SUPPLEMENTARY MATERIAL

Conservation of Cdc14 phosphatase specificity in plant fungal pathogens: implications for antifungal development

Andrew G. DeMarco[1], Kedric L. Milholland[1], Amanda L. Pendleton[1,4], John J. Whitney[1], Peipei Zhu[1,2], Daniel T. Wesenberg[1], Monessha Nambiar[2], Antonella Pepe[3,†], Stefan Paula[1,2,‡], Jean Chmielewski[2,3], Jennifer H. Wisecaver[1,4], W. Andy Tao[1,2,3], Mark C. Hall[1,3]

[1]Department of Biochemistry, [2]Department of Chemistry, [3]Center for Cancer Research, and [4]Center for Plant Biology, Purdue University, West Lafayette, IN 47907

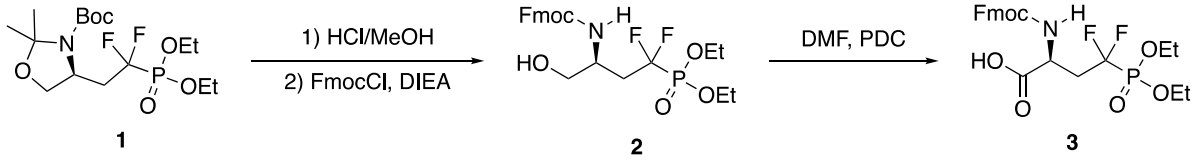
[†]Current Address: Institute of Chemical Biology and Drug Discovery, Stony Brook University, Stony Brook, NY, 11794-3400
[‡]Current Address: Department of Chemistry, California State University, Sacramento, 6000 J Street, Sacramento, CA 95819

Contents:

SUPPLEMENTARY METHODS

pCF$_2$Ser synthesis.

pCF$_2$Ser was prepared as shown in Scheme 1 and following synthetic protocols published by Berkowitz *et al.* [59] and Arrendale *et al.* [49].



Scheme 1:Synthesis of non-hydrolyzable phosphonate analog of phosphoserine

General procedure

tert-Butyl (S)-4-(2-(diethoxyphosphoryl)-2,2-difluoroethyl)-2,2-dimethyloxazolidine-3-carboxylate (**1**) was prepared as reported by Berkowitz *et al.* [49]. All reagents were used as obtained from commercial sources and used directly. ^{1}H and ^{13}C NMR spectra were recorded on Bruker 500 MHz Avance III HD spectrometer. ^{1}H chemical shifts values (δ) are referenced to the residual nondeuterated components of the NMR solvents (δ = 7.26 ppm for $CHCl_3$ and δ = 2.50 ppm for DMSO-d_5. The ^{13}C chemical shifts (δ) are referenced to $CDCl_3$ (central peak, δ = 77.0 ppm), DMSO-d6 (central peak, δ = 39.5 ppm). Signals are listed in ppm, and multiplicity identified as s = singlet, br = broad, d = doublet, t = triplet, q = quartet, m = multiplet. Automated flash chromatography was performed on Yamazen W-Prep 2XY using Yamazen Universal Premium silica gel (30 mm 60 Å) columns. Analytical thin-layer chromatography (TLC) was carried out on silica gel 60 F254 aluminum plates and eluted plates were visualized with nihydrin stain. Mass spectral data were obtained from the Purdue University Metabolite Profiling Facility, West Lafayette, IN on an Agilent 6550 QTOF mass spectrometer coupled to an Agilent 1200 series high-performance liquid chromatography system.

Synthesis of (9*H*-fluoren-9-yl)methyl (*S*)-(4-(diethoxyphosphoryl)-4,4-difluoro-1-hydroxybutan-2-yl)carbamate (2) [59]

