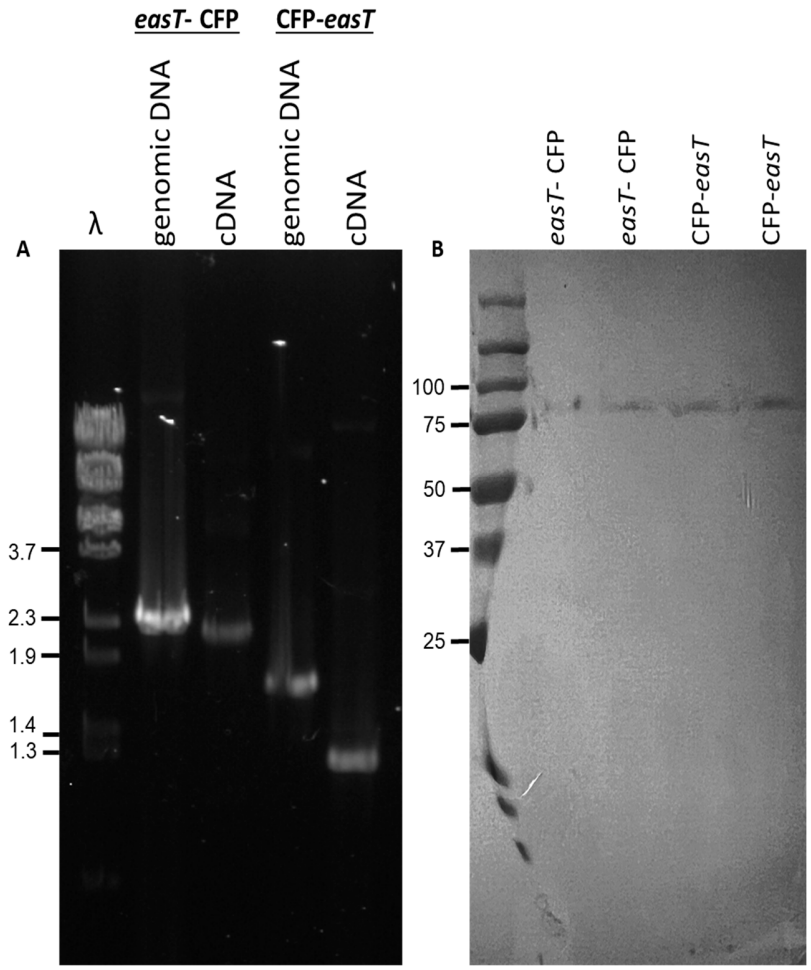

Figure S3. Confirmation of mRNA and protein expression. (A) DNA gel showing products amplified from genomic DNA and cDNA of *A. fumigatus* mutants expressing easT-CFP and CFP-easT fusions using primer combinations 12-13 (listed in Table S1), respectively. Sizes of relevant fragments from *Bst*EII-digested bacteriophage lambda DNA are indicated to the left. Gel was stained with ethidium bromide. (B) Western blot of duplicate membrane fractions from the same mutants as panel A. Sizes of Precision Plus pre-stained protein marker (Bio-Rad, Hercules, CA, USA) fragments in the first lane are indicated to the left of the blot. Size marker lane is distorted due to high amounts of detergent in membrane samples.

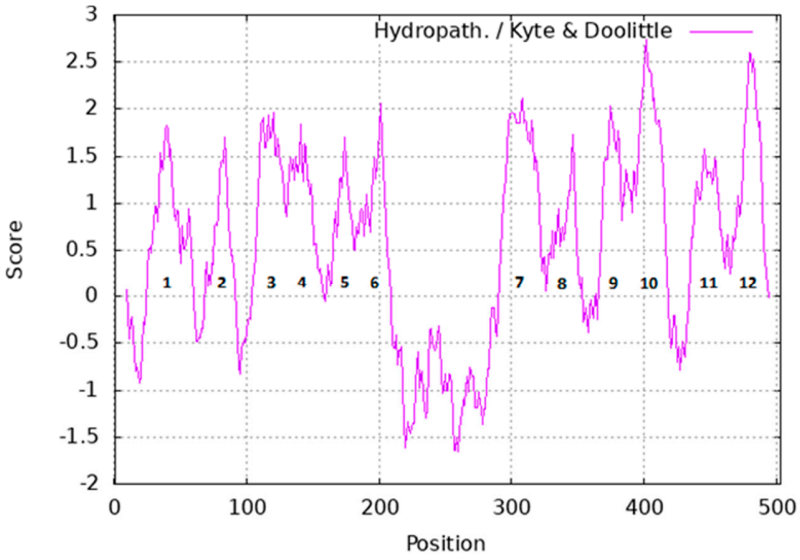

Figure S4. Kyte-Doolittle hydropathy plot derived from EasT amino acid sequence. A window size of 19 amino acids was used to search for transmembrane regions in this protein. The hydrophobic residues are shown above zero, whereas the hydrophilic residues are below zero. Derived hydrophobic regions are numbered. Plot was generated with ProtScale (web.expasy.org, accessed on 10 July 2023).

Figure S5. PCR and DNA sequence analyses of *easT* knockout in *A. leporis*. In the left panel, PCR products from a transformant (ko) and *A. leporis* strain NRRL 3216 (wt) were derived from reactions with primer combination 14 (Table S1). Relative mobility of relevant fragments (in kb) of *Bst*EII-digested bacteriophage λ are shown to the left of the gel. The right panel presents the DNA sequence of the *easT* locus after CRISPR-Cas9 mutagenesis. Unhighlighted sequence are part of *easT*. Sequences highlighted yellow are nucleotides from easT incorporated into the sgRNA, and the target

sequence PAM site is highlighted red. Sequences of the pBCphleo selectable marker construct (incorporated into the locus during repair) are shaded gray. The abbreviation [*NNNNNNNN*] represents approximately 1000 nt of the insert that were omitted to simplify the presentation.