Figure S1 The proline catabolism-associated genes are located in a cluster on a single chromosome in *A. nidulans,* however in *S. cerevisiae* and *C. neoformans,* the genes are unlinked and located on different chromosomes. *prnA* encodes the pathway-specific transcription factor of *prn* expression, *prnD, PUT1* and *PUT5* encode proline oxidase, *prnB* and *PUT4* encode the proline-specific permease, while *prnC* and *PUT2* encode P5C dehydrogenase. The multiple candidates of proline permease-encoding genes in *C. neoformans* are designated using the Broad Institute annotations.
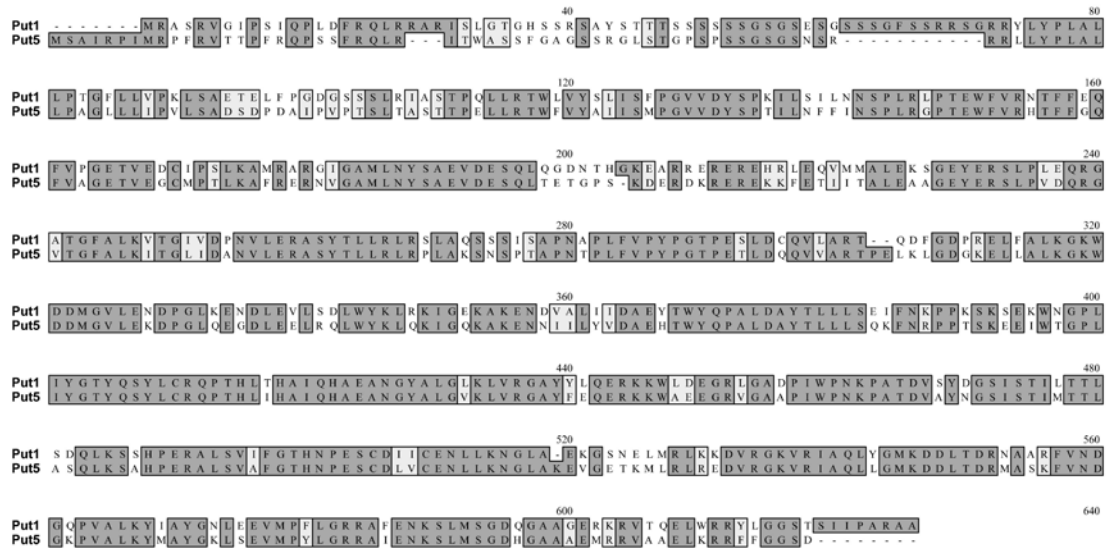
