Polyketide synthases of *Diaporthe helianthi* and involvement of DhPKS1 in virulence on sunflower

Michelina Ruocco1*, Riccardo Baroncelli2, Santa Olga Cacciola3, Catello Pane4, Maurilia Maria Monti1, Giuseppe Firrao5, Mariarosaria Vergara6,7, Gaetano Magnano di San Lio8, Giovanni Vannacci7 and Felice Scala1,2,3,4,5,6,7,8,9

**Abstract**

**Background:** The early phases of *Diaporthe helianthi* pathogenesis on sunflower are characterized by the production of phytotoxins that may play a role in host colonisation. In previous studies, phytotoxins of a polyketidic nature were isolated and purified from culture filtrates of virulent strains of *D. helianthi* isolated from sunflower. A highly aggressive isolate (7/96) from France contained a gene fragment of a putative nonaketide synthase (*lovB*) which was conserved in a virulent *D. helianthi* population.

**Results:** In order to investigate the role of polyketide synthases in *D. helianthi* 7/96, a draft genome of this isolate was examined. We were able to find and phylogenetically analyse 40 genes putatively coding for polyketide synthases (PKSs). Analysis of their domains revealed that most PKS genes of *D. helianthi* are reducing PKSs, whereas only eight lacked reducing domains. Most of the identified PKSs have orthologs shown to be virulence factors or genetic determinants for toxin production in other pathogenic fungi. One of the genes (*DhPKS1*) corresponded to the previously cloned *D. helianthi lovB* gene fragment and clustered with a nonribosomal peptide synthetase (NRPS) -PKS hybrid/lovastatin nonaketide like *A. nidulans* LovB. We used *DhPKS1* as a case study and carried out its disruption through *Agrobacterium*-mediated transformation in the isolate 7/96. *D. helianthi DhPKS1* deleted mutants were less virulent to sunflower compared to the wild type, indicating a role for this gene in the pathogenesis of the fungus.

**Conclusion:** The PKS sequences analysed and reported here constitute a new genomic resource that will be useful for further research on the biology, ecology and evolution of *D. helianthi* and generally of fungal plant pathogens.

**Keywords:** Polyketide synthases, *Diaporthe helianthi*, Plant pathogen, Pathogen virulence, toxins

**Background**

Polyketides are a large and diverse group of secondary metabolites with different biological activities, including pathogenicity, such as T-toxin produced by *Cochliobolus heterostrophus* [1] and melanin, a pigment essential for plant pathogenesis by many fungi [2, 3]. Biosynthesis of these metabolites is accomplished by polyketide synthases [4]. Polyketide synthases (PKSs) are modular enzymes classified on the basis of their molecular architecture and operating mechanisms into types I, II and III [5–8]. Fungal PKs show a structural diversity that can vary from simple aromatics to highly modified complex reduced-type compounds [9]. Many fungal PKSs have an architecture based on single modular iterative type I polyketide synthases (iPKSs), containing ketosynthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) domains. In addition to these, other functional domains, such as ketoreductase (KR), dehydratase (DH), enoylreductase (ER), methyltransferase (MeT) and thioesterase (TE), may be present in PKSs [10].

*Diaporthe helianthi* Munt.-Cvetk., Mihaljč. & M. Petrov (syn. *Phomopsis helianthi* Munt.-Cvetk., Mihaljč. &
M. Petrov) is a phytopathogenic fungus which causes stem canker and leaf shedding in sunflower (Helianthus annuus L.). The fungus invades and spreads through the leaves, progresses towards the petioles along foliar veins, and finally enters the stem, where cankers form in the advanced stage of pathogenesis [11]. D. helianthi is an important pathogen with a worldwide distribution. First reported in the former Yugoslavia [12], it subsequently spread to several other countries [13–15]. It can cause significant losses in yield and reduction of oil content when environmental conditions are favourable for disease development [16]. In Italy this disease has been observed since 1987, but even when climatic conditions are favourable to the disease [17, 18], its appearance is sporadic [19]. Epidemiological differences of this widespread disease could be explained by a high genetic diversity occurring in the D. helianthi populations depending on their geographic origin. Intraspecific variability has been previously displayed by isolates representative of diverse sunflower-growing areas [20–23]. All the isolates collected in France and in the former Yugoslavia, where epidemics of sunflower stem canker are severe, formed a monophyletic clade clearly distinct from all other isolates, while all the Italian isolates were phylogenetically distant from this cluster, evidencing a clear link between genetic biotype and pathogenic behaviour [21].

It has been nowadays accepted that sunflower stem canker is associated with a complex of Diaporthe species with different levels of pathogenicity. The main causal agent of the disease has been identified as D. gulyae sp. nov. in association with two less virulent species, D. kochmanii sp. nov. and D. kongii sp. nov. in Australia [23], whereas in the United States, D. gulyae and D. helianthi were both identified as causal agents of the disease having similar levels of aggressiveness [24].

The highly virulent French isolate 7/96 can be referred as D. helianthi sensu stricto. A DNA region of 532 bp from this isolate, shared with all highly virulent strains but not with the Italian mildly virulent isolates, was identified and sequenced [25]. This sequence, named lovB (accession number: AJ512137) showed a high similarity to genes encoding polyketide synthases (PKSs) from several species of filamentous fungi, including mlcA and mlcB of Penicillium citrinum, lovB of Aspergillus terreus, fun5 of Gibberella moniliformis, pks1 of Cochliobolus heterostrophus and pks1 of G. fujikuroi.

The mechanisms of pathogenicity and symptom induction are poorly understood in D. helianthi. Mazars et al. [26, 27] have demonstrated the production of a polyketidic phytotoxin, named phomozin, during pathogenesis on sunflower leaves and in culture filtrates of a French D. helianthi strain. The purified toxin produced symptoms comparable to those caused by pathogen infection. Subsequently, Avantaggiato et al. [28] purified other two phytotoxic metabolites, identified as cis- and trans-4,6-dihydroxymellein, from cultures of French and Italian isolates with different degrees of virulence. These toxins show a structure similar to phomozin, sharing with it the same precursor, known as orsellinic acid [28].

