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NOTE

Cloning of *Sal1*, a scytalone dehydratase gene involved in melanin biosynthesis in *Cochliobolus heterostrophus*

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Abstract A melanin biosynthetic gene, *Sal1*, in the southern corn leaf blight fungus *Cochliobolus heterostrophus*, was identified by degenerate and inverse PCR. Structural analysis revealed that the open reading frame is 555 bp in length, interrupted by one intron. Melanin deficiency of the *sal1* mutant strain, which accumulates the intermediate scytalone, was successfully complemented by introducing the gene fragment. These results suggest that *Sal1* is a scytalone dehydratase gene of *C. heterostrophus*.

Keywords *Bipolaris maydis* · *Cochliobolus heterostrophus* · Color mutant · 1,8-Dihydroxynaphthalene · SCD

Many ascomycetous fungi produce melanin (Bell and Wheeler 1986), a polymer of 1,8-dihydroxynaphthalene (DHN). This secondary metabolite is considered to be important for their survival and essential for host infection by plant or animal pathogenic fungi (Bell and Wheeler 1986; Sussman 1986; Langfelder et al. 2003).

Recent molecular genetic and genome-based studies have indicated that there are likely more than two different biosynthetic pathways for 1,8-DHN in ascomycetous fungi. For example, a rice blast fungus, *Magnaporthe grisea*, and a cucumber anthracnose, *Colletotrichum orbiculare* (syn: *C. lagenarium*), produce 1,8-DHN via the pentaketide, 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN), whereas a human pathogen, *Aspergillus fumigates*, produces 1,8-DHN via a heptaketide, naphthopyrone (Howard and Valent 1996; Watanabe et al. 2000; Tsai et al. 2001; Tsuji et al. 2003). To understand the melanin biosynthetic pathways in ascomycetous fungi, additional studies on the genes, metabolites, and mutant strains associated with melanin biosynthesis are required.

A southern corn leaf blight fungus, *Cochliobolus heterostrophus*, also produces DHN melanin (Tanaka et al. 1991). Seven genes, i.e., *Alb1*, *Alb3*, *Brn1*, *Cal1*, *Cal2*, *Pgr1*, and *Sal1*, have been identified that are involved in melanin biosynthesis in *C. heterostrophus* using melanin-deficient chemical mutants (Tanaka et al. 1991; Shimizu et al. 1996). Shimizu et al. (1997) also determined a nucleotide sequence of the *Brn1* gene and revealed that the gene encodes a 1,3,8-trihydroxynaphthalene reductase. However, there still remain six genes to be identified and functionally analyzed.

A *sal1* mutant forms a salmon-colored colony (Tanaka et al. 1991). Chemical analyses revealed that the *sal1* mutants accumulated scytalone, an intermediate of DHN melanin biosynthesis (Tanaka et al. 1991). In this pathway, scytalone is converted to 1,3,8-trihydroxynaphthalene by a scytalone dehydratase. The *Sal1* gene is therefore predicted to encode a scytalone dehydratase.

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To determine the structure and function of *Sal1*, we cloned a putative scytalone dehydratase gene from *C. heterostrophus*. Genomic DNA was isolated from *C. heterostrophus* wild-type strain HITO7711 (Tanaka et al. 1991) by the method of Nakada et al. (1994). We used degenerate primers ScdFW1 (5′-TACGAGTGGGCNGACWSCTAYGA-3′) and ScdREV1 (5′-AACTTCCASACGCCGTYGAYCTT-3′), which were designed based on highly conserved regions among fungal scytalone dehydratases, i.e., YEWADSYD and KIDGEWKF, respectively, and amplified to an approximately 340-bp fragment. The amplified fragment was cloned into the *Eco*RV site of pZErO-2 vector (Invitrogen). The inserted fragments were sequenced using primers M13-20 (5′-CGACGTTGTAAAACGACGGCCAGT-3′) and M13-RVM (5′-GAGCGGATAACAATTTCACACAGG-3′). The 5′- and 3′-flanking regions of the obtained sequence were amplified by inverse polymerase chain reaction (PCR) (Ochman et al. 1988) using a restriction enzyme *Eco*RI and the primer pair Sal1invF (5′-TTGTCCAAGAAGGACCGGTAGTCGAT-3′) and Sal1invR (5′-TTCAACACGCACTGGTACAAGAAGAT-3′), and an entire sequence of this gene was determined by primer-walking analysis. To map an intron within this gene, we also performed poly(A)$^+$ RNA isolation and reverse transcriptase (RT)-PCR by the method described in Yoshimi et al. (2004). A primer 5′-CGACGGCCAGTGCCAAGCTTTTTTTTTTT-3′ was used for the first-strand cDNA synthesis, and a primer 5′-CGACGGCCAGTGCCAAGC-3′ as an adaptor primer.