Figure S2 ClustalW multiple sequence alignment of *C. neoformans* Put1 and Put5. Identical amino acid residues are shaded dark grey while similar residues are shaded light grey.
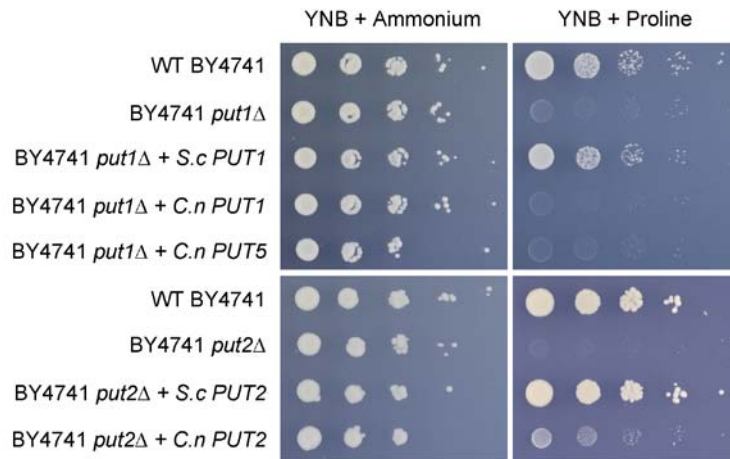
Figure S3 Cross-species complementation using *C. neoformans PUT2* partially restored the proline utilization ability of the *S. cerevisiae put2Δ* mutant. Tenfold spot dilution assays for nitrogen source utilization showed that *C. neoformans PUT2* complemented the *S. cerevisiae put2Δ* mutant's proline assimilation defect, albeit to a poorer extent than an *S. cerevisiae PUT2.* On the other hand, neither *C. neoformans PUT1* or *PUT5* restored the proline utilization defect of the *S. cerevisiae put1Δ* strain.
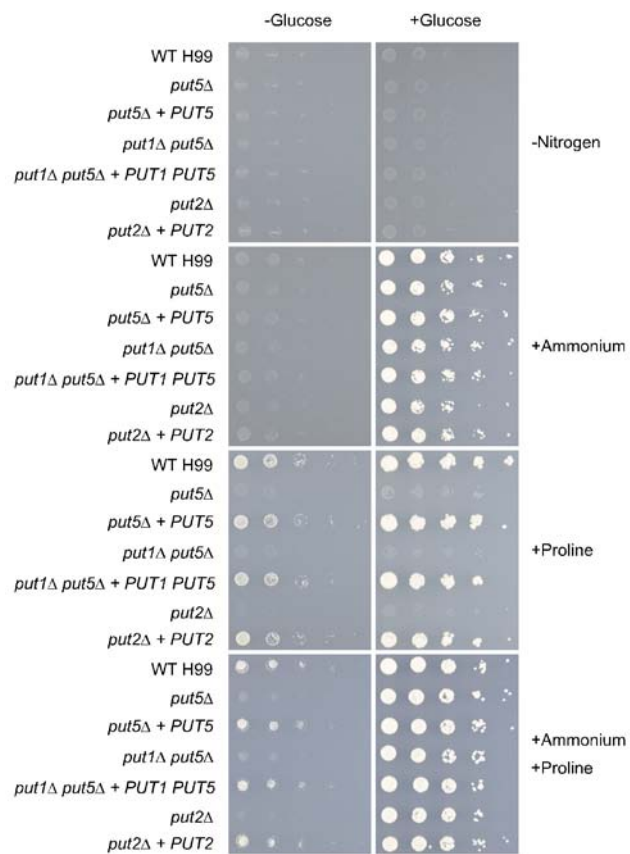
I. M. R. C. Lee *et al.*

