Supplementary material (ESI) for Chemical Communications

Supporting Information for *Equisetin biosynthesis in* Fusarium heterosporum

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Experimental Procedures

Strains and Culture Conditions

F. heterosporum ATCC 74349 was maintained on Difco potato dextrose agar (PDA) and grown in Difco potato dextrose broth (PDB). Production of equisetin was achieved by growing *F. heterosporum* for 3 weeks (27 ^{o}C) on semi-solid corn grit agar (CGA): corn grits (Arrowhead Mills; 200 g), Bacto agar (10 g), H_2O (100 ml).

Purification of Equisetin

Equisetin was purified from 3-week-old *F. heterosporum* on corn grits (200 g) following a previously reported procedure,[1] except that a C_{18} Sep Pak (Waters) was used as the final step with a methanol/water gradient. Equisetin: colorless powder; $[\alpha]_D$ –147.8; ^{13}C NMR [$CDCl_3$, 25 °C] 190.5, 177.0, 130.9, 130.0, 127.0, 126.6, 77.3, 77.0, 76.7, 70.2, 66.8, 66.3, 65.1, 60.5, 60.2, 48.7, 45.0, 42.2, 39.9, 38.6, 38.5, 35.7, 34.1, 33.5, 31.9, 29.7, 29.3, 29.1, 28.3, 27.3, 24.9, 22.4, 17.9, and 14.1; CIMS (isobutene) *m/z* = 374.5 (calcd for $C_{22}H_{32}NO_4$ 374.2).

Bioassay Conditions

B. subtilis ATCC 6633 was grown on Difco Antibiotic I plates. Crude extract and pure equisetin (1 ng-1 mg) were loaded onto 6 mm paper disks, and zones of inhibition were measured after three days of growth (30 °C).

Genomic DNA and Cosmids

F. heterosporum samples were grown for three days in PDB (50 ml) in a rotary incubator (30 °C, 200 rpm). Mycelia were harvested by centrifugation (3550x*g*) and the resulting pellet was lyophilized. The dried pellet was ground under liquid nitrogen, and DNA was extracted following a mammalian tissue protocol from Sambrook and Russell.[2] A cosmid library was constructed in the filamentous fungal vector pMOcosX.[3] Genomic DNA was digested to ~40 kbp size with Sau3AI and ligated to an XhoI digested, dephosphorylated, partially end-filled pMOcosX. The ligation mix was packaged with the Gigapack III XL packaging kit (Stratagene), and clones were selected for ampicillin resistance.

cDNA Preparation

mRNA was prepared from *F. heterosporum* samples grown on CGA and PDA. Fungal mass was peeled from the plates, ground in liquid nitrogen, and extracted following the method of McCloskey *et al*.,[4] except that a 4 h incubation was used. The resulting enriched RNA was processed with the RNeasy Kit (Qiagen). The mRNA was then further purified using the Oligotex Kit (Qiagen), and cDNA was produced with Thermoscript reverse transcriptase (Invitrogen).

PCR for PKS Genes

Partially reducing fungal PKS genes were identified in GenBank and aligned with ClustalX. CODEHOP was used to design random primers that were situated over conserved regions of the KS and AT domains: EQCH F (5'-CAAGTGCTAATGGTTATGCTAGAGGNGARGSNRT); and EQCH R (5'

CCACACCATTGAGCACCTTGNCCNGTRAA). These primers were then used for PCR with *F. heterosporum* DNA to yield a ~1 kbp product, which was cloned with the pCR2.1-TOPO kit (Invitrogen). Resulting colonies were screened by RFLP analysis and by sequencing.

RT-PCR

Primers were designed to detect the three reducing PKS homologs: *pks1* (pks1F-5' GGGTGTCCTCGTGTTGAAACG, pks1R-5' CGCCTCTGACTGTAGCCACTGTC); *pks2* (pks2F-5' TGCTTCCGTCGTCCTCAAGA, pks2R-5' TGGCTCTTTTGACTCCTCCATCT); and *pks3* (pks3F-5' GGATAAAAGATGGTCGGTCCTTG, pks3R-5' TGTTGCTCAGGCTTGGGAAG). These primers were used to screen cDNA from *F. heterosporum* grown on CGA and PDA.