Our results show that the open reading frame (ORF) of this gene is composed of 552 bp and is interrupted by a 60-bp intron (Fig. 1). The putative polypeptide sequence of this gene compared with those of other fungal scytalone dehydratases were 49% homologous with ARP1 of *Aspergillus fumigatus* (Tsai et al. 1999), 98% with SCD1 of *Bipolaris oryzae* (Kihara et al. 2004), 74% with SCD1 of *Colletotrichum orbiculare* (Kubo et al. 1996), and 74% with OSD1 of *Ophiostoma floccosum* (Wang et al. 2001). We deposited this sequence in DDBJ/EMBL/Genbank under accession no. AB587821.

To confirm whether this putative scytalone dehydratase gene is functional and identical to *Sal1*, we introduced the entire region of this gene into a *sal1* mutant strain (Fig. 2). We amplified an approximately 2.8-kb fragment of this gene including 1.2-kb 5′- and 1.0-kb 3′-regions with primers Sal1-f0 (5′-CAGGAGCCTCTACTGTATATTCAATCA-3′) and Sal1-r0 (5′-GCCCAGCCAAGCTGACAGAGACAT-3′), using the PrimeStar GXL DNA polymerase (Takara). The amplified fragment was cloned into the *Eco*RV site of a plasmid pCB1004 containing a hygromycin B phosphotransferase (*Hph*) gene cassette as a selection marker (Carroll et al. 1994). The resulting plasmid pCBSal1 was used for transformation of the *C. heterostrophus sal1* mutant strain 823-1. Transformation experiments were performed using the method described by Izumitsu et al. (2007). We obtained three transformants: CSal-1, CSal-2, and CSal-3.

Colonies of the wild-type, the *sal1* mutant, and the resultant transformant are shown in Fig. 3a. A *sal1* mutant 823-1 formed a salmon-colored colony; the resultant transformant CSal-1 formed a dark green colony similar to the wild-type strain. The other transformants, CSal-2 and CSal-3, also formed dark green colonies (data not shown). This result suggests that DHN melanin biosynthesis was restored in transformants. To confirm whether the recovery of melanization in CSal-1 was caused by the integration of the putative scytalone dehydratase gene, we also performed PCR analysis using primers Hph-FW (5′-GTGCTTCTCAAATGCCTGAG-3′) and Hph-REV (5′-CGAAGAACGTTTTCCAATG-3′), which anneal the region within the *Hph* cassette of the plasmid (Fig. 2). Amplicon of the *Hph* gene was detected from a genomic DNA obtained from CSal1-1 strain whereas no amplicons were detected from the DNA of wild-type and *sal1* mutant strains. To check whether DNAs isolated from these three strains are amenable to PCR amplification, we also amplified a region within the *C. heterostrophus* glyceraldehyde-3-phosphate dehydrogenase gene *gpd1* (GenBank accession number X63516) using the primer pair GpdFW1 (5′-ACATGCTCAAGTATGACAGCACACA-3′) and GpdREV1 (5′-TTGTCGTACCAGGAGACGAGCTTGA-3′). We obtained amplicons of *gpd1* (predicted size, 861 bp) from all three strains (Fig. 3b). These results indicate that abnormal melanin deficiency (i.e., accumulation of scytalone) of *sal1* mutant strain 823-1 was recovered by the integration of the putative scytalone dehydratase sequence in the strain CSal-1. Our results also suggest that this putative scytalone dehydratase gene is identical to *Sal1*.