Figure S4 Restoration of proline utilization ability upon the re-introduction of *PUT5, PUT1 PUT5* and *PUT2* into the *put5Δ,* double *put1Δ put5Δ* and *put2Δ* mutants, respectively. Tenfold spot dilution assays for nitrogen or nitrogen and carbon source utilization showed that the complemented *put5Δ + PUT5, put1Δ put5Δ + PUT1 PUT5* and *put2Δ + PUT2* strains exhibited wild-type growth on YNB supplemented with 10 mM proline (with or without 2% glucose as a carbon source).

A Menadione stress assays
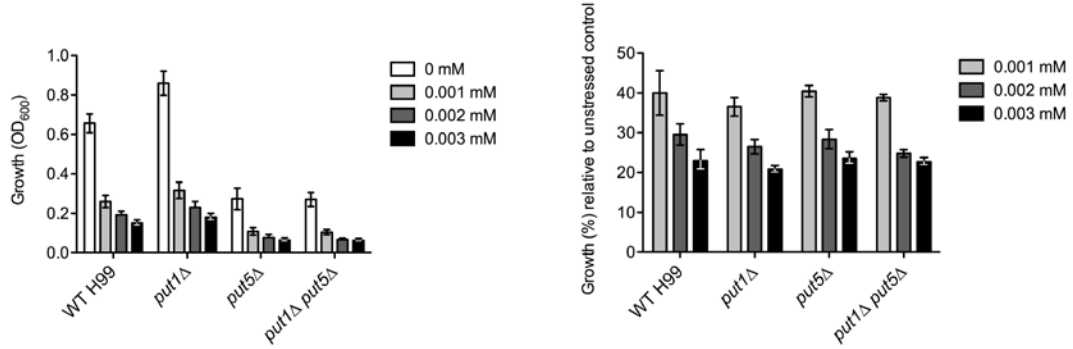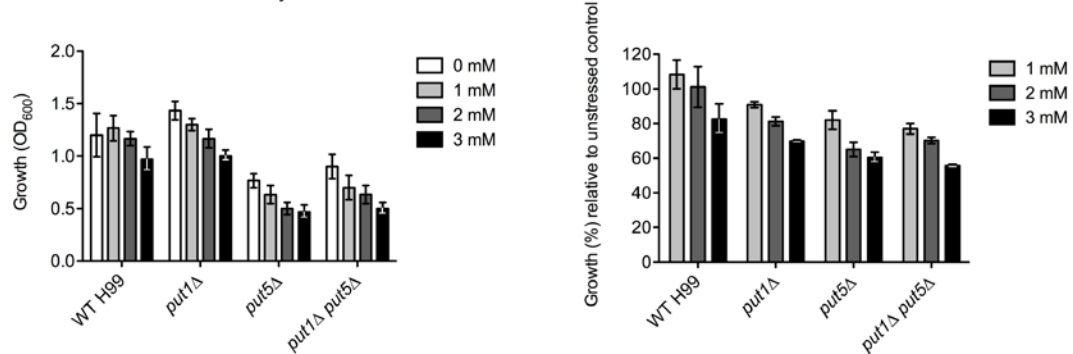

B Sodium nitrite stress assays

Figure S5 Tolerance against the oxidative stressor menadione and nitrosative stressor sodium nitrite is equivalent between the wild-type and proline oxidase(s) deletion mutant strains. Wild-type H99, *put1Δ, put5Δ* and double *put1Δ put5Δ* strains were grown in YNB [proline and glutamate (10 mM each)] supplemented with (A) 0 – 0.003 mM menadione, or (B) 0 – 3 mM sodium nitrite. All strains displayed relatively similar growth percentage relative to the unstressed control when subjected to these individual stressors. Growth percentage relative to unstressed control is defined by dividing each stressed strain's growth in OD_{600} with the same strain's growth in OD_{600} when unstressed. Error bars represent standard errors across three biological replicates.