tert-Butyl (*S*)-4-(2-(diethoxyphosphoryl)-2,2-difluoroethyl)-2,2-dimethyloxazolidine-3-carboxylate (**1**) [59] (1.35 g, 3.37 mmol) was dissolved in anhydrous MeOH (30 mL) and the resulting clear solution was cooled in ice/water bath. Conc. HCl (3.00 mL) was added dropwise with vigorous stirring and then the cold bath was removed and the reaction mixture was stirred at room temperature for 2 hours. Upon disappearance of the starting material, the solvent was evaporated under reduced pressure and water was stripped by azeotropic evaporation with CH_3CN (2x 30 mL) and toluene (1x 30 mL). Dichloromethane (45 mL) was added to the crude mixture, and the solution was cooled in ice/water bath. Then *N*,*N*-diisopropylethylamine (1.76 mL, 10.1 mmol) and fluorenylmethyloxycarbonyl chloride (945 mg, 3.71 mmol) were added. The reaction mixture was stirred for 18 h with slow warming up to room temperature and then it was quenched with a saturated aqueous solution of $NaHCO_3$ (75 mL). The aqueous layer was extracted with CH_2Cl_2 (3 x 90 mL) and the combined organic layers

were washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated and the crude material was purified by automated flash chromatography using a 55 g silica gel column, and a mixture of ethyl acetate in hexanes with a gradient of 20-70% (R_f = 0.3, 100% ethyl acetate). The title compound was isolated as a foaming colorless solid in 51% yield (unoptimized yield, 840 mg).
^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.77 (ddd, *J* = 7.5, 0.89, 0.89 Hz, 2H), 7.60 (ddd, *J* = 7.5, 2.1, 1.1 Hz, 2H), 7.40 (ddd, *J* = 7.5, 7.5, 0.89 Hz, 2H), 7.32 (ddd, *J* = 7.5, 7.5, 1.1 Hz, 2H), 5.38 (d, *J* = 7.7 Hz, 1H), 4.40 (d, *J* = 7.1 Hz, 2H), 4.35 – 4.18 (m, 5H), 4.07 (brs, 1H), 3.77 (bs, 2H), 2.44 (m, 3H), 1.38 (td, *J* = 7.1, 2.1 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 156.1, 143.8, 141.3, 127.7, 127.1, 125.1, 123.5 – 116.7 (m, CF_2), 120.0, 66.9, 64.9 (t, *J* = 6.2 Hz), 64.7, 47.9, 47.2, 35.0 (m), 16.4, 16.37. ^{31}P NMR (203 MHz, Chloroform-*d*) δ 6.48 (t, *J* = 106.4 Hz). Spectral data are in agreement with those reported.

Synthesis of (*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-4-(diethoxyphosphoryl)-4,4-difluorobutanoic acid (3) [49]

Pyridinium dichromate (3.92g, 6.00 equiv.) was added to a colorless, solution of **(2)** (840 mg, 1.74 mmol) in DMF (8.00 mL) and the bright orange suspension was stirred overnight. After 16 h, the reaction mixture appeared as a dark brown suspension and it was diluted with water (70 mL) and ethyl acetate (100 mL). The aqueous layer was separated and extracted with ethyl acetate (3 x70 mL). The combined organic layers were washed with brine and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the resulting yellow oil was purified by flash column chromatography on silica gel. Ethyl acetate in hexanes was used as eluant with a gradient of 10-100% to elute the less polar fractions and then methanol in ethyl acetate with a gradient of 1-5% was used to elute **3**. The title compound was isolated as a white solid in 33% yield (unoptimized yield) [49, 60] (291 mg). The optical purity of **3** was assessed by measuring its specific optical rotation, which was slightly higher than the value reported for the same compound. [49] In addition, **3** was reacted with (*S*)-α-methylbenzylamine and separately with racemic α-methylbenzylamine. Comparing ^{1}H and ^{19}F NMR of the resulting amides it was possible to confirm that **3** was essentially optically pure.
$[\alpha]^{23}_D$= + 5.6 (c = 1.15, $CHCl_3$), lit. $[\alpha]^{25}_D$= + 4.3 (c = 0.923, $CHCl_3$). [49]
^{1}H NMR (500 MHz, DMSO-d_6, 65 °C) δ 7.86 (ddd, *J* = 7.5, 1.0 Hz, 2H), 7.68 (d, *J* = 7.5 Hz, 2H), 7.40 (dd, *J* = 7.5, 7.5 Hz, 2H), 7.30 (ddd, *J* = 7.4, 2.0, 1.0 Hz, 2H), 4.41 – 4.10 (m, 7H), 3.43 – 2.89 (m, 3H), 2.64 – 2.40 (m, 1H), 1.29 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (126 MHz, DMSO, 65 °C) δ 172.9, 156.0, 144.3, 141.2, 128.0, 127.5, 125.6, 121.6 (d, *J* = 47.7 Hz) 120.5, 66.2, 64.7, 48.9, 47.3, 35.6, 16.63, 16.59. ^{19}F NMR (471 MHz, DMSO-d_6, 65 °C) δ -111.98 (ddd, *J* = 293.4, 104.3, 26.1 Hz), -113.29 (dddd, *J* = 297.4, 106.6, 28.7, 10.0 Hz). ^{31}P NMR (203 MHz, DMSO-d_6) δ 6.39 (t, *J* = 103.3 Hz). HRMS (ESI), $[M+Na]^+$ calculated for $C_{23}H_{26}F_2N_1O_7PNa$ 520.1307, found 520.1311. Spectral and analytical data are in agreement with those reported.