In the present study we generated a draft genome of the highly virulent isolate D. helianthi 7/96 and annotated 40 genes coding for putative PKSs [29]. By using the Pathogen-Host Interaction database (PHI-base) (http://www.phi-base.org) [30], orthologs of genes known to be involved in biosynthesizing PKs, which are virulence factors in other fungal species, were identified. Furthermore, the role in virulence of the gene DhPHS1 (= D. helianthi polyketide synthase 1) was evaluated through a gene disruption approach.

Methods

Fungal isolate and media

D. helianthi highly virulent French isolate 7/96 belongs to the fungal collection of Department of Agriculture, Food and Environment, University of di Pisa (Italy) [25] and was maintained on slants of PDA (potato dextrose agar, Difco) under mineral oil at 4 °C.

Diaporthe helianthi strain 7/96 draft genome sequence v2

Based on raw data available from a previous project [29], we generated a new D. helianthi strain 7/96 genome assembly. Paired end reads of 90 bp (1.80 Gbp) were assembled using SPAdes 3.11.0 [31]. The genome of D. helianthi consists of 7376 sequence scaffolds with a total assembly length of 63.67 Mbp (N50 = 20,184 and L50 = 860), 43.99% GC-content, and a maximum scaffold size of 151,286 bp. The completeness of the assembly was assessed using BUSCO v1.2 [32], which estimated the genome sequence to be 99.65% complete. The genome was annotated using the MAKER2 pipeline [33]. Overall, 13,139 protein-coding gene models were predicted.

The new genome assembly of D. helianthi strain 7/96 is present in GenBank with accession number: MAVT00000000.2.

Genomic characterization of putative PKSs genes

Putative PKS genes were identified according to Klarsson et al. [34] and manually inspected for conserved domain (acyl transferase [AT] – InterPro domain IPR014043, acyl carrier protein or phosphopantetheine attachment site [ACP or PP] – IPR009081, beta-ketoacyl synthase N-terminal domain [KS-N] – IPR014030, beta-ketoacyl synthase C-terminal domain [KS-C] – IPR014031, Ketoreductase [KR] – IPR013968, polyketide synthase dehydratase [DH] – IPR020807, polyketide synthase, enoylreductase domain [ER] – IPR020843, methyltransferase [MT] – IPR013217, thioesterase [TE] –
IPR001031) using InterProScan [35]. Moreover, putative D. helianthi PKSs sequences were aligned with references of other ascomycetes using MAFFT 7.310 [36] and a phylogenetic analysis was performed with PhyML 3.0 [34].

**DNA molecular techniques**

Total DNA was obtained from D. helianthi grown on PDA plates overlaid with a cellophane membrane. Plates were inoculated with mycelial plugs and incubated at 24 °C for 5 days. Mycelium mats were peeled from PDA plates overlaid with a cellophane membrane. Plates and a phylogenetic analysis was performed with PhyML leaving blunt ends and ligated to adaptors. For each fragment library, two primary PCR amplifications were carried out using an adaptor primer provided with the kit and an outer, gene-specific primer for downstream and upstream walking, 5'-AAG GTG GAC ACC GCA TAC CAC TCA TT-3' and 5'-CCA AGT CTT CAG CAG GAA TAT CAA CCA C-3', respectively. The primary PCR product was then diluted and used as a template for a secondary PCR amplification using a nested adaptor primer and nested gene-specific primers (5'-AGC TGC AAG TAG GGT GAC ACG GCA TAC-3' for downstream walking and 5'-ATG GGT GTG GTG CCG TGT CCA CCT TC-3' for upstream walking). The resulting DNA, flanking LovB, of 548 and 1256 bp were singly cloned in p-GEM-T Easy vector system (Promega) to form clones and DL2, respectively. To generate pUR5750-DhPKS1, the two previously cloned regions were excised from p-GEM-T Easy vector and fragments of about 548 bp (5' flanking gene fragment, called hereafter “A”) and 944 bp (3’ flanking gene fragment, called hereafter “B”), were inserted in pUR5750 KpnI and HindIII restriction sites, respectively, at the sides of (hph)-resistance cassette (Fig. 1).

In detail, the A fragment was excised from the plasmid by EcoRI digestion, and PCR-amplified with specific primers (5' AGGTACCTATGACTATAGGGCAGC 3’ and 5'AGGTACCTATGACTATAGGGCAGC 3') carrying at ends the KpnI cutting site sequence. The B fragment, was excised from the plasmid pGEM-T easy by EcoRI digestion, and inserted in the EcoRI site of pBlue-script KS; from this construct a smaller fragment of about 944 bp was excised, with HindIII restriction enzyme. The two DhPKS1 gene fragments (KpnI 548 bp and HindIII 944 bp) flanking the designed knock-out site, were inserted in the corresponding cutting-site in the plasmid pUR5750, upstream and downstream of the hph resistance cassette, to form a new plasmid named pUR5750-DhpkS1. Finally, pUR5750-DhPKS1 was transferred into A. tumefaciens LBA1100 by electroporation [40]; electroporation conditions were 25 μF, 200 Ω, 2.5 kV (0.2 cm cuvettes) in Gene Pulser® electroporator (Bio-Rad, USA), and transformants were selected on LB agar with and kanamycin (100 μg/ml). Agrobacterium strains containing the binary vectors were identified by PCR.