I. M. R. C. Lee *et al.*
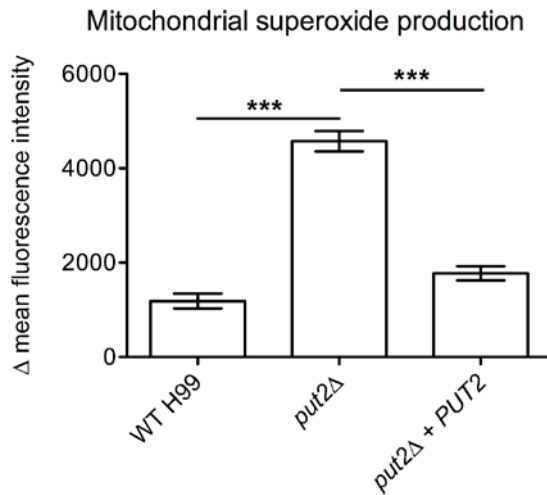
Mitochondrial superoxide production

Figure S6 Restoration of mitochondrial superoxide ions to wild-type levels upon the re-introduction of *PUT2* into the *put2Δ* mutant. Wild-type H99, *put2Δ* and complemented *put2Δ + PUT2* strains were briefly subjected to culture in YNB supplemented with 10 mM proline, stained with MitoSOX, and the accumulation of ROS in the mitochondria was assessed by flow cytometry. Bar chart presented as average $\pm$ standard error of triplicate experiments showed that the *put2Δ* mutant had significantly enhanced Δ mean fluorescence intensity (Δ MFI) of oxidized MitoSOX relative to the wild-type or *put2Δ + PUT2* strains (*** denotes $P < 0.001$). Δ MFI is defined by subtracting the background MFI (unstained cells) from the MFI generated from MitoSOX stained cells.
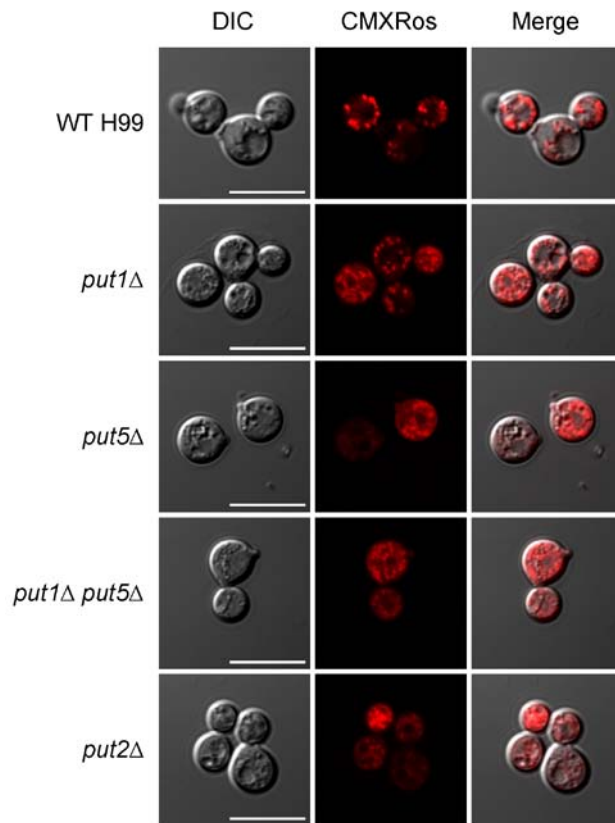
Figure S7 None of the proline catabolic deletion mutants display a defect in mitochondrial membrane potential. Wild-type H99 and proline catabolic deletion mutant strains were briefly subjected to culture in YNB supplemented with 10 mM proline, stained with MitoTracker Red CMXRos, and cells viewed under a confocal microscope. The staining profile of the mutants was similar to that of wild-type showing accumulation of the fluorescence dye that is dependent on normal mitochondrial membrane permeability. Scale bar, 10 μm.

I. M. R. C. Lee *et al.*
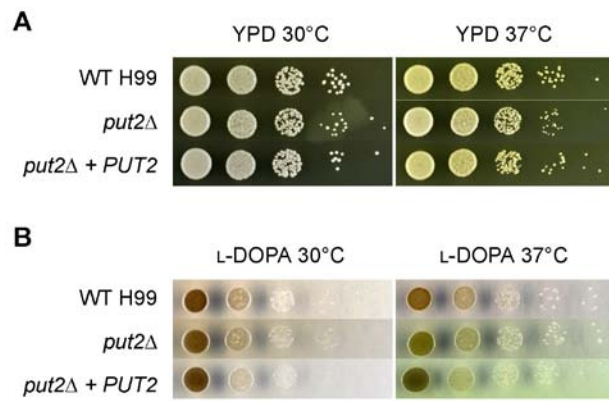
Figure S8 Restoration of virulence factor expression to wild-type levels upon the re-introduction of *PUT2* into the *put2Δ* mutant. (A) Tenfold spot dilution assays on YPD medium demonstrated that the complemented *put2Δ + PUT2* strain exhibited wild-type growth at both 30 and 37°C. (B) Tenfold spot dilution assays on L-DOPA medium showed that the *put2Δ + PUT2* strain produced equal amount of melanin as the wild-type strain at both 30 and 37°C.