***eqi* Cluster Identification and Sequencing**

pks2-containing clones were identified by arrayed library screening,[5] in which 10 clones per well were grown up in 96-well plates. Columns and rows were pooled to yield 20 samples from each plate. Cosmids or fosmids were purified from these samples by Miniprep (Qiagen) and screened by PCR using the *pks2* primer set. A cosmid, designated F4, was sequenced by a shotgun method (2x coverage), and the resulting gaps were filled by cosmid walking.

Transformation of *F. heterosporum*

A 2.5 kbp XbaI fragment from the N-terminus of *eqiS* was ligated to the XbaI / SpeI site of pCR2.1 (Invitrogen). The hygromycin resistance cassette was excised from vector AN26 with XbaI / AvrII and ligated into an AvrII site in the middle of the *eqiS* fragment, to create pKOE. *F. heterosporum* was transformed to hygromycin resistance using a protoplasting method developed by Shim *et al.*,[6] except that a different growth media were used (PDB and PDA) and protoplasts were formed in a different lysis buffer: 0.8N NaCl (20 ml), 1M NaH_2PO_4 (400 μl), Lysing enzyme (Sigma) (1 g), and Yatalase (TaKaRa) (100 mg). Vector pKOE (10 μg) was linearized with XbaI / BamHI and added to the PEG solution for a 50-min incubation. Colonies displaying strong mycelial growth on sucrose plates and wild-type growth rates on PDA supplemented with hygromycin B (150 μg/ml) were used for further studies.

Chemical Analysis of Transformants

Purified equisetin and acetone extracts of *F. heterosporum* wild-type and mutants were used for all chemical experiments. For TLC, equisetin and extracts were spotted onto aluminum-backed TLC plates, and compounds were resolved with 50/50 hexane/ethyl acetate containing a few drops of acetic acid. Equisetin was observed by UV and using an $FeCl_3$ (0.5 g) solution (H_2O:ethanol 1:1, 50 mL).

DNA Analysis of Transformants

Transformants were analyzed by PCR using primers derived from the *hyg* cassette and from *eqiS*. For Southern blotting, DNA (~5 μg) of each transformant and wild type was digested with XbaI. After electrophoresis on 1% agarose, DNA was transferred via Turboblotter to a NYTRAN SuPerCharge membrane (Schleicher & Schuell) and crosslinked (Stratalinker). Probes were synthesized using undisrupted regions of *eqiS* and *hygR* with the Klenow fragment (Promega) following the manufacturer's Klenow labeling protocol. Hybridization was then achieved following standard protocols.[2]

Phylogenetic Analysis. Sequences from GenBank and genome sequencing projects were aligned in ClustalX,[7] and gapped or poorly aligned regions were excised. Trees were generated by neighbor-joining and 1,000x boostrapping in ClustalX. Trees were viewed using Phylodendron (http://www.es.embnet.org/Doc/phylodendron/treeprint-form.html).

References

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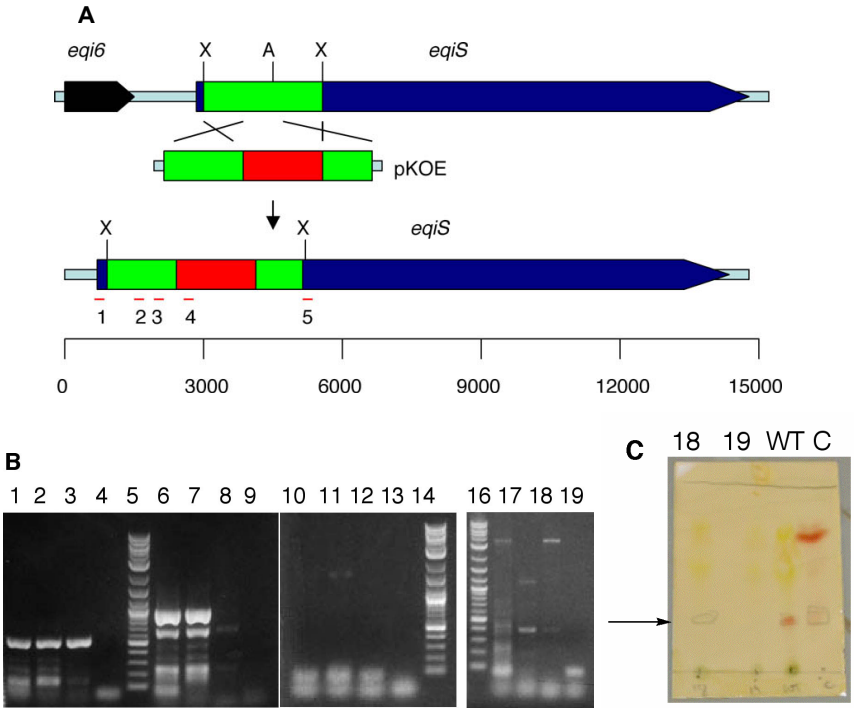
eqi predicted open reading frames and protein functions