**Agrobacterium tumefaciens-mediated gene disruption**

The LBA1100-derived strain transformed with pUR5750-DhPKS1 was grown at 28 °C for 3 days in Petri dishes containing LB medium supplemented with flaving regions of the previously isolated sequence LovB [25] were identified and cloned with GenomeWalker kit (Clontech Laboratories, Palo Alto, CA) as follows: Separate fungal DNA aliquots were digested with four different restriction enzymes (EcoRV, DraI, PvuII, Stul) and a phylogenetic analysis was performed with PhyML 3.0 [34].
kanamycin (100 μg/ml). One hundred milliliter of liquid LB supplemented with kanamycin (100 μg/ml) were inoculated with a single bacterial colony and incubated at 28 °C overnight on an orbital shaker at 150 rpm. Bacterial cells were harvested by centrifugation at 12000 g at 4 °C and re-suspended in 5 ml of IM (1 mM KH2PO4, pH 4.8; 2.4 mM MgSO4·7H2O; 5 mM NaCl; 0.068 mM CaCl2; 0.03 mM FeSO4·7H2O; 0.0015 mM ZnSO4·7H2O; 0.002 mM CuSO4·5H2O; 0.008 mM H3BO3; 0.003 mM MnSO4·H2O; 0.002 mM Na2MoO4·2H2O; 6.25 mM NH4NO3; 54.2 mM glucose; 40 mM 2-(N-morpholino)ethanesulfonic acid; 0.05 mM glucose). The bacterial suspension was distributed in aliquots of 1 ml and further incubated at 30 °C for 6 h under stirring. Cultures of the fungal isolate 7/96b were grown on IM-agar supplemented with acetosyringone (AS, 0.2 mM) and incubated at 24 °C. After 4–5 days, mycelial plugs of these colonies were used to inoculate 10 ml of liquid medium in 2 L volume Erlenmeyer flasks containing 1 L of substrate made with: 3 g/L L-asparagine, 15 g/L sucrose, 1 g/L K2HPO4, 0.5 g/L MgSO4·7 H2O, 0.5 g/L KCl, 0.018 g/L FeSO4·7H2O, 5 g/L fresh sunflower tissue. The same liquid medium without fungal inoculation was used as a control. After 28 days, the liquid cultures were vacuum filtered, sterilised through Millipore filters of 0.2 μm and used in phytotoxicity tests, performed with Millipore filters of 0.2 μm and used in phytotoxicity tests, performed with cuttings of 15-day-old sunflower seedlings according to Avantaggiato et al. [28]. Ten sunflower cuttings were used per experiment and the experiment was replicated three times.

Phytotoxicity and virulence assays

For phytotoxicity tests, mycelial plugs from actively growing colonies of D. helianthi isolate 7/96 and transformants were grown, in static condition, on liquid medium in 2 L volume Erlenmeyer flasks containing 1 L of substrate made with: 3 g/L L-asparagine, 15 g/L sucrose, 1 g/L K2HPO4, 0.5 g/L MgSO4·7 H2O, 0.5 g/L KCl, 0.018 g/L FeSO4·7H2O, 5 g/L fresh sunflower tissue. The liquid medium without fungal inoculation was as a control. After 28 days, the liquid cultures were vacuum filtered, sterilised through Millipore filters of 0.2 μm and used in phytotoxicity tests, performed with cuttings of 15-day-old sunflower seedlings according to Avantaggiato et al. [28]. Ten sunflower cuttings were used per experiment and the experiment was replicated three times.

For virulence tests inoculum was prepared by growing D. helianthi strain 7/96 and transformants in Petri dishes containing different parts of sunflower (stems, leaves and flowers). Fresh plant tissues, surface sterilized with sodium hypochlorite (2.5%/vol.) and washed in sterile water, were dried, chopped (250 g/L) and mixed with agar (15 g/L). The fungus inoculated on the sunflower-
agar substrate was left to grow in the dark for 1 week at 25 °C. Seventy-eight-day-old sunflower plants (very susceptible cv Ala) at the flower bud stage were inoculated by placing a mycelial plug both on the upper surface of leaves and at the insertion point of the leaf petiole (sticking with a wooden toothpick). The diseased area was measured recording two diameters of the necrotic spot, by using a digital caliper. Inoculated plants were kept in a moist chamber for 7 days at 24 °C. The bioassay was carried out on five plants for each fungal strain and was repeated twice. Data were analysed by ANOVA test with Welsch modification, because data were normally distributed but not homoscedastic. Different means were separated by T3 Dunnett test. Statistical analyses were performed by SPSS 20.0 software.

Results
PKS genes in Diaporthe helianthi genome
By searching the draft nuclear genome of D. helianthi isolate 7/96 [29], we found a large number of putative PKS homologues which have been deposited in GenBank (Additional file 1). Most PKS genes coded by D. helianthi were reducing PKSs, whereas only eight PKSs lacked reducing domains and clustered with non-reducing PKSs (Additional file 1). DhPKS8, DhPKS18, DhPKS27 proteins did not cluster into any of the clades indicated in Fig. 2. DhPKS1 was placed in nonribosomal peptide synthetase (NRPS)-PKS clade, with the closest sequence being ATEG_00325 protein from A. terreus involved in isoflavipucine biosynthesis.

Homologies of DhPKSs with other genes involved in host-pathogen interaction
By whole genome sequencing of D. helianthi, we were able to decipher the complete sequence of all DhPKS genes and in silico deduced amino acid sequences (Additional file 1). Through a PHI-base interrogation we identified homologs for all DhPKS genes, except for two (DhPKS11 and DhPKS35), with experimentally verified pathogenicity, virulence and effector genes from fungal, oomycete and bacterial pathogens, infecting animal, plant, fungal and insect hosts. Results of this search are reported in Table 1.

Replacement of DhPKS1
Using oligonucleotides designed at 5’ and 3’ ends of genomic sequence of DhPKS1 we sequenced the complete gene (7877 bp) confirming its in silico prediction. In silico analysis showed that the coded protein corresponded to a highly reducing (HR) type I iPKS, containing the full set of domains, ketoacyl synthase (KS), acyl transferase, (AT), ketoreductase (KR), dehydratase (DH) and acyl carrier protein (ACP) (Additional file 1). Due to the identity of DhPKS1 with lovB (AN AJ512137) fragment identified by Vergara et al. [25] we decided to test its potential involvement in pathogenicity of D. helianthi.

Transformation of D. helianthi 7/96 with the gene disruption cassette in DhPKS1 yielded 160 hygromycin-resistant colonies. These putative transformants were purified by successive transfers of mono-hyphal and single protoplasts to selective medium amended with hygromycin 100 μg/L. Hygromycin resistance of transformants was not lost upon successive transfers; 74 of the hygromycin resistant colonies were screened by two PCR analyses. The first PCR was performed on the genomic DNA of each transformant, with the hph2for – trpc2rev primers specific to amplify the hygromycin resistance cassette (Fig. 3). Hph-containing transformants were then screened with a second PCR, to verify the homologous integration of the construct. In this case, we used Dhpk537for - Dhpk1326rev primers, designed on sequences internal to the replacement site of DhPKS1 (Fig. 4). This second PCR showed that 13 out of the 74 (18%) transformants integrated the ADhPKS1 construct at the homologous site. The number of integrated copies of the construct in the genome of D. helianthi transformants was ascertained by Southern blot analysis with a probe specific for the hygromycin resistance cassette (Fig. 5). Single copy integration of the construct occurred in five transformants designated as Tr1-5, as the enzymes used for genomic DNA digestion do not cut the Agrobacterium-transferred construct.