SUPPLEMENTARY FIGURE LEGENDS

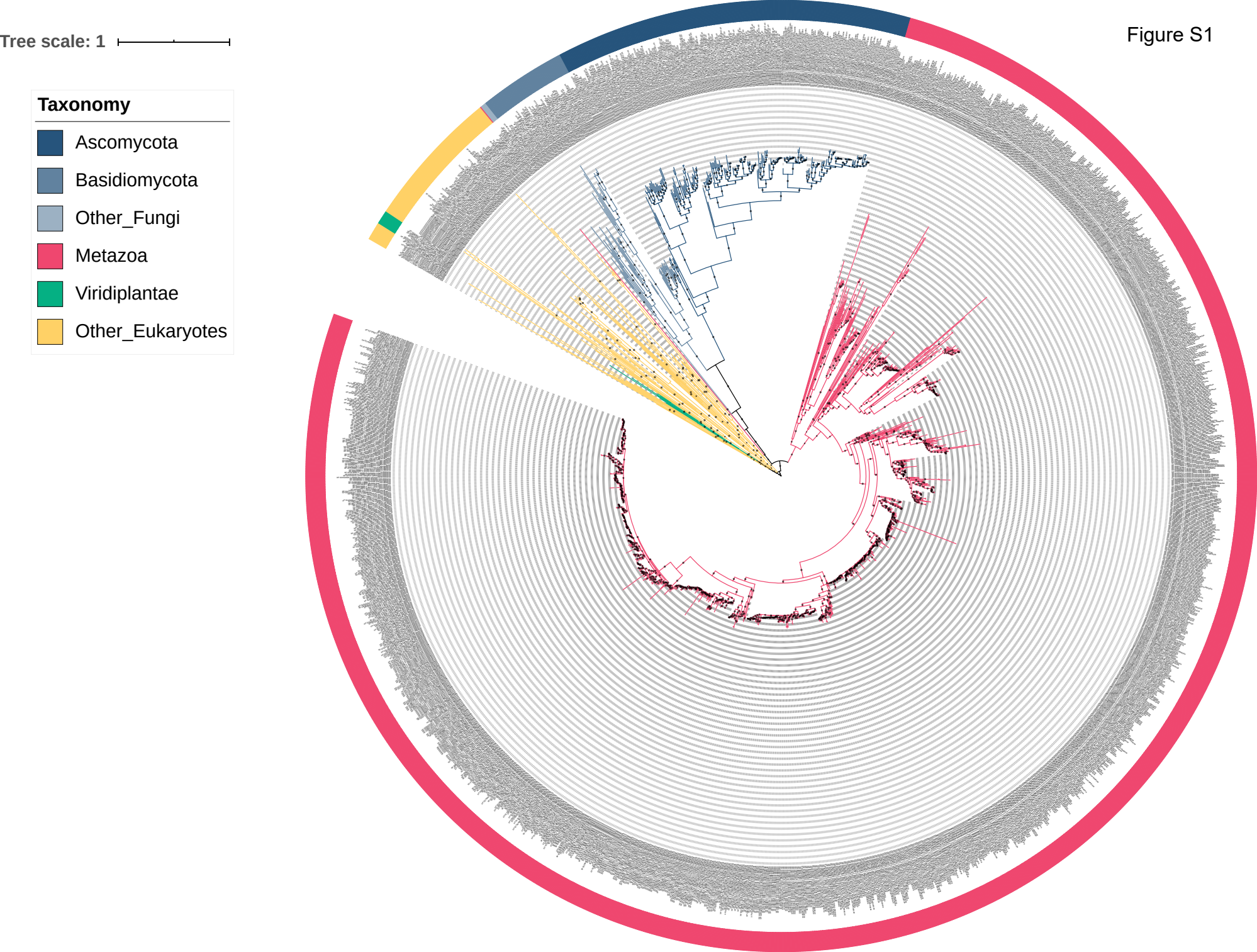
Figure S1. Maximum likelihood phylogeny of Cdc14 sequences. Branches are color coded to indicate their taxonomic association. The size of the grey circles on branches indicate the strength of the SH-like support values; the larger the circle, the greater the support. If no circle is present, the branch has > 0.95 support.