Table S1 Fungal strains used in this study

Strain	Genotype	Original source
H99	*Cryptococcus neoformans MATα*	John Perfect
EL13	H99 *put1::NEO*	This study
EL1	H99 *put5::NEO*	This study
EL9	H99 *put1 put5::NEO*	This study
EL18	H99 *put2::NEO*	This study
EL22	H99 *put1::NEO + PUT1 NAT*	This study
EL20	H99 *put5::NEO + PUT5 NAT*	This study
EL21	H99 *put1 put5::NEO + PUT1 PUT5 NAT*	This study
EL23	H99 *put2::NEO + PUT2 NAT*	This study
BY4741	*Saccharomyces cerevisiae MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Carrie Brachmann
4099B7	BY4741 *put1::KanMX*	Thermo Scientific
1000H4	BY4741 *put2::KanMX*	Thermo Scientific

Table S2 Primers used in this study

Primer	Purpose	Sequence (5'-3')
UQ1070	Cn *PUT1* qRT-PCR F	AAAAGCTAAAGAGAACGACGTTGC
UQ1071	Cn *PUT1* qRT-PCR R	TCTCACTCTTTGACTTTGGAGGTTT
UQ1072	Cn *PUT5* qRT-PCR F	AGAAGGCTAAGGAGAACAACATCATT
UQ1073	Cn *PUT5* qRT-PCR R	GTCCAGATCTCCTCCTTGGAAG
UQ1074	Cn *PUT2* qRT-PCR F	TCATTAACGGTGAGGAGGTCAAG
UQ1075	Cn *PUT2* qRT-PCR R	GCAAGAGCACCGTCAATGG
UQ1300	Cn *PUT1* deletion 5' F / genomic DNA generation F	CTGCCATGTACAATCCATAAT
UQ1301	Cn *PUT1* deletion 5' R	CTCTCCAGCTCACATCCTCGCAGCGCGATTTCGTTA
UQ1302	Cn *PUT1* deletion 3' F	TGTTAATACAGATAAACCGTTGGTTCAAGATACTTT
UQ1303	Cn *PUT1* deletion 3' R / genomic DNA generation R	TCTCTACATTACGCAACATGG
UQ1304	Cn *PUT1* deletion *NEO* F	TAACGAAATCGCGCTGCGAGGATGTGAGCTGGAGAG
UQ1305	Cn *PUT1* deletion *NEO* R	AAAGTATCTTGAACCAACGGTTTATCTGTATTAACA
UQ1306	Cn *PUT5* deletion 5' F / genomic DNA generation F	GCACGCCTTCCGAAGTGAAAG
UQ1307	Cn *PUT5* deletion 5' R	AGCTCACATCCTCGCAGCCGTTGTGGATCGTTGGTT
UQ1308	Cn *PUT5* deletion 3' F	TGTTAATACAGATAAACCAACGCTTAAGTGGAGATG
UQ1309	Cn *PUT5* deletion 3' R / genomic DNA generation R	CCTCGTTCGGCTAAAGCTGAC