Phytotoxicity and virulence bioassays
ΔDhPKS1 mutants showed regular in vitro development and their growth rates were not significantly different from that of the wild type. Sunflower cuttings steeped in liquid cultures of the D. helianthi isolate 7/96 showed, after 5 days, brown marginal necrotic lesions and leaf chlorosis attributable to filtrate phytotoxicity. Cuttings steeped in culture extracts of DhPKS1 knock-out mutants, did not show clear evidence of phytotoxicity (Fig. 6).

Inoculation of sunflower plants with D. helianthi strain 7/96 wild type resulted in typical symptoms of stem cancer disease when observed 20 days after inoculation (Fig. 7a). Plants inoculated with transformants showed less intense symptoms (Fig. 7b). The disease severity in terms of necrotic area was measured in cm² on leaf blade and stem, in sunflower plants inoculated with the parental strain and transformants. For each time point, infection was markedly less severe with ΔDhPKS1 mutant than with the other strains. In particular the average necrotic area (cm²) at 84 h was 1.3 ± 0.28, 8.0 ± 1.65 and 8.5 ± 1.56, for ΔDhPKS1, wild type and empty vector transformant strains, respectively (Fig. 7c).
Discussion

The management of sunflower stem canker requires the identification of diverse species of *Diaporthe* associated with it, the determination of their aggressiveness and the study of pathogenicity mechanisms. In this paper we analysed the genome of the highly aggressive isolate 7/96 of *D. helianthi*, regarded as the main causal agent of sunflower stem canker epidemics in Europe [25], on the basis of its putative polyketide synthase genes. We found in its genome at least 40 PKS genes, more than those reported in other ascomycetes: 15 in *G. moniliformis*, 16 in *G. zeae*, 20 in *B. cinerea*, 25 in *C. heterostrophus* [41] and 27 in *Aspergillus nidulans* [42]. Such a repertoire of PKSs makes possible the synthesis of almost all known types of polyketide compounds by *D. helianthi* 7/96.

The PHI-base search demonstrated that all the putative 40 PKS proteins found in *D. helianthi*, with the exception of DhPKS11 and DhPKS35, have at least one ortholog in other pathogenic fungi or bacteria (Table 1). The majority of the reported orthologous genes have been demonstrated to be important factors in pathogenicity or virulence by gene deletion experiments.

A fragment of *LovB*-like coding sequence was isolated by Vergara et al. [25] from the genome of the aggressive isolate of *D. helianthi* 7/96, and proved to be present in the genome of the most aggressive isolates of *D. helianthi*, such as the French and Yugoslavian ones. In the present work this gene, named *DhPKS1*, was fully characterized. In silico sequence analysis revealed that *DhPKS1* belongs to subclade II of type I reducing PKS.
Table 1 List of all DhPKS genes, with the exclusion of two (DhPKS11 and DhPKS35), with experimentally verified pathogenicity, virulence and effector genes from fungal, oomycete and bacterial pathogens, which infect animal, plant, fungal and insect hosts, obtained by searching PHI-base (http://www.phi-base.org)