Figure S2. Alignment of Cdc14 homologs from plant fungal pathogens. The expressed sequence of ScCdc14 and Cdc14 homologs from 8 plant pathogenic fungi (initially identified by BLAST using ScCdc14 as query) representing diverse ascomycete and basidiomycete lineages were aligned in Clustal Omega using default settings. The aligned sequences reflect the truncated coding sequences included in the pET15b expression constructs for purification from *E. coli*, with the exception of the sequence from *U. maydis.* The *CDC14* gene from *U. maydis* has an unusual 5' extension, which was also truncated, and the 3' tail following the catalytic domain was truncated at a different site. The truncation sites for UmCdc14 are indicated by the gray highlighting. The common truncation site for the other homologs was chosen based on unpublished data from our lab indicating that all sequence following the conserved QPRK motif at residues 434-437 of ScCdc14 are dispensable for full catalytic activity. Cyan highlighting indicates amino acids contributing to substrate recognition around the Cdc14 active site, as in Figure 2d.

Figure S3. Purification of plant pathogenic fungal Cdc14 homologs. SDS-PAGE analysis, with Coomassie blue staining, of identical amounts of recombinant plant pathogen Cdc14 homolog catalytic domains purified from E. coli by nickel affinity chromatography.

Figure S4. Substrate specificity profile of PsCdc14. Data are equivalent to those for the other homologs in Figure 5A. However, PsCdc14 was recovered at relatively low concentration, apparently due to limited solubility. As a result, we were unable to perform assays at all the same enzyme concentrations used for the other homologs. This prevented the generation of suitable substrate:product ratios for accurate calculation of k_{cat}/K_M for some of the peptides. However, the relative reaction rates are still appropriate for comparison to each other to establish the PdCdc14 specificity profile, which is clearly similar to the other Cdc14 homologs. Data points are the average rates from 3 independent experiments at a single enzyme concentration and error bars are standard deviations.