UQ1310	Cn *PUT5* deletion *NEO* F	CAACCAACGATCCACAACGGCTGCGAGGATGTGAGC
UQ1311	Cn *PUT5* deletion *NEO* R	CATCTCCACTTAAGCGTTGGTTTATCTGTATTAACA
UQ1312	Cn *PUT2* deletion 5' F / genomic DNA generation F	CTCCGGTGTCCATTATTCCTA
UQ1313	Cn *PUT2* deletion 5' R	AGCTCACATCCTCGCAGCTTTTGGTAAGGATGAAGA
UQ1314	Cn *PUT2* deletion 3' F	TGTTAATACAGATAAACCGTCAATGAGAATAGAAAA
UQ1315	Cn *PUT2* deletion 3' R / genomic DNA generation R	TTCTGACGGTGCAGGCGAAGA
UQ1316	Cn *PUT2* deletion *NEO* F	TCTTCATCCTTACCAAAAGCTGCGAGGATGTGAGCT
UQ1317	Cn *PUT2* deletion *NEO* R	TTTTCTATTCTCATTGACGGTTTATCTGTATTAACA
UQ1036	Universal deletion diagnostic (*NEO* marker specific) R	TGTGGATGCTGGCGGAGGATA
UQ1410	Cn *PUT1* deletion diagnostic F	AAACGCTTAAGTGGAGATGAA
UQ1411	Cn *PUT5* deletion diagnostic F	AGGCTGAAGATGATGCGAGAA
UQ1412	Cn *PUT2* deletion diagnostic F	CGATGACAGCCGATACCTACA
UQ18	Sequencing (M13F pCR2.1-TOPO)	GTAAAACGACGGCCAG
UQ19	Sequencing (M13R pCR2.1-TOPO)	CAGGAAACAGCTATGAC
UQ1594	Cn *PUT1* genomic DNA sequencing	GCTGTTCCGAAAGCTACCAGA
UQ1595	Cn *PUT1* genomic DNA sequencing	CCTCCTCGTCCTCTGGTTCT
UQ1596	Cn *PUT1* genomic DNA sequencing	TGGCGTTGGAAAAGAGCGGAG
UQ1597	Cn *PUT1* genomic DNA sequencing	AAAGAGAACGAGTAGACACAT
UQ1598	Cn *PUT1* genomic DNA sequencing	GCGTCATTTTCGGTACTCACA
UQ1599	Cn *PUT1* genomic DNA sequencing	AACTTGATTTCATTCGTGCTC
UQ1600	Cn *PUT5* genomic DNA sequencing	TCCCCTGCTATTTTTTATCGT
UQ1601	Cn *PUT5* genomic DNA sequencing	GACAACTCCTTTTCGACAACC
UQ1602	Cn *PUT5* genomic DNA sequencing	TGAAACCGTGGAGGGATGTAT
UQ1603	Cn *PUT5* genomic DNA sequencing	GAAGGGCAAGTGGGATGACAT
UQ1604	Cn *PUT5* genomic DNA sequencing	CATACAACACGCCGAAGCCAA
UQ1605	Cn *PUT5* genomic DNA sequencing	GGCGGCAGAGATGAGGCGAGT
UQ1606	Cn *PUT2* genomic DNA sequencing	TGGGTGTTGAAGATTTGGTCG

