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Cloning and characterization of novel methylsalicylic acid synthase gene involved in the biosynthesis of isoasperlactone and asperlactone in *Aspergillus westerdijkiae*

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A B S T R A C T

Aspergillus westerdijkiae is the main producer of several biologically active polyketide metabolites including isoasperlactone and asperlactone. A 5298 bp polyketide synthase gene "*aomsas*" has been cloned in *Aspergillus westerdijkiae* by using gene walking approach and RACE-PCR. The predicted amino acid sequence of *aomsas* shows an identity of 40–56% with different methylsalicylic acid synthase genes found in *Byssochlamys nivea*, *P. patulum*, *A. terreus* and *Streptomyces viridochromogenes*. Based on the reverse transcription PCR and kinetic secondary metabolites production studies, *aomsas* expression was found to be associated with the biosynthesis of isoasperlactone and asperlactone. Moreover an *aomsas* knockout mutant "*aoΔmsas*" of *A. westerdijkiae*, not only lost the capacity to produce isoasperlactone and asperlactone, but also 6-methylsalicylic acid. The genetically complemented mutant *ao+msas* restored the biosynthesis of all the missing metabolites. Chemical complementation through the addition of 6-methylsalicylic acid, aspyrone and diepoxide to growing culture of *aoΔmsas* mutant revealed that these compounds play intermediate roles in the biosynthesis of asperlactone and isoasperlactone.

1. Introduction

Aspergillus westerdijkiae is a producer of several biologically active polyketide metabolites including isoasperlactone and asperlactone (Atoui et al., 2006; Balcells et al., 1995; Gaucher and Shepherd, 1968; Torres et al., 1998). These two metabolites belong to the partially reduced or methylsalicylic acid (MSA) type polyketide group and have same chemical structure but are different stereochemically. Isoasperlactone and asperlactone are on one hand efficient antimicrobial agents (Rosenbrook and Carney, 1970; Torres et al., 1998), and on the other hand they contain ovicidal activities against *Nezara viridula* (Balcells et al., 1995, 1998). It has been reported that asperlactone and isoasperlactone presented a strong anti-bacterial and anti-fungal activities when compared with other antimicrobial agents (Balcells et al., 1998).

It has been previously demonstrated that during the biosynthesis of MSA type polyketides, the first step is the formation of 6-methylsalicylic acid (6-MSA) by the condensation of one molecule of acetyl-coA and three molecules of malonyl-coA (David et al., 1995). This reaction is catalyzed by a multifunctional enzyme system known as methylsalicylic acid synthase (MSAS). This enzyme has been reported to catalyze several MSA type polyketide compounds like patuline in *Penicillium patulum* (Beck et al., 1990) and *Byssochlamys fulva* (Puel et al., 2007) and avilamycin in *Streptomyces viridochromogenes* (Gaisser et al., 1997).

Not much is known about the biosynthetic pathways of isoasperlactone and asperlactone, except for a hypothetical scheme proposed by James and Andrew (1991). According to this scheme, the isomeric metabolites aspyrone, isoasperlactone and asperlactone are derived from a common biosynthetic precursor, the diepoxide. James and Andrew (1991) further stated that asperlactone is formed directly from the diepoxide, while isoasperlactone is formed from diepoxide via aspyrone pathway (Fig. 1).

Many PKS genes have been sequenced in filamentous fungi including *A. fumigatus* and *A. niger* (Metz et al., 2001; Niermanm et al., 2005; Pel et al., 2007; Sebastian et al., 2007). It is therefore possible to use the most conserved region among these PKS genes (Fig. 2) as primers for a PCR based cloning strategy. For this purpose, mainly the ketosynthase (KS) and acyle transferase (AT) domains, i.e. the most conserved domains among different PKSs (Fig. 2), have been utilized. The previously designed pairs of degenerated primers KS1/KS2, LC1/LC2c and LC3/LC5c (Bingle et al., 1999; Nicholson et al., 2001) has been already utilized in