Protein	Amino acids	Proposed Function	Sequence similarity	Identity/Similarity	Accession #
Eqi1	747	Monooxygenase	Hypothetical Protein; *M. grisea* MG00031.4	37/54 %	EAA48373.1
Eqi2	490	Metal-dependent hydrolase	Hypothetical Protein; FG11158.1 *G. zeae*	38/59 %	EAA75368.1
Eqi3	452	Dimethylallylpyrophosphate transferase	Hypothetical Protein; *A. nidulans* AN6784.2	39/58%	EAA58602.1
Eqi4	247	Methyltransferase	Hypothetical Protein; *L. maculans*	36/50%	AAR11079
Eqi5	528	Cytochrome P450 monooxygenase	Hypothetical Protein; MG08494.4 *M. grisea* 70-15	48/67%	EAA49579.1
Eqi6	511	ABC transporter	Hypothetical Protein; AN7972.2 *A. nidulans* FGSC A4	49/65%	EAA59626.1
EqiS	3953	Equisetin synthetase hybrid PKS-NRPS	Hypothetical Protein; *A. nidulans* FGSC A4 AN8412.2	34/52%	EAA67034.1
Eqi7	355	unknown	Unknown *F. sporotrichioides*	92/96%	AAO27747.1
Eqi8	642	unknown	Hypothetical Protein; FG02689.1 *G. zeae* PH-1	48/65%	EAA67554.1
Eqi9	335	Zn oxidoreductase	Hypothetical Protein; *G. zeae* FG03515.1	76/86%	EAA72481.1
Eqi10	1314	Ankyrin-repeat domain containing protein	Hypothetical Protein; AN3543.2 *A. nidulans* FGSC A4	24/44%	EAA58868.1

Mutational Analysis



A. Construction of a hygromycin resistance vector for insertional mutagenesis. X and A indicate XbaI and AvrII sites, respectively. pKOE consists of a hyg cassette (red) and a homologous region (black). Primer regions used to detect insertion events are shown in red and numbered 1-5. B. PCR of mutants JWS 18 (lanes 1, 6, 10, 16) and JWS 19 (lanes 2, 7, 11, 17), wild type (lanes 3, 8, 12, 18), and no DNA controls (lanes 4, 9, 13, 19). 2-log ladders (100 bp-10 kbp) are in lanes 5, 14, 15. Primers used are (from Fig. 2A) 2-3 for lanes 1-4; 2-4 for lanes 6-9; 1-4 for lanes 10-13; and 1-4 for lanes 16-19. Lanes 1-4 show that the *eqiS* sequence is present in all strains, while lanes 6-9 show that hyg-resistant colonies (but not wild type) contain the pKOE fragment. Lanes 10-13 show that JWS19 contains a ~1.5 kbp band for a specific integration of pKOE into *eqiS*, while JWS18 is a nonspecific integrant. Lanes 16-19 reveal that a ~4 kbp band for primers 2-4 is present in JWS18 and wild type, but no band is present in JWS19, since following specific insertion the product could not be obtained in several PCR reactions. C. TLC showing equisetin production (arrow) by wild type and JWS 18, but not by JWS19. 18 = JWS18, 19 = JWS19, WT = wild type, C = enriched equisetin control. TLC plate was stained with $FeCl_3$.