Eliahu et al. (2007) recently reported that the transcription factor Cmr1, involved in melanin biosynthesis, regulates the expression of *Scd1* (putative scytalone dehydratase), *Brn1*, and *Brn2* (putative tetrahydroxynaphthalene reductase) in *C. heterostrophus*. The predicted amino acid sequence of *Sal1* gene in our study is identical (185 aa/185 aa) to that of *Scd1* (DDBJ/EMBL/Genbank accession nos. EF060261 and ABK63478). Our results provide new evidence that *Sal1*/*Scd1* encodes a functional scytalone dehydratase and has essential roles for melanin biosynthesis in *C. heterostrophus*. Our results, combined with previous reports (Shimizu et al. 1997; Eliahu et al. 2007), indicate that *C. heterostrophus* biosynthesizes 1,8-DHN via scytalone by scytalone dehydratase *Sal1*/*Scd1* and 1,3,8-trihydroxynaphthalene reductase *Brn1*.

In fungi, genes encoding proteins involved in the same metabolic process, especially secondary metabolisms, are often clustered (Keller and Hohn 1997). In *A. fumigatus*,

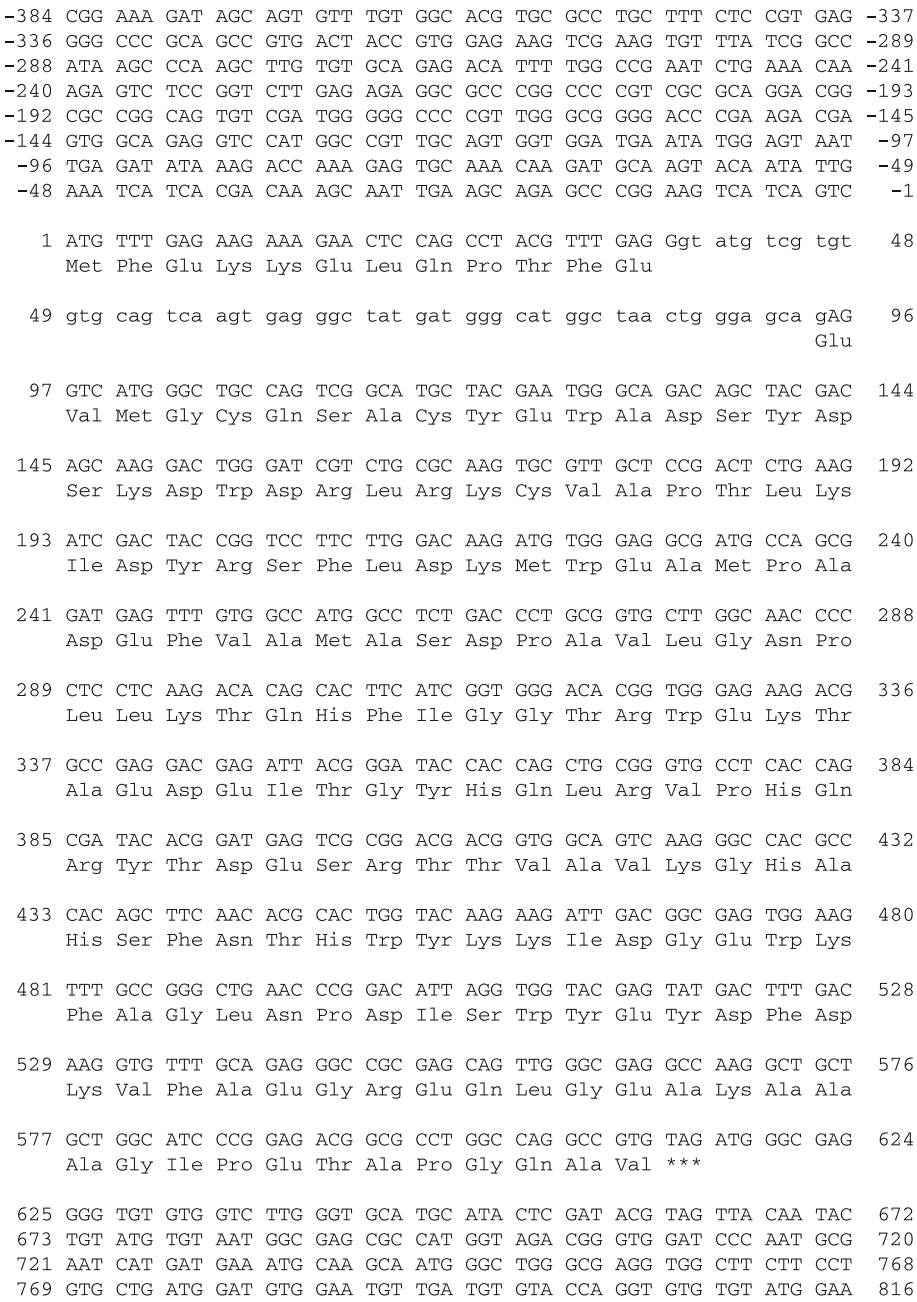

Fig. 1 Nucleotide and deduced amino sequences of the *Cochliobolus heterostrophus* scytalone dehydratase gene *Sal1*. An intron is indicated by *lowercase letters*

genes encoding polyketide synthase (PKS) for naphthopyrone, scytalone dehydratase, tetrahydroxynaphthalene reductase, polyketide-shortening enzyme, and DHN melanization class metallo-oxidase and laccase are clustered within a 19-kb chromosomal region (Tsai et