Figure S1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Basidiomycota
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Metazoa
Viridiplantae
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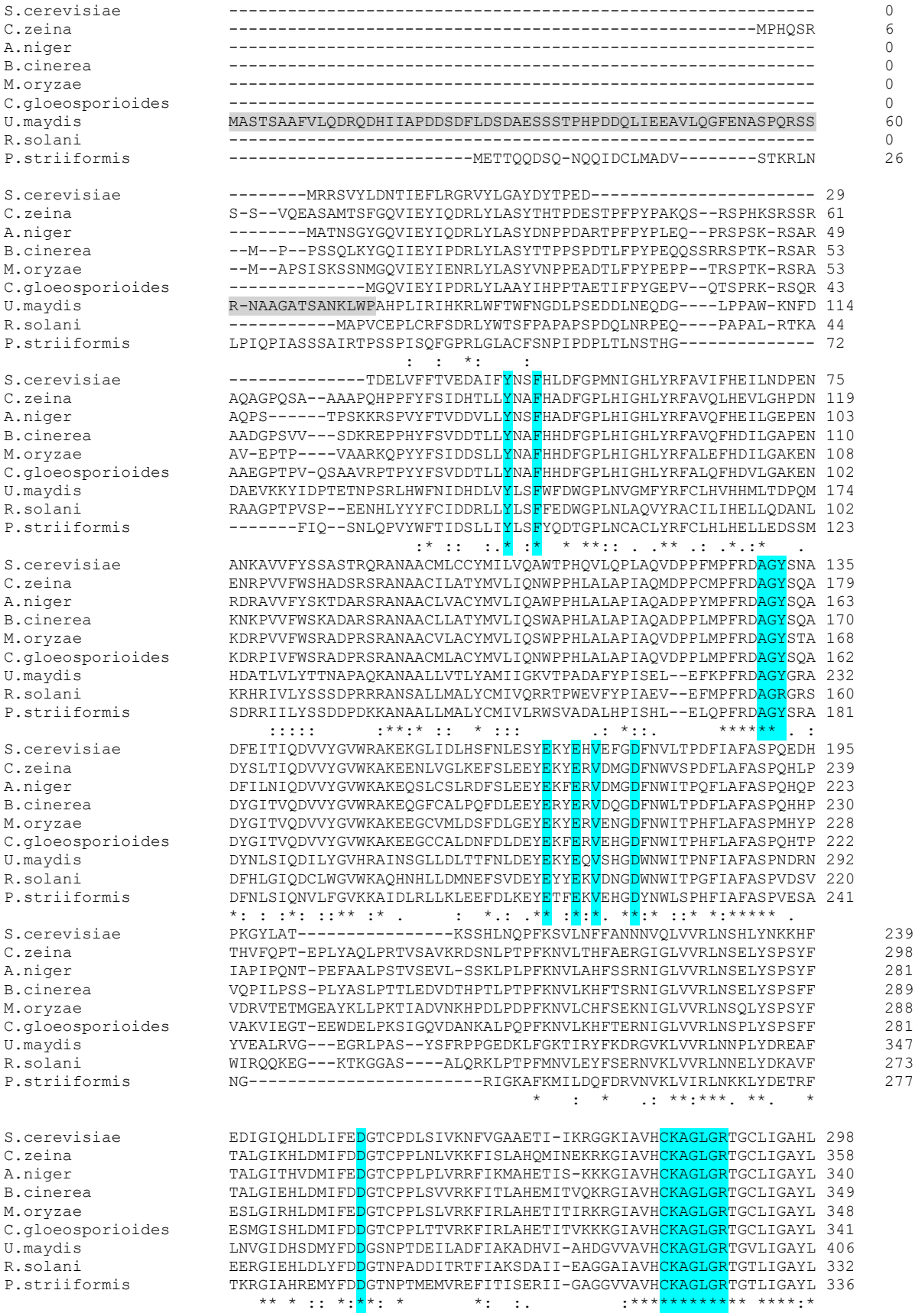
Figure S2


S.cerevisiae
C.zeina
A.niger
B.cinerea
M.oryzae
C.gloeosporioides
U.maydis
R.solani
P.striiformis

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S.cerevisiae           IYTYGFTANECIGFLRFIRPGMVVGPQQHWLYLHQNDFREWKYTTRISLKPSEAIGGLYP       330
C.zeina                IYRYGFTANEVIAFMRFMRPGMVVGPQQHWLHLNQGTFREWWFEDTMREKLMASMQPATP       390
A.niger                IYRYGFTANEIIAFMRFMRPGMVVGPQQHWLHLNQGSFREWWYEDSMKEKLAQMQAA---       372
B.cinerea              IYRYGFTANEIIAYMRFMRPGMVVGPQQHWLHLNQGTFREWWIEEQFEIRMKEKLANMAP       381
M.oryzae               IYRHGFTADEVISYMRFMRPGMVVGPQQHWLHLNQGVFREWWVEERVARKLRKELAAAAQ       380
C.gloeosporioides      IYRHGFTANEIIAFMRFMRPGMVVGPQQHWLHINQGIFREWWIEERIERKLRKEMAA---       373
U.maydis               VWKHGFSAGEAIGFMRFMRPGCVVGPQQHFMYQNFAEWIKWGVRDHAMKEARQLIAEERT       438
R.solani               IYKYGFTASEAIGFMRIVRPGSVVGPQQQFMYMKQLEWAKWAAIDEIQRAEAQRVEVP--       364
P.striiformis          IYKYRFTAEEAVGFMRIMRPGTCVGPQQHFLYENQLTWIEWSARDELLAEQQAQISPSKT       368
                       :: : *:* * :.::*::***  *****:::: :   : :*