UQ1607	Cn *PUT2* genomic DNA sequencing	ACCAAAAATGTCTTCCCAACT
UQ1608	Cn *PUT2* genomic DNA sequencing	GCCGAAATCGACGCTGCCGCT
UQ1609	Cn *PUT2* genomic DNA sequencing	CCCAAAACCTCGACATCTACA
UQ1610	Cn *PUT2* genomic DNA sequencing	TACGCTTTTCATTTCCGGCCT
UQ1611	Cn *PUT2* genomic DNA sequencing	AGCGATTAGGGTTTACGACAT
UQ1612	Cn *PUT2* genomic DNA sequencing	AGAAGAAGAAGTCAGTCAGCA
UQ270	GenomeWalker adaptor	GTAATACGACTCACTATAGGGC
UQ271	GenomeWalker nested adaptor	ACTATAGGGCACGCGTGGT
UQ272	GenomeWalker 3' gene specific	CTGAATGGCGAATGAGCTTGAGCTTGG
UQ273	GenomeWalker 3' nested gene specific	ATCAGATTGTCGTTTCCCGCCTTCAG
UQ274	GenomeWalker 5' gene specific	GAGAGGCGGTTTGCGTATTGGCTAGAG
UQ275	GenomeWalker 5' nested gene specific	ACGTCCGCAATGTGTTATTAAGTTGTC
UQ1719	Sc *PUT1* genomic DNA / promotor generation F	AGACAAAACTGAGCGAGACAG
UQ1756	Sc *PUT1* genomic DNA / terminator generation R	GCACCTACACTAACAGCTTCT
UQ1729	Sc *PUT2* genomic DNA / promotor generation F	TCACCGCATCGTTCAACTGTC
UQ1760	Sc *PUT2* genomic DNA / terminator generation R	TGCAATGCCAAAAGTGTCTGT
UQ1720	Cn *PUT1* cDNA generation F	GGCATCCCATCCATACAACCTCTAGATTTTAGACA
UQ1761	Cn *PUT1* cDNA generation R	GACCAAAGTATCTTGAACCAA
UQ1725	Cn *PUT5* cDNA generation F	CACTAACGCACGCTAGAAATGTCAGCAATCCGACC
UQ1762	Cn *PUT5* cDNA generation R	TTAATCAGAGCCTCCAAAGAA
UQ1730	Cn *PUT2* cDNA generation F	AATTCACAGGAATTGCTTTTCTTTCTTCATCCTTA
UQ1763	Cn *PUT2* cDNA generation R	GATACGAGACATTATGTATGA
UQ1814	Sc *PUT1* promotor generation R for Cn *PUT1*	GCGGGAGGCACGCATTTCTAGCGTGCGTTAGTGTT
UQ1758	Sc *PUT1* promotor generation R for Cn *PUT5*	GGTCGGATTGCTGACATTTCTAGCGTGCGTTAGTG
UQ1815	Sc *PUT1* terminator generation F for Cn *PUT1*	CAAGATACTTTGGTCGAGGACTATAATATATACTC
UQ1728	Sc *PUT1* terminator generation F for Cn *PUT5*	AGATTCTTTGGAGGCTCTGATTAAGAGGACTATAA
UQ1759	Sc *PUT2* promotor generation R for Cn *PUT2*	TAAGGATGAAGAAAGAAAAGCAATTCCTGTGAATT
UQ1816	Sc *PUT2* terminator generation F for Cn *PUT2*	ATGTCTCGTATCAAAAATTTTTGTGGAATAGAACC
UQ1770	Sc *PUT1* genomic DNA sequencing	CAGCTATGACCATGATTACGC
UQ1769	Sc *PUT1* genomic DNA sequencing	GTCGCTACACACGTACATTGC
UQ1818	Sc *PUT1* genomic DNA sequencing	AGTGAAGGTGAAAGCGAGGCA
UQ1771	Sc *PUT1* genomic DNA sequencing	CGCCACTCATCAATTGTTTAT
UQ1772	Sc *PUT1* genomic DNA sequencing	TCCAAAAGCTCCTTATTAGTT
UQ1819	Sc *PUT1* genomic DNA sequencing	ACAACATCCTACTGCCCAATA
UQ1773	Sc *PUT1* genomic DNA sequencing	ATGAATATAAGCACCACGAAC
UQ1774	Sc *PUT1* genomic DNA sequencing	TCACTTGGTTGTCGCCTCTCA
UQ1775	Sc *PUT1* genomic DNA sequencing	AAGATTTCCGTTTGAACTAGC
UQ1776	Sc *PUT2* genomic DNA sequencing	ATGACCATGATTACGCCAAGC
UQ1777	Sc *PUT2* genomic DNA sequencing	AAGATGGCAAGGGATATGTTT
UQ1778	Sc *PUT2* genomic DNA sequencing	CATATCCCTTGCCATCTTCAC
UQ1820	Sc *PUT2* genomic DNA sequencing	GACTTTCTCAGTAGGGGTAGA
UQ1779	Sc *PUT2* genomic DNA sequencing	AAACCTTCACAAACCGCTGCC
UQ 1821	Sc *PUT2* genomic DNA sequencing	CCATGGATATACTTCTGGAGT
UQ1780	Sc *PUT2* genomic DNA sequencing	AGCCTTATCGTCGGTACCACT
UQ1822	Sc *PUT2* genomic DNA sequencing	GCCACTTTGTATTTTGCCATA
UQ1781	Sc *PUT2* genomic DNA sequencing	GGCGAGCAGTCCCTGACAAGA