al. 1999). In *C. heterostrophus*, different from the case of *A. fumigatus*, only three genes (*Alb1*, *Alb3*, *Brn1*) are located together (Tanaka et al. 1991). Genome sequences of *C. heterostrophus* revealed that genes encoding trihydroxynaphthalene reductase (*Brn1*), melanin-specific transcription factor (*Cmr1*), and PKS (*Pks18*) are located on a 30-kb chromosomal fragment (http://genome.jgi-psf.org/cgi-bin/browserLoad/?db=CocheC5_1&position=scaffold_10:1065532-1094452). This result implies that *Alb1* and *Alb3* would be synonymous with *Pks18* and *Cmr1*, respectively. Another chromosomal linkage of the melanin biosynthetic genes of *C. heterostrophus*, identified by crossing experiments, has demonstrated that *Sal1* and *Pgr1* (involved in DHN oxidation) are in the same linkage group (Tanaka et al. 1992). Saitoh et al. (2009, 2010) reported that two genes, *ChMco1*, encoding DHN melanization class metallo-oxidase, and *ChCcc2*, encoding heavy-metal ATPase, which delivers copper ions to ChMco1 and laccases, play roles in DHN oxidation. Genome sequences of *C. heterostrophus*

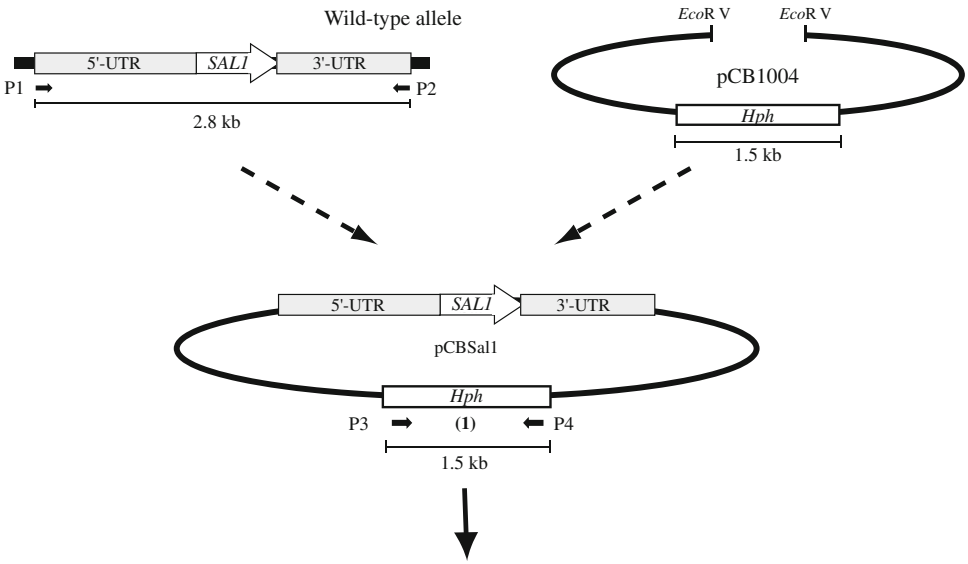

Fig. 2 Construction scheme of a plasmid pCBSal1 containing a 2.8-kb fragment of *Sal1* ORF with its 5′- and 3′-untranslated regions (*UTR*), and the hygromycin B phosphotransferase (*Hph*) gene cassette as a selection marker. *Arrows* show primers used in this experiment. Primer codes: *P1*, Sal1-f0; *P2*, Sal1-r0; *P3*, Hph-FW; *P4*, Hph-REV