| Name     | GenBank Acc. Num. | Putative protein length (aa) | Organism                                      | Disease                          | Protein name | Protein ID | Identity (%) | Similarity (%) | Ref. |
|----------|------------------|-------------------------------|-----------------------------------------------|----------------------------------|--------------|------------|--------------|---------------|------|
| DhPKS1   | KR153185         | 2501                          | *M. oryzae*                                   | Rice blast                      | ACE1         | Q6ZX14     | 33.98        | 50.56         | [50] |
|          |                  |                               | *B. cinerea*                                  | Gray mould                       | BcBOA6       | B1GVX7     | 34.76        | 53.57         | [44] |
|          |                  |                               | *A. brassicicola*                             | Black spot                       | DEPS         | D2E9X0     | 32.68        | 50.45         | [52] |
| DhPKS2   | KR153184         | 2635                          | *G. fujikuroi*                                | Foolish seedling                 | FUM1 (FUM5)  | Q9Y8A2     | 32.53        | 49.48         | [66] |
| DhPKS3   | KR153183         | 1798                          | *A. oligospora*                               | Nematophagous fungus             | AoMls        | G1XL72     | 46.59        | 63.93         | [67] |
| DhPKS4   | KR153182         | 3071                          | *F. verticillioides*                          | Ear and stalk rot of maize       | FVEG_12528   | W7N3C7     | 28.69        | 45.46         | [68] |
|          |                  |                               | *F. graminearum*                              | Fusarium ear blight              | PKS4 (ZEA1)  | Q2VLJ2     | 28.14        | 45.46         | [69] |
| DhPKS5   | KR153181         | 2585                          | *C. heterostrophus*                           | Leaf blight                      | PKS1         | Q92217     | 31.58        | 48.37         | [3]  |
| DhPKS6   | KR153180         | 3013                          | *M. oryzae*                                   | Rice blast                       | ACE1         | Q6ZX14     | 35.17        | 53.24         | [50] |
|          |                  |                               | *B. cinerea*                                  | Gray mould                       | BcBOA6       | B1GVX7     | 38.13        | 56.32         | [44] |
| DhPKS7   | KR153179         | 2140                          | *C. graminicola*                              | maize anthracnose                | CgPKS1       | C9W7X1     | 32.98        | 49.38         | [70] |
|          |                  |                               | *F. graminearum*                              | Fusarium ear blight              | PKS13 (ZEA2) | Q2VLJ3     | 36.24        | 55.66         | [71] |
| DhPKS8   | KR153178         | 2193                          | *C. alternata*                                | Leaf spot                        | ACRTS2       | F8R4Y0     | 36.75        | 53.86         | [63] |
|          |                  |                               | *C. neoformans*                               | Leaf spot                        | ACRTS2       | F8R4Y0     | 38.87        | 55.41         | [63] |
|          |                  |                               | *C. heterostrophus*                           | Facultative plant and animal fungal pathogen | Cln1         | J9W14      | 38.57        | 55.52         | [72] |
| DhPKS9   | KR153177         | 2519                          | *A. fumigatus*                                | Respiratory disease              | ALB1         | O59897     | 38.17        | 56.14         | [73] |
|          |                  |                               | *A. fumigatus*                                | Respiratory disease              | PKSP         | O60026     | 38.08        | 56.09         | [74] |
|          |                  |                               | *C. lagenarium*                               | Anthracnose                      | PKS1         | P79068     | 37.07        | 54.01         | [62] |
|          |                  |                               | *E. dermatitidis*                             | Phaeohyphomycosis                | WdPKS1       | Q9Y7A7     | 36.64        | 53.72         | [75] |
| DhPKS10  | KR153176         | 2209                          | *F. verticillioides*                          | Ear and stalk rot of maize       | FVEG_12528   | W7N3C7     | 38.91        | 54.24         | [68] |
|          |                  |                               | *C. heterostrophus*                           | Leaf blight                      | PKS1         | Q92217     | 38.05        | 54.73         | [3]  |
| DhPKS11  | KR153175         | 171                           | *A. alternata*                                | Leaf spot                        | ACRTS2       | F8R4Y0     | 31.21        | 47.02         | [63] |
| DhPKS12  | KR153174         | 2492                          | *M. oryzae*                                   | Rice blast                       | ACE1         | Q6ZX14     | 39.13        | 57.03         | [50] |
| DhPKS13  | KR153173         | 4088                          | *B. cinerea*                                  | Gray mould                       | BcBOA6       | B1GVX7     | 39.88        | 57.66         | [44] |
| DhPKS14  | KR153172         | 2191                          | *A. fumigatus*                                | Respiratory disease              | ALB1         | O59897     | 42.51        | 60.58         | [73] |
|          |                  |                               | *A. fumigatus*                                | Respiratory disease              | PKSP         | O60026     | 42.46        | 60.48         | [74] |
|          |                  |                               | *C. nicotianae*                               | Leaf spot                        | CTB1         | Q6DQW3     | 42.97        | 59.11         | [64] |
|          |                  |                               | *C. sativus*                                  | Spot blotch disease              | PKS1         | G8DNT0     | 39.75        | 58.54         | [76] |
|          |                  |                               | *A. alternata*                                | Leaf spot                        | ACRTS2       | F8R4Y0     | 39.62        | 57.97         | [63] |
|          |                  |                               | *C. neoformans*                               | Facultative plant and animal fungal pathogen | Cln1         | J9W14      | 38.06        | 56.65         | [72] |
| Name          | GenBank Acc. Num. | Putative protein length (aa) | Organism            | Disease                     | Protein name | Protein ID | Identity (%) | Similarity (%) | Ref. |
|---------------|-------------------|------------------------------|---------------------|-----------------------------|--------------|------------|--------------|----------------|------|
| DHPKS15      | KRI53171          | 1613                         | B. cinerea          | Gray mould                  | BcBOA6       | B1GVX7    | 34.61         | 50.99          | [44] |
| DHPKS16      | KRI53170          | 3886                         | M. oryzae           | Rice blast                  | ACE1         | Q6ZX14    | 34.17         | 52.24          | [50] |
| DHPKS17      | KRI53169          | 2616                         | G. fujikuroi        | Foolish seedling            | FUM1        | Q9Y8A2    | 31.55         | 48.7           | [66] |
| DHPKS18      | KRI53168          | 2161                         | A. brassicicola     | Black spot                  | DEPS         | D2E9X0    | 36.67         | 53.72          | [52] |
| DHPKS19      | KRI53167          | 2647                         | F. graminearum      | Fusarium ear blight         | PKS13       | Q2VLJ3    | 29.11         | 45.31          | [71] |
| DHPKS20      | KRI53166          | 1996                         | A. fumigatus        | Respiratory disease         | ALB1         | O59897    | 44.37         | 60.66          | [73] |
| DHPKS21      | KRI53165          | 814                          | A. brassicicola     | Black spot                  | DEPS         | D2E9X0    | 41.09         | 57.14          | [52] |
| DHPKS22      | KRI53164          | 2505                         | A. brassicicola     | Black spot                  | DEPS         | D2E9X0    | 32.25         | 50.16          | [52] |
| DHPKS23      | KRI53163          | 7787                         | B. cinerea          | Gray mould                  | BcBOA6       | B1GVX7    | 53.44         | 68.48          | [37] |
| DHPKS24      | KRI53162          | 2545                         | C. heterostrophus   | Leaf blight                 | PKS1         | Q92217    | 32.44         | 50.48          | [3]  |
| DHPKS25      | KRI53161          | 2590                         | F. verticillioides  | Ear and stalk rot of maize  | FVEG_12528  | W7NCN7    | 35.75         | 53.85          | [68] |
| DHPKS26      | KRI53160          | 2543                         | F. graminearum      | Fusarium ear blight         | PKS4        | Q2VLJ2    | 34.1          | 50.4           | [71] |
| DHPKS27      | KRI53159          | 2064                         | A. alternata        | Leaf spot                   | ACRTS2       | F8R4Y0    | 47.12         | 64.5           | [63] |
| DHPKS28      | KRI53158          | 2169                         | C. lagenarium       | Anthracnose                 | PKS1         | P79068    | 71.73         | 83.2           | [62] |
| DHPKS29      | KRI53157          | 1813                         | C. graminicola      | maize anthracnose           | CgPKS1       | C9W7X1    | 32.98         | 49.38          | [70] |
| DHPKS30      | KRI53156          | 2624                         | C. graminicola      | maize anthracnose           | CgPKS1       | C9W7X1    | 32.98         | 49.38          | [70] |
| DHPKS31      | KRI53155          | 2753                         | A. brassicicola     | Black spot                  | DEPS         | D2E9X0    | 36.4          | 53.12          | [52] |
| DHPKS32      | KRI53154          | 2484                         | A. oligospora       | Nematophagous fungus        | AoMls        | G1XL2T    | 46.59         | 63.93          | [63] |
| DHPKS33      | KRI53153          | 2254                         | M. oryzae           | Rice blast                  | ACE1        | Q6ZX14    | 39.74         | 56.57          | [50] |
| DHPKS34      | KRI53152          | 2365                         | G. fujikuroi        | Foolish seedling            | FUM1        | Q9Y8A2    | 28.72         | 45.55          | [66] |
|              |                   |                              | C. heterostrophus   | Southern corn leaf blight   | PKS2         | Q6RGK2    | 32.0          | 49.87          | [1]  |
[41], having the typical conserved domain of this protein family KS-AT-DH-(ME)PP-(CON)-(AMP-PP). Reducing PKS subclade II is characterized by enzymes missing the ER domain; PKs synthesized by PKSs of this subclade are predicted to either lack reduced alkyl groups or to contain alkyl groups whose reduction is completed by the product of an external ER domain-containing gene, as A. terreus lovC [43] and P. citrinum mlcG. The PKSs of this clade were also found to have either a condensation (CON) domain typical of nonribosomal peptide synthetases (NPSs) [43] or an entire NPS module consisting of a CON domain, and an acyl carrier or phosphopantetheine attachment site (ACP or PP) domain. The absence of a DhPKS1 homologous gene in the less aggressive Italian isolates prompted us to further investigate the possible significance of this gene as a virulence determinant. Results from PHI-base search revealed that DHPKS1 is very similar to PKSs from filamentous fungi known to be involved in the production of important pathogenicity factors. Among these, it showed 35% identity with BcPKS6 gene of B. cinerea which encodes a key enzyme for botcinic acid biosynthesis, a phytotoxin involved in virulence of the fungus on tomato [44]. Moreover, BcPKS6 belongs to a PKS cluster co-regulated by the Gα subunit BCG1, which, in turn, is essential for pathogenicity of B. cinerea on bean