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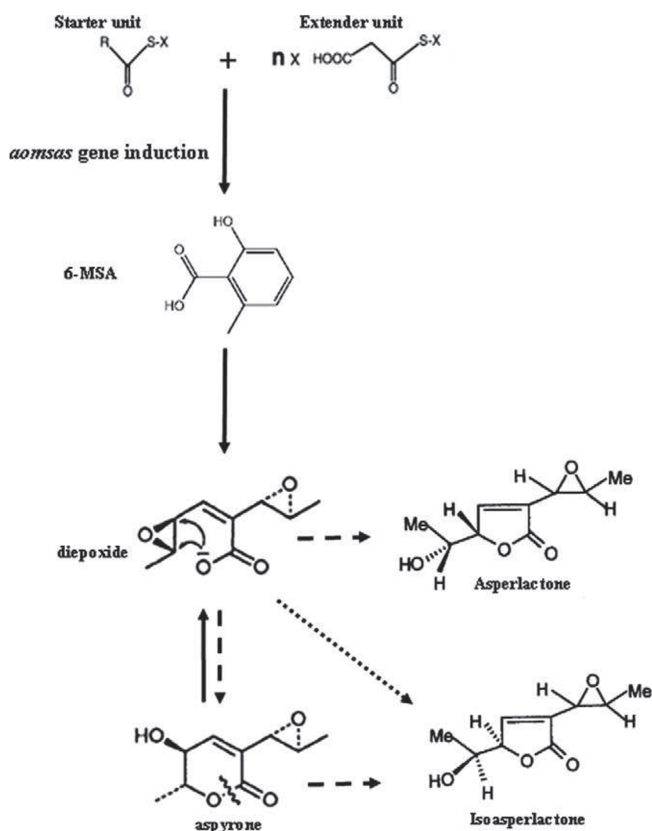

Fig. 1. Proposed biosynthetic scheme of isoasperlactone and asperlactone in *A. westerdijkiae*. Based on our results and the study of James and Andrew (1991), we proposed a hypothetical biosynthetic scheme of isoasperlactone and asperlactone biosynthesis. Straight arrow lines indicate our proposed scheme, dotted arrow lines indicate biosynthetic scheme of James and Andrew (1991), while dashed arrow line indicate steps that are shared in our proposed biosynthetic scheme and in the scheme of James and Andrew (1991).

our laboratory to clone eight KS domains from different PKSs (Atoui et al., 2006), including one from MSAS in *A. westerdijkiae*. Recently one of these PKS genes has been found to be involved in the biosynthesis of ochratoxine A (Bacha et al., 2009).

In this paper, we report sequencing and functional characterization of the first MSAS-type PKS gene "*aomsas*" identified in *A. westerdijkiae*. It is involved in the biosynthesis of two pharmacologically important lactonic metabolites, i.e. isoasperlactone and asperlactone. We also demonstrate that 6-methylsalicylic acid (6-MSA), aspyrone and diepoxide are intermediates in their biosynthetic pathway.

2. Materials and methods

2.1. Fungal strain and culture conditions

Aspergillus westerdijkiae NRRL 3174 strain was grown for sporulation at 25 °C on potato dextrose agar for 7 days. Spores were collected using a solution of 0.01% (v/v) Tween 80, counted by using Thoma Bright line counting chamber (Optick labor), and stored at −20 °C in 25% (v/v) glycerol before use. Conidia were inoculated (density $\sim 10^6$/mL) into 250 mL Erlenmeyer flasks containing 100 mL synthetic medium (SAM) at 25 °C for 2–18 days, without shaking. The composition of SAM (per liter of distilled water) was: 3 g NH_4NO_3, 26 g K_2HPO_4, 1 g KCl, 1 g $MgSO_4 \cdot 7H_2O$, 10 mL mineral solution (composition per liter of distilled water: 70 mg $Na_2B_4O_7 \cdot 10H_2O$, 50 mg $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$, 1000 mg $FeSO_4 \cdot 7H_2O$, 30 mg $CuSO_4 \cdot 5H_2O$, 11 mg $MnSO_4 \cdot H_2O$, 1760 mg $ZnSO_4 \cdot 7H_2O$), and 50 g glucose. The pH of the medium was adjusted to 6.5 by the addition of 2 N HCl. Mycelium was harvested by filtration through a 0.45-μM filter, grounded in liquid nitrogen and then stored at −80 °C before nucleic acid extraction. Secondary metabolites were extracted from filtrates of 2–18 days old cultures medium. Three replications of each sample were analyzed.

2.2. Nucleic acid extraction

Rapid method of genomic DNA extraction (Lui et al., 2000) was used for transformants screening by PCR. Large quantity genomic DNA was extracted by CTAB extraction method (Gardes and Bruns, 1993). The quality and quantity of DNA were estimated by measuring OD 260 nm/OD 280 nm and OD 260 nm, respectively.