I. M. R. C. Lee *et al.*

UQ1782	Sc *PUT2* genomic DNA sequencing	TTCCACATGGCAAAGAATAAA
UQ1595	Cn *PUT1* cDNA sequencing	CCTCCTCGTCCTCTGGTTCT
UQ1596	Cn *PUT1* cDNA sequencing	TGGCGTTGGAAAAGAGCGGAG
UQ1598	Cn *PUT1* cDNA sequencing	GCGTCATTTTCGGTACTCACA
UQ1601	Cn *PUT5* cDNA sequencing	GACAACTCCTTTTCGACAACC
UQ1602	Cn *PUT5* cDNA sequencing	TGAAACCGTGGAGGGATGTAT
UQ1603	Cn *PUT5* cDNA sequencing	GAAGGGCAAGTGGGATGACAT
UQ1604	Cn *PUT5* cDNA sequencing	CATACAACACGCCGAAGCCAA
UQ1605	Cn *PUT5* cDNA sequencing	GGCGGCAGAGATGAGGCGAGT
UQ1607	Cn *PUT2* cDNA sequencing	ACCAAAAATGTCTTCCCAACT
UQ1608	Cn *PUT2* cDNA sequencing	GCCGAAATCGACGCTGCCGCT
UQ1609	Cn *PUT2* cDNA sequencing	CCCAAAACCTCGACATCTACA
UQ1611	Cn *PUT2* cDNA sequencing	AGCGATTAGGGTTTACGACAT

Table S3 Plasmids used in this study

Plasmid	Detail	Original source
pJAF1	*NEO* selectable marker in pCR2.1-TOPO	James Fraser
pCH233	*NAT* selectable marker in pCR2.1-TOPO	Christina Hull
pELYL1	H99 *PUT1* genomic DNA including promotor and terminator in pCR2.1-TOPO	This study
pELYL2	H99 *PUT5* genomic DNA including promotor and terminator in pCR2.1-TOPO	This study
pELYL4	H99 *PUT1 PUT5* genomic DNA including promotor and terminator in pCR2.1-TOPO	This study
pELYL3	H99 *PUT2* genomic DNA including promotor and terminator in pCR2.1-TOPO	This study
pELYL5	H99 *PUT1* genomic DNA including promotor and terminator in pCH233	This study
pELYL6	H99 *PUT5* genomic DNA including promotor and terminator in pCH233	This study
pELYL8	H99 *PUT1 PUT5* genomic DNA including promotor and terminator in pCH233	This study
pELYL7	H99 *PUT2* genomic DNA including promotor and terminator in pCH233	This study
YCplac111	YC-type (centromeric) shuttle vector with *LEU2* and ampR markers	Daniel Gietz
pEWC38	H99 *PUT1* cDNA in pCR2.1-TOPO	This study
pEWC39	H99 *PUT5* cDNA in pCR2.1-TOPO	This study
pEWC37	H99 *PUT2* cDNA in pCR2.1-TOPO	This study
pEWC45	H99 *PUT1* cDNA flanked by BY4741 *PUT1* promotor and terminator in pCR2.1-TOPO	This study
pEWC46	H99 *PUT5* cDNA flanked by BY4741 *PUT1* promotor and terminator in pCR2.1-TOPO	This study
pEWC40	H99 *PUT2* cDNA flanked by BY4741 *PUT2* promotor and terminator in pCR2.1-TOPO	This study
pEWC35	BY4741 *PUT1* genomic DNA including promotor and terminator in pCR2.1-TOPO	This study
pEWC36	BY4741 *PUT2* genomic DNA including promotor and terminator in pCR2.1-TOPO	This study
pEWC48	H99 *PUT1* cDNA flanked by BY4741 *PUT1* promotor and terminator in YCplac111	This study
pEWC49	H99 *PUT5* cDNA flanked by BY4741 *PUT1* promotor and terminator in YCplac111	This study
pEWC47	H99 *PUT2* cDNA flanked by BY4741 *PUT2* promotor and terminator in YCplac111	This study
pEWC50	BY4741 *PUT1* genomic DNA including promotor and terminator in YCplac111	This study
pEWC51	BY4741 *PUT2* genomic DNA including promotor and terminator in YCplac111	This study