S.cerevisiae           LISL---EEY--------------RLQKKKLK----DDKRVAQNNIEGELRDLTMTPPS       396
C.zeina                TRLQ-----------------------------------QSNSRKLAS--NGQTFTPPN       440
A.niger                -PIT---PG---------------------RPT----TRQR-------A--NGPVATPPN       419
B.cinerea              VTPR---KG---------------------HYT-------------------SKSQVVTP       426
M.oryzae               NTAN---TG---------------------VPS----TPIRAMQKASLG--RTSATQAST       438
C.gloeosporioides      ---N---AA---------------------VPS----TPIRAMQKTSLR--NGQASTPPH       425
U.maydis               KLEAATAAARAAAAAAEAANAATAAVALPMIPTTRASTKRRAAGSDAAKCAEADRFDQPD       526
R.solani               -TPRTP-PE---------------EIQPMTTGSTNGTSSSAVRSGNSAAPSSPRATTPP       432
P.striiformis          ERPITPPPE---------------NLAPGITIS-----------------STTSTPPP       422

S.cerevisiae           NGHG--------A----LSARNSSQP---STAN--------------------------       414
C.zeina                GDRH--------Q----TSPRRALGE---ITNNEGA-----------------------       461
A.niger                N-----------G----HSKRAALGE---IDHNEG-----------------------       436
B.cinerea              P-----------N----GNQRTPLGE---VDNERS-----------------------       443
M.oryzae               P-----------P----SKSRTPLGE---MDTES-----------------------       454
C.gloeosporioides      R-----------S----TSNRTPLSE---VDHDR-----------------------       441
U.maydis               ADVSLDEHIKHAPQLSRNTRRKPTSEHSDSDDDEGELQRPRQKRKSAAAAAAAAAAAAAA       586
R.solani               G-------LMSSPVI---------------------------------------------       440
P.striiformis          K-------SHMAPTM---------------------------------------------       430

S.cerevisiae           ----------------------NGSNSFKSSAVPQTSPGQPRKGQNGSNTIEDIN     447
C.zeina                PASTYTD-------------HMKSSVGVADENLPAPTPGQPRKTNKVYGGRR---     500
A.niger                ------------------------A-GTQAEEYLPAPTPGQPRKSHRKDSRHH---     464
B.cinerea              ------------------------HSIGAQEDYLPAPTPGQPRKTGRIDR--HH--     471
M.oryzae               ------------------------NTVGVQEDYLPAPTPGQPRKTGRNPSGRHH--     484
C.gloeosporioides      ----------------------NNIGAQEDYLPAPTPGQPRKTNRAGADRHH--     471
U.maydis               AAADYIDGSGPVTPIAQRASSRAVPASAAPVSKPAPCVGQPRKSPSPSRKR----     637
R.solani               -----NGGAVPVTPHKARP--------TTPIKT-DAPVLQPRKTPAAKRA-----     476
P.striiformis          -----PTSAVPG-----------------------------QPRKTPGAKTRH----     449
                                                               ****
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Figure S3