Total RNA was extracted from *A. westerdijkiae* using the Tri-reagent (Euromedex France) DNA/RNA/Protein extraction kit. The quality and quantity of RNA was checked by the OD 260 nm/OD 280 nm ratio and agarose gel electrophoresis according to standard protocols (Sambrook et al., 1989).

2.3. Cloning of aomsas gene in A. westerdijkiae

Degenarated primers based Gene walking approach and RACE-PCR was used to clone *aomsas* gene in *A. westerdijkiae*. Fig. 2 marks positions while Table 1 lists all the primers used during this study. The degenerated primer pair LC3/LC5c (Bingle et al., 1999) designed for ketosynthase (KS) domains of MSAS-type PKSs was used to isolate putative PKS gene fragment of 700 bp (Atoui et al., 2006). The degenerated primers MS2 and MS4 targeting the conserved regions GVSAMGFPW and GVVHAAGV were further used with specific primer MS1 allowed by PCR to clone an additional 2 kb of the gene. Using the same strategy, an additional 1.3 kb downstream sequence of the *aomsas* gene was subsequently cloned. Finally the 5′ and 3′ extremity of the *aomsas* gene were subsequently amplified by using a RACE-PCR approach on cDNA (kit supplied by Invitrogen with 5′-primer and 3′-primer). Each time the PCR products obtained were cloned in pCR2.1-Topo vector (Invitrogen) and sequenced. Alignments of the sequenced fragments were performed to search for consensus.

2.4. PCR and sequencing

PCR was performed with the *Taq* recombinant polymerase (Invitrogen, USA). Amplification was carried out in a 50-μL reaction mixture containing: 5 μL of *Taq* polymerase 10× buffer, 1.5 μL of 50 mM $MgCl_2$, 1 μL of dNTP 10 mM of each (Promega), 1 μM of each primer, 1.5 U of *Taq*, about 200 ng of DNA genomic, H_2O up to 50 μL. Reaction conditions were: 94 °C for 4 min, (94 °C for 45 s, 53 °C for 45 s and 72 °C for 1 min) × 30 cycles followed by an incubation at 72 °C for 10 min. The amplified products were examined by 1% (w/v) agarose gel. The PCR products were cloned into pCR2.1-Topo vector according to the supplier's instructions. Sequencing of the fragments was performed by Genomexpress (Grenoble, France).

2.5. Data analysis

The deduced amino acid sequence was determined using the http://www.expasy.org/tools/dna.html site while protein–protein Blast (Blastp) searches were conducted at the GenBank database: http://www.ncbi.nlm.nih.gov. The alignments were conducted using the website http://prodes.toulouse.inra.fr/multalin/multalin.html.