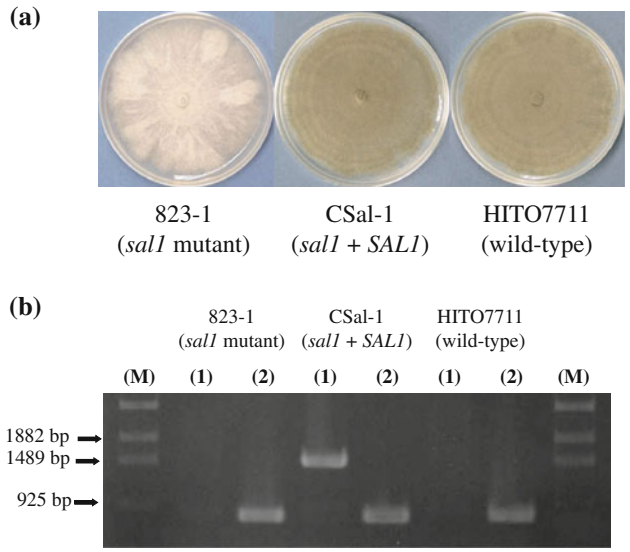

Fig. 3 **a** Colonial growth of *823-1*, *CSal-1*, and *HITO7711* on cornmeal agar (CMA) 10 days after inoculation. **b** Electrophoresis of polymerase chain reaction (PCR) products from the *sal1* mutant 823-1, *SAL1*-complemented strain CSal-1, and wild-type strain HITO7711 using the primer pair *(1)* specific to sequences of the *Hph* gene within the pCBSal1 (see Fig. 2). The primer pair *(2)* that is specific to a *C. heterostrophus* glyceraldehyde-3-phosphate dehydrogenase gene *Gpd1* and amplifies an approximately 0.9-kb fragment was also used to check the quality of DNA samples used in this experiment. λDNA *Sty*I digestions were used as size markers (*lanes M*)

also elucidated that *ChCcc2*, but not *ChMco1*, is located near *Sal1*, 40.6 kb apart on the same scaffold (*ChMco1* http://genome.jgi-psf.org/cgi-bin/browserLoad/?db=CocheC5_1&position=scaffold_9:1164348-1166362; *ChCcc2*: http://genome.jgi-psf.org/cgi-bin/browserLoad/?db=CocheC5_1&position=scaffold_28:132324-136022; *Sal1*/*Scd1* http://genome.jgi-psf.org/cgi-bin/browserLoad/?db=CocheC5_1&position=scaffold_28:172171-172973). This result also suggests that *Pgr1* is likely identical with *ChCcc2*. However, further evidence from genetic complementation or by sequence elucidation of mutant alleles is needed to address the hypothesis.

References

Bell AA, Wheeler MH (1986) Biosynthesis and function of fungal melanins. Annu Rev Phytopathol 24:411–451

Carroll AM, Sweigard JA, Valent B (1994) Improved vectors for selecting resistance to hygromycin. Fungal Genet Newsl 41:22

Eliahu N, Igbaria A, Rose MS, Horwitz BA, Lev S (2007) Melanin biosynthesis in the maize pathogen *Cochliobolus heterostrophus* depends on two mitogen-activated protein kinases, Chk1 and Mps1, and the transcription factor Cmr1. Eukaryot Cell 6:421–429

Howard RJ, Valent B (1996) Breaking and entering: host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. Annu Rev Microbiol 50:491–512

Izumitsu K, Yoshimi A, Tanaka C (2007) Two-component response regulators Ssk1p and Skn7p additively regulate high-osmolarity adaptation and fungicide sensitivity in *Cochliobolus heterostrophus*. Eukaryot Cell 6:171–181

Keller NP, Hohn TM (1997) Metabolic pathway gene clusters in filamentous fungi. Fungal Genet Biol 21:17–29