| Name     | GenBank Acc. Num. | Putative protein length (aa) | Organism                  | Disease                      | Protein name | Protein ID | Identity (%) | Similarity (%) | Ref. |
|----------|------------------|-------------------------------|---------------------------|------------------------------|--------------|------------|--------------|----------------|------|
| C. heterostrophus | Q92217          | 36.83                       | Leaf blight               | PKS1                         |              |            | 36.83        | 54.98          | [3]  |
| F. verticillioides | W7NCN7          | 36.16                       | Ear and stalk rot of maize| FVEG,12528                   |              |            | 36.16        | 53.24          | [68] |
| F. graminearum   | Q2VLJ2           | 35.14                       | Fusarium ear blight       | PKS4 (ZEA1)                  |              |            | 35.14        | 52.15          | [71] |
| C. heterostrophus | Q92217          | 34.46                       | Leaf blight               | PKS1                         |              |            | 34.46        | 53.1           | [3]  |
| G. fujikuroi (FUM5) | Q9Y8A2          | 33.81                       | Foolish seedling          | FUM1 (FUM5)                  |              |            | 33.81        | 51.81          | [66] |
| C. heterostrophus | Q92217          | 32.04                       | Leaf blight               | PKS1                         |              |            | 32.04        | 49.81          | [3]  |
| G. fujikuroi (FUM5) | Q9Y8A2          | 32.04                       | Foolish seedling          | FUM1 (FUM5)                  |              |            | 32.04        | 49.81          | [3]  |
| A. brassicicola | D2E9X0           | 30.69                       | Black spot                | DEPS                         |              |            | 30.69        | 47.58          | [52] |
| C. heterostrophus | Q92217          | 30.69                       | Leaf blight               | PKS1                         |              |            | 30.69        | 47.58          | [52] |
| G. fujikuroi (FUM5) | Q9Y8A2          | 30.69                       | Foolish seedling          | FUM1 (FUM5)                  |              |            | 30.69        | 47.58          | [52] |
| A. brassicicola | D2E9X0           | 30.69                       | Black spot                | DEPS                         |              |            | 30.69        | 47.58          | [52] |
| F. verticillioides | W7NCN7          | 30.69                       | Ear and stalk rot of maize| FVEG,12528                   |              |            | 30.69        | 47.58          | [52] |

Table 1 List of all DhPKS genes, with the exclusion of two (DhPKS11 and DhPKS35), with experimentally verified pathogenicity, virulence and effector genes from fungal, oomycete and bacterial pathogens, which infect animal, plant, fungal and insect hosts, obtained by searching PHI-base (http://www.phi-base.org) (Continued)

**Fig. 3** PCR analysis of Diaporthe helianthi strain 7/96 (lane 3) and ΔDhPKS1 putative transformants (lanes 4–8) with hph2-for/trpc2-rev primers. Lanes 1 and 2 correspond to 1 kb molecular weight ladder and positive control, respectively

**Fig. 4** PCR analysis of Diaporthe helianthi strain 7/96 (lane 2) and ΔDhPKS1 putative transformants (lanes 4–15) with Dhpks537-for/ Dhpks1326-rev primers. Lanes 1 and 3 correspond to 1 kb molecular weight ladder and negative control, respectively
leaves [45] and is part of a clade including several PKSs responsible for the synthesis of cyclic polyketides like: LovB (LNKS, nonaketide part of lovastatin [46]); MlcA (nonaketide part of citrinin; [47]), EQS (equisetin; [48]) and FusS (fusarin; [49]). DHPKS1 has also a relatively high homology (Table 1) with the *Magnaporthe grisea* gene *ACE1* encoding a putative PKS expressed exclusively during penetration of *M. grisea* into leaves and involved in the recognition of the fungus by resistant rice varieties carrying the resistance gene *Pi33*, thus revealing a role for avirulence [50, 51]. Orthologous to DHPKS1 is also *AbPKS9* (*DEPS*) gene involved in biosynthesis of depudecin, an 11 linear polyketide inhibitor of histone deacetylase (HDAC) virulence factor of the fungus *A. brassicicola* [52].

Among the functionally characterized PKSs DhpKS1 orthologs, there is also *lovB* of *A. terreus*, which synthesizes the nonaketide chain of lovastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor [53]. *A. terreus* has been known to carry an unusual PKS gene cluster for the polyketides in which two PKS genes (*lovB* and *lovF*) are closely linked in the cluster and are required for the biosynthesis of the nonaketide and the diketide moieties of the compound, respectively [54]. Interestingly, we also found that LovF of *A. terreus* was homologous to DhPKS24, suggesting the presence in *D. helianthi* 7/96 of a similar PKS gene cluster.