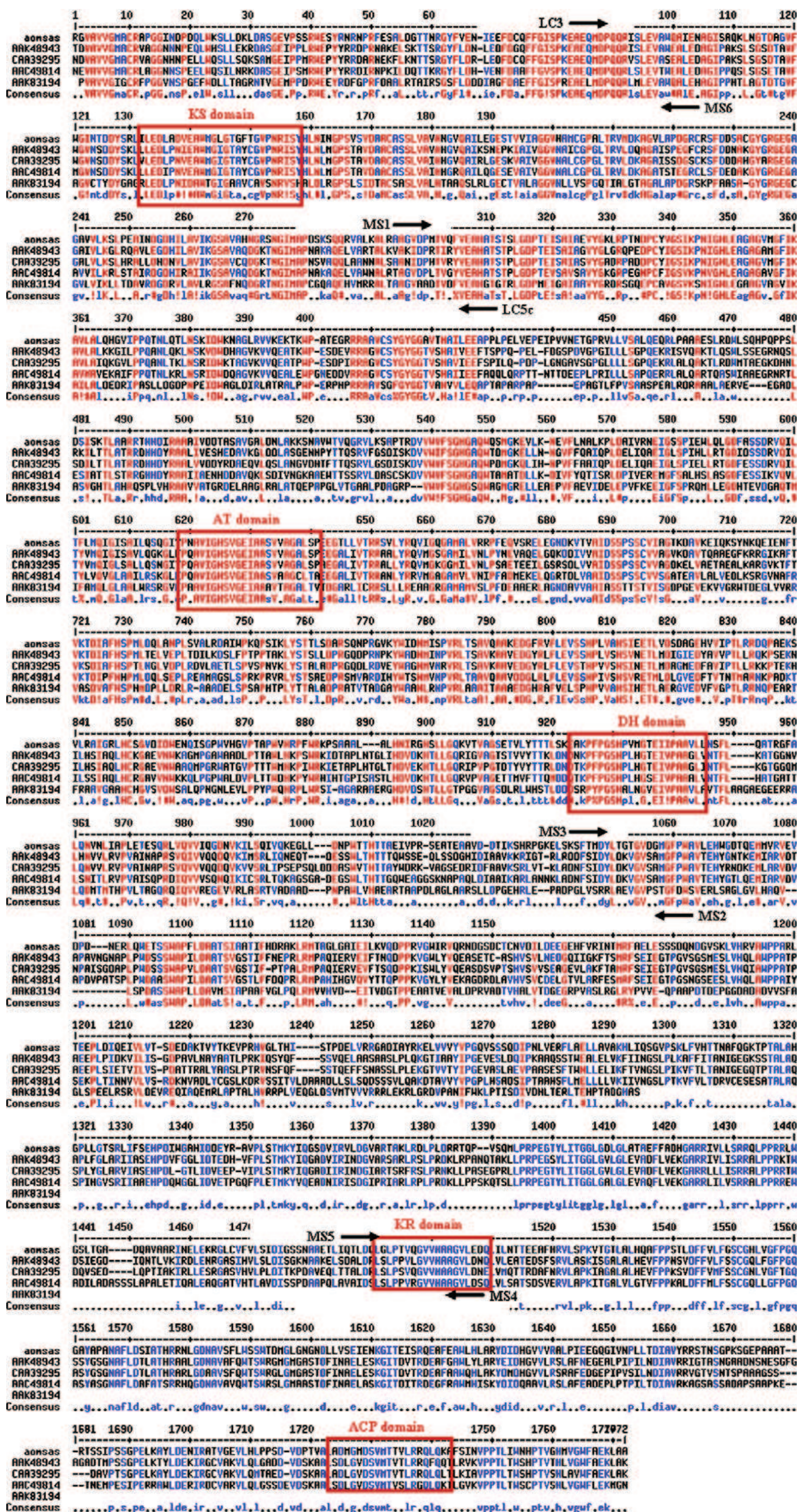

Fig. 2. Alignment of the deduced amino acid sequence of *aomsas* gene with other MSAS-type PKS gene. 6-MSAS involved in the biosynthesis 6-MSA in *Byssochlamys nivea* (Accession No.: AAK48943), 6-MSAS involved in the biosynthesis of patulin in *P. patulum* (Accession No.: CAA39295), 6-MSAS in *A. terreus* (Accession No.: AAC49814) and PKS responsible for orsellinic acid biosynthesis in *Streptomyces viridochromogenes* (Accession No.: AAK83194). Red boxes indicate conserved regions of different functional domains. Flash arrows indicate positions of different specific and degenerated primers (Table 1) used in gene walking experiment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Oligonucleotide primers.

Primer name	Sequence (5′–3′)
LC3	GCIGA(A/G)CA(A/G)ATGGA(T/C)CCICA
LC5c	GTIGAIGTIGC(G/A)TGIGC(T/C)TC
MS1	GCCGCCGGCGTTGACCCGATGAC
MS2	CGGCCIAAGGIAA(T/C)ICCITG
MS3	CTTAGCAAATCATTCACCATGGAC
MS4	(C/T)T(G/C)(A/G)(T/C)(C/T)(A/T)TCCAGCACGCCIGC
MS5	ATCCAAACGCTAGACCAACTCGGC
MS6	CATTTTCGATCGCCTGCCATGCC
MSdF1	ATGCCTTTCTTGGATCCGTCCTCG
MSdR1	CTAGGCAGCTAGTTTCTCCGCGAAC
MSdF	CCGATCGTGTTCAGATCTTGACCTTCCTGATG
MSdR	ACTAAGTTGGGGATGTCCTGAGATGACGAGA
AoLC35-2R	CTGGAGGATCTCGCTGATGT
AoLC35-2L	TTTGATCGACCATTGTGTGC
3′MsR	CGGGGGCAATGAGATATGAAAAAG
3′MsF	GAACCCGCTCGTCTGGCTAAG
hph2R	CTGGATGCAGCAACCTCTAT
hph2F	CTGAGATGACGAACTTGAC
TubF	CTCGAGCGTATGAACGTCTAC
TubR	AAACCCTGGAGGCAGTCGC