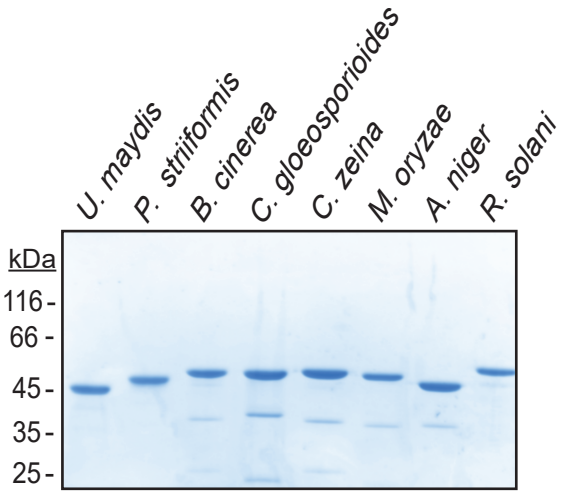
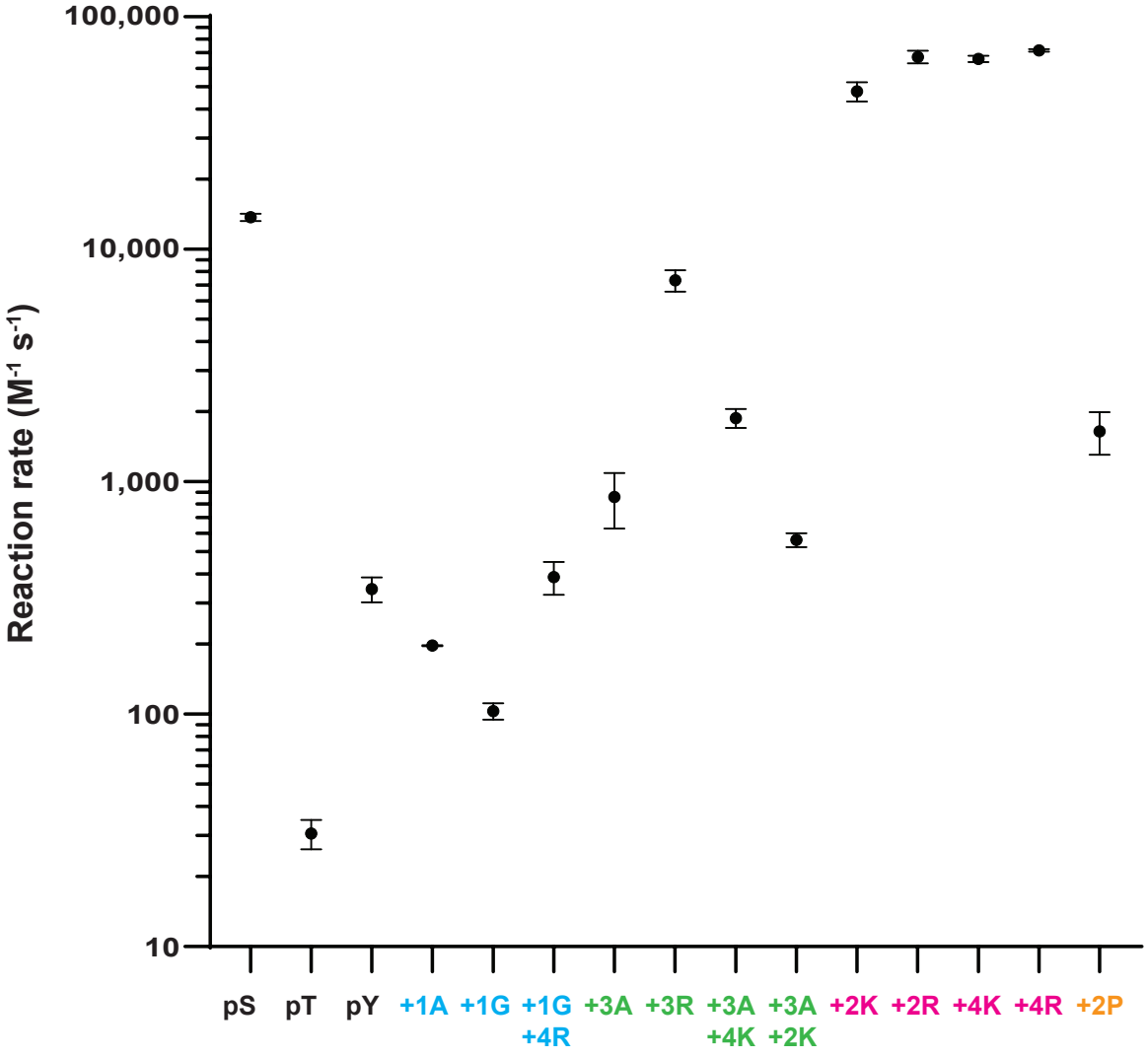

U. maydis
P. striiformis
B. cinerea
C. gloeosporioides
C. zeina
M. oryzae
A. niger
R. solani
kDa
116
66
45
35
25

Figure S4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1,000
100
10
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pT
pY
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+1G
+1G +4R
+3A
+3R
+3A +4K
+3A +2K
+2K
+2R
+4K
+4R
+2P