In our work, as a case study, knock-out mutants of DhpKS1 were produced by targeted DNA integration through *Agrobacterium*-mediated transformation. This method has been successfully applied to study gene functions in other phytopathogenic and toxigenic fungi [2, 56]. The presence of a single copy of the T-DNA made the analysis of the transformants straightforward. Unfortunately, it was not possible to confirm the above results by performing experiments with complementation mutants. The reason why no double mutants were obtained is unknown, but it seems that this fungal strain cannot be doubly transformed. As a result of DhpKS1 gene inactivation, reduced fungal virulence on a susceptible sunflower cultivar was observed. Indeed, in contrast with the wild type, mutants caused less visible symptoms after artificial inoculation of fungal mycelia on stem and leaves. This indicates that DhpKS1 could be involved in virulence of the sunflower stem canker agent. The DhpKS1 gene disruption also seems to affect in vitro production of toxic secondary metabolites by *D. helianthi*. Liquid filtrates of ΔDhpKS1 mutants showed a
reduced phytotoxicity on sunflower seedlings. The highly reducing synthase DhPKS1 probably produces a metabolite, not yet identified, which could be involved in the D. helianthi 7/96 strain virulence on sunflower. However, for a better understanding of the possible role of DhPKS1, more studies should be carried out considering also the role of the other PKSs genes that cluster with it.

In previous work [26] the polyketidic metabolite phomozin, an ester of orsellinic acid, was isolated both from culture filtrates of D. helianthi and from infected plants, and its possible role in development of symptoms was suggested. Orsellinic acid synthase is the simplest tetra-ketide synthase and is grouped with the nonreducing PKSs (NR-PKSs). Our results indicate that, in the case of DhPKS7 due to its high homology with the A. nidulans gene EAA59563 coding for orsellinc acid synthase [57]. The toxinic theory is supported by the evidence that other phytopathogenic species related to the genus Phomopsis produce toxic metabolites involved in pathogenesis [58–60]. For a disease caused by a Phomopsis species affecting soybean, similar to stem canker of sunflower, the possible involvement of a phytoxin was also implied [61]. Polyketide synthases have been reported as important virulence factors in other several phytopathogenic fungi such as C. heterostrophus [3], Colletotrichum lagenarium [62] and A. alternata [63]. In C. nicotianae, the genes CTB1 and CTB3 encode two polyketide synthases, involved in the biosynthesis of cercosporin, a photoactivated perylene-quinone toxin, which play a key role in fungal pathogenesis [64, 65].

Conclusions
The PKS sequences reported here are a new important resource that will be useful for further research in the biology, ecology and evolution of D. helianthi and in general of fungal plant pathogens. Further investigation is necessary to fully understand the role of D. helianthi PKS genes. Moreover, it will be very important to verify if the PKSs identified in D. helianthi sensu stricto are also present in other species of Diaporthe, which have been associated with sunflower stem canker.

Additional file

Abbreviations
ACP: Acyl carrier protein; AT: Acyltransferase; DH: Dehydratase; EDC: Equisetin; ER: Enoylreductase; HDAC: Histone deacetylase; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; Hph: Hygromycin phosphotransferase; KR: Ketoreductase; LS: Ketosynthase; LNKS: Nonaketide part of lovastatin; Lov: Lovastatin; MeT: Methyltransferase; NRPS: Nonribosomal peptide synthetase; PKS: Polyketide synthases; PP: Phosphopantetheine; TE: Thioesterase

Acknowledgements
The Authors take this opportunity to remember prof. Giovanni Del Sorbo who started this study and to thanks dr. Paolo Alfonso Pedata who helped with statistical analysis.

Funding
This project was financially supported by the projects: BIP Biindustrial Processes, CUP: B25C13000290007 and CARINA, CUP: B25B09000080007 and by the PhD program of the University of Palermo.

Availability of data and materials
The reassembled WGS genome of D. helianthi strain 7/96 is present in NCBI GenBank with accession number: MAVT00000000.2. The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
MR and FS designed the main aims of this work and wrote the manuscript. MR and RB performed genomic characterization of putative PKSs genes, RB conducted the different bioinformatic analyses; CP and MR performed gene disruption and virulence assays, DNA isolation and purification, and the different molecular biology techniques used in this study. MR, FS, RB, CP, SOC, MMM, GF, MV, GMSL and GV provided guidance in the drafting of the manuscript and contributed to acquisition, analysis and interpretation of data. All authors read, corrected and approved the final manuscript.
40. Mozo T, Hooiyyaas PJJ. Electroporation of megaplasmids into Agrobacterium. Plant Mol Biol. 1991;16(5):917–8.

41. Kocen S, Glass NL, Taylor JW, Yoder OC, Turgeon BG. Phylogenomic analysis of type I polyketide synthase genes in pathogenic and saprobic ascomycetes. Proc Natl Acad Sci U S A. 2003;100(26):15670–5.

42. Galagan JE, Calvo SE, Borkovich KA, Selker EU, Reid ND, Jaffe D, FitzHugh W, Ma LJ, Srinivas S, Pocciell S, et al. The genome sequence of the filamentous fungus Neurospora crassa. Nature. 2003;422(6934):859–68.

43. Kennedy J, Auclair K, Kendrew SG, Park C, Vederas JC, Hutchinson CR. Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. Science. 1999;284(5418):358–72.

44. Dalmair B, Schumacher J, Mosaga J, LEP P, Tuzdynski B, Collado K, Vlaud M. The Botrytis cinerea pytoxacin boric acid requires two polyketide synthases for production and has a redundant role in virulence with botrydial. Mol Plant Pathol. 2011;12(6):564–79.

45. Schumacher J, Vlaud M, Simon A, Tuzdynski B. The G subunit BCG1, the phosphotyrosine phosphatase co-ordinately regulate gene expression in the grey mould fungus Botrytis cinerea. Mol Microbiol. 2008;67(1):1027–50.

46. Sutherland A, Auclair K, Vederas JC. Recent advances in the biosynthetic studies of lovastatin. Curr Opin Drug Discov Dev. 2001;4(12):229–36.

47. Abe Y, Suzuki T, Ono C, Iwamoto K, Hosobuchi M, Yoshikawa H. Molecular cloning and characterization of an ML-236B (compactin) biosynthetic gene cluster in Penicillium. Mol Gen Genom. 2002;267(5):636–46.

48. Sims JW, Fillmore JP, Warner DD, Schmidt EW. Equisetin biosynthesis in Fumonisin heterosporum. Chem Commun (Camb). 2005;2:186–8.

49. Song Z, Cox RJ, Simpson TP, Fusarin C biosynthesis in Fusarium Grisea. Proc Natl Acad Sci U S A. 2009;106(34):14558–63.