Kihara J, Moriwaki A, Ueno M, Tokunaga T, Arase S, Honda Y (2004) Cloning, functional analysis and expression of a scytalone dehydratase gene (*SCD1*) involved in melanin biosynthesis of the phytopathogenic fungus *Bipolaris oryzae*. Curr Genet 45:197–204

Kubo Y, Takano Y, Endo N, Yasuda N, Tajima S, Furusawa I (1996) Cloning and structural analysis of the melanin biosynthesis gene *SCD1* encoding scytalone dehydratase in *Colletotrichum lagenarium*. Appl Environ Microbiol 62:4340–4344

Langfelder K, Streibel M, Jahn B, Haase G, Brakhage AA (2003) Biosynthesis of fungal melanins and their importance for human pathogenic fungi. Fungal Genet Biol 38:143–158

Nakada M, Tanaka C, Tsunewaki K, Tsuda M (1994) RFLP analysis for species separation in genera *Bipolaris* and *Curvularia*. Mycoscience 35:271–278

Ochman H, Gerber AS, Hartl DL (1988) Genetic applications of an inverse polymerase chain reaction. Genetics 120:621–623

Saitoh Y, Izumitsu K, Tanaka C (2009) Phylogenetic analysis of heavy-metal ATPases in fungi and characterization of the copper-transporting ATPase of *Cochliobolus heterostrophus*. Mycol Res 113:737–745

Saitoh Y, Izumitsu K, Morita A, Shimizu K, Tanaka C (2010) ChMCO1 of *Cochliobolus heterostrophus* is a new class of metallo-oxidase, playing an important role in DHN-melanization. Mycoscience 51:327–336

Shimizu K, Tanaka C, Tsuda M (1996) Genetic analyses of *Cochliobolus heterostrophus* albino mutant with deficiencies at two loci. Mycoscience 37:345–349

Shimizu K, Tanaka C, Tsuda M (1997) Cloning of *Brn1*, a reductase gene involved in melanin biosynthesis in *Cochliobolus heterostrophus*. J Gen Appl Microbiol 43:145–150

Sussman AS (1986) Longevity and survivability of fungi. In: Ainsworth GC, Sussman AS (eds) The fungi. An advanced treatise, vol 3. Academic Press, New York, pp 447–486

Tanaka C, Kubo Y, Tsuda M (1991) Genetic analysis and characterization of *Cochliobolus heterostrophus* colour mutants. Mycol Res 95:49–56

Tanaka C, Tajima S, Furusawa I, Tsuda M (1992) The *Pgr1* mutant of *Cochliobolus heterostrophus* lacks a *p*-diphenol oxidase involved in naphthalenediol melanin synthesis. Mycol Res 96:959–964

Tsai H-F, Wheeler MH, Chang YC, Kwon-Chung KJ (1999) A developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. J Bacteriol 181:6469–6477

Tsai H-F, Fujii I, Watanabe A, Wheeler MH, Chang YC, Yasuoka Y, Ebizuka Y, Kwon-Chung KJ (2001) Pentaketide melanin biosynthesis in *Aspergillus fumigatus* requires chain-length shortening of a heptaketide precursor. J Biol Chem 276:29292–29298

Tsuji G, Sugahara T, Fujii I, Mori Y, Ebizuka Y, Shiraishi T, Kubo Y (2003) Evidence for involvement of two naphthol reductases in the first reduction step of melanin biosynthesis pathway of *Colletotrichum lagenarium*. Mycol Res 107:854–860

Wang HL, Kim SH, Breuil C (2001) A scytalone dehydratase gene from *Ophiostoma floccosum* restores the melanization and pathogenicity phenotypes of a melanin-deficient *Colletotrichum lagenarium* mutant. Mol Genet Genomics 266:126–132

Watanabe A, Fujii I, Tsai H, Chang Y, Kwon-Chung KJ, Ebizuka Y (2000) *Aspergillus fumigatus alb1* encodes naphthopyrone synthase when expressed in *Aspergillus oryzae*. FEMS Microbiol Lett 192:39–44

Yoshimi A, Tsuda M, Tanaka C (2004) Cloning and characterization of the histidine kinase gene *Dic1* from *Cochliobolus heterostrophus* that confers dicarboximide resistance and osmotic adaptation. Mol Genet Genomics 271:228–236