50. Bohnert HU, Fudal I, Dioh W, Tharreau D, Notteghem JL, Lebrun MH. A functional genomics approach for the identification of fumonisin biosynthesis genes in Fusarium heterosporum. Mol Genet. 2002;5(13):1196–203.

51. Fudal I, Collemare J, Bohnert HU, Melayah D, Lebrun MH. Expression of the polyketide synthase gene ESt111 in Fusarium moniliforme and Fusarium venenatum. Chembiochem. 2004;5(13):1196–203.

52. Bohnert HU, Fudal I, Dioh W, Tharreau D, Notteghem JL, Lebrun MH. A putative polyketide synthase/peptide synthetase from Magnaporthe grisea signals pathogen attack to resistant rice. Plant Cell. 2004;16(19):2499–513.

53. Fudal I, Collemare J, Bohnert HU, Melayah D, Lebrun MH. Expression of Magnaporthe grisea avirulence gene ACE1 is connected to the initiation of appressorium-mediated penetration. Euycukoar Cell. 2007;63(3):546–54.

54. Wight WD, Kim KH, Lawrence CB, Walton JD. Biosynthesis and role in virulence of the histone deacetylase inhibitor depudecin from Alternaria brassicicola. Mol Plant-Microbe Interact. 2009;22(10):1258–67.

55. Hendrickson L, Davis CR, Roach C, Nguyen DK, Aldrich T, McAda PC, Reeves CD. Lovastatin biosynthesis in Aspergillus terreus: characterization of the multifunctional polyketide synthase gene. Chem Biol. 1999;6(7):429.

56. Ma SM, Tang Y. Biochemical characterization of the minimal polyketide synthase domain loci of the lovastatin nonaketide synthase LovB. FEBS J. 2007;274(11):2854–64.

57. Kim YT, Lee YR, Jin J, Han KH, Kim H, Kim JC, Lee T, Yun SH, Lee YW. Two different polyketide synthase genes are required for synthesis of zearealenone in Gibberella zeae. Mol Microbiol. 2005;58(4):1102–13.

58. Schummann J, Hertweck C. Advances in cloning, functional analysis and heterologous expression of fungal polyketide synthase genes. J Biotechnol. 2006;124(4):690–703.

59. Schroeder V, Scherlach K, Nuttmann HW, Shelest E, Schmidt-Heck W, Schuemann J, Martin K, Hertweck C, Brakhage AA. Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in Aspergillus nidulans. Proc Natl Acad Sci U S A. 2009;106(34):14558–63.

60. Evidente A, Rodeva R, Andolfi A, Sotolaiva Z, Porecne C, Motta A.负责任 for biosynthesis of host-selective ACR-toxin in the rough lemon pathotype of Alternaria alternata. Mol Plant-Microbe Interact. 2012;25(11):1419–29.

61. Choquer M, Dekkers KL, Chen HQ, Cao L, Ueng PP, Dabro ME, Chung KR. The CTI1 gene encoding a fungal polyketide synthase is required for cercosporin biosynthesis and fungal virulence of Cercospora nicotianae. Mol Plant-Microbe Interact. 2005;18(5):468–76.

62. Dekkers KL, You BJ, Gowda VS, Liao HL, Lee MH, Bai HL, Ueng PP, Chung KR. The Cercospora nicotianae gene encoding dual O-methyltransferase and FAD-dependent monooxygenase domains mediates cercosporin biosynthesis. Fungal Genet Biol. 2007;44(5):444–54.

63. Dejardins AE, Munkvold GP, Platner RD, Proctor RH. Ful1 -- a gene required for fumonisin biosynthesis but not for maize ear rot and ear infection by Gibberella moniliformis in field tests. Mol Plant-Microbe Interact. 2002;15(11):1157–64.

64. Zhao X, Wang Y, Zhao Y, Huang Y, Zhang KQ, Yang J. Malate synthase gene AoMIs in the nematode-trapping fungus Arthrobotrys oligospora contributes to conditio, trap formation, and pathogenicity. Appl Microbiol Biotechnol. 2014;98(6):2555–63.

65. Brown DW, Lee S-H, Kim L-H, Ryu J-G, Lee S, Seo Y, Kim YH, Busman M, Yun S-H, Proctor RH, Lee T. Identification of a 12-gene fusaric acid biosynthetic gene cluster in Fusarium species through comparative and functional genomics. Mol Plant-Microbe Interact. 2015;28(3):319–32.

66. Gaffoor I, Malli DW, Platner R, Proctor RH, Qi W, Trail F. Functional analysis of the polyketide synthase genes in the filamentous fungus Gibberella zeae (anamorph Fusarium graminearum). Fungal Ecol Rev. 2005;9(11):1926–33.

67. Garcia-Rodas R, Trevijano-Contador N, Roman E, Janbon G, Moyrand F, Pla J, Casadevall A, Zaragoza O. Role of Cln1 during melanization of Cryptococcus neoformans. Front Microbiol. 2015;6:798.

68. Waniare A, Fuji I, Tsai H, Wang YC, Kwon-Chung KJ, Ebuzuka Y, Aspergillus fumigatus alb1 encodes naphthopyrone synthase when expressed in Aspergillus oryzae. FEMS Microb Lett. 2000;192(1):39.

69. Yoon S-H, Proctor RH, Lee T. Identification of a 12-gene fusaric acid biosynthetic gene cluster in Fusarium species through comparative and functional genomics. Mol Plant-Microbe Interact. 2014;27(4):315–27.

70. Ludwig N, Lohrer M, Hempel M, Mathea S, Schleiber L, Menzel M, Kiesow A, Schaffrath O, Deising HB, Hoborh B. Melanin is not required for turgor generation but enhances cell-wall rigidity in appressoria of the corn pathogen Colletotrichum graminicola. Mol Plant-Microbe Interact. 2014;27(4):315–27.

71. Chooi YH, Muria-Gonzalez MJ, Mead OL, Solomon PS. SnPKS19 encodes the primary subunit BCG1, the polysaccharide synthase gene ACRTS2, is responsible for biosynthesis of host-selective ACR-toxin in the rough lemon pathotype of Alternaria alternata. Mol Plant-Microbe Interact. 2012;25(11):1419–29.

72. Fumonisin biosynthesis. Science. 1999;284(5418):1368–70.

73. Ruocco et al. BMC Genomics (2018) 19:27

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