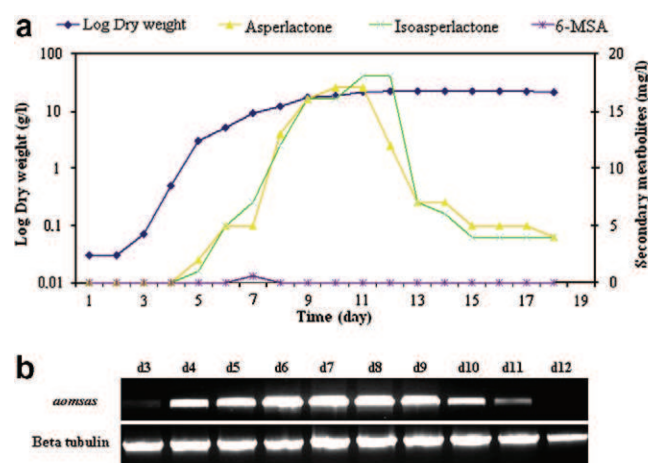

Fig. 3. Kinetic production of secondary metabolites and expression of *aomsas* gene in *A. westerdijkiae*. (a) Kinetic production of isoasperlactone, asperlactone and 6-MSA in a growing culture of *A. westerdijkiae* at 25 °C in synthetic medium during a time course of 2–18 days. (b) Profile of *aomsas* gene expression (upper panel) by RT-PCR. Beta tubuline was used as loading control (bottom panel).

2.6. Reverse transcription PCR

The expression of *aomsas* gene in *A. westerdijkiae* was examined by using reverse transcription PCR (RT-PCR) with two specific primers AoLC35-2L and AoLC35-2R designed from the KS domain of *aomsas* gene (Table 1). For RT-PCR, total RNA was treated with DNase I (Promega) to remove DNA contamination. cDNA was synthesized from each sample with Advantage RT-for-PCR Kit (BD Biosciences) according to the supplier's manual. cDNA amplification were performed using *Taq* recombinant polymerase (Invitrogen, USA). Beta tubulin was used as positive control using primers TubF and TubR (Table 1).

2.7. Plasmid construction and transformation

To construct TopoMShph, the *aomsas* gene replacement vector, a 1995 bp amplified fragment containing SalI restriction site was obtained using MSdF and MSdR primers (Fig. 4a and Table 1). This fragment was ligated into pCR2.1-Topo plasmid generating the TopoMS plasmid. TopoMS and pID2.1 plasmids were restricted using SalI enzyme. The *hph* cassette (2.4 kb) obtained from pID2.1 was ligated with the restricted TopoMS plasmid to produce TopoMShph vector, in which the 5′ and 3′-ends of *hph* cassette are flanked by *aomsas* gene fragments. To construct the complementation vector pAN-MSAS, PCR primers MSdF1 and MSdR1 were designed to amplify a 5-kb *aomsas* gene fragment. The amplified fragment was cloned in pAN8-1 plasmid containing phleomycine resistance cassette.

The *aomsas* gene inactivation and complementary mutant production were achieved by preparing protoplasts of the corresponding strains and transforming with TopoMShph (Fig. 3b) and pAN-MSAS, respectively, as previously described (Jaoanne et al., 2007; O'Callaghan et al., 2003). Lysing enzyme (40 mg/mL) (Sigma) was used for the preparation of protoplasts.

2.8. Screening of the transformants

The *aomsas* disrupted mutants (*aoΔmsas*) were initially selected on YES medium (20 g/L of yeast extract, 1 M sucrose) supplemented with 150 µg/mL of hygromycin B, while the genetically complemented mutants (*ao+msas*) were selected on YES medium supplemented with 120 µg/mL phleomycin. These mutants were further screened through PCRs, using two *hph* gene specific primers hph2R and hph2F (Table 1) and *aomsas* gene specific primers MSdF1, MSdR1 and MSdR. Total genomic DNA from wild type *A. westerdijkiae*, *aoΔmsas* and *ao+msas* strains were digested with enzymes BalI and SmaI and were subjected to southern hybridization (Southern, 1975) to confirm *aomsas* gene disruption and genetic complementation. These restriction enzyme were chosen because they cut the 5′-flanking region of the *aomsas* (position 2054 bp and 2850 bp) but do not cut the *hph* cassette or 3′-flanking end of the *aomsas* gene (Fig. 4b). The digested DNA was then transferred to nylon membrane (Amersham, France) and probed with radioactively labeled DNA fragments of *aomsas1* gene "PM" amplified through primer pair 3′MsF/3′MsR (probe position is shown in Fig. 4b).

2.9. Extraction of secondary metabolites

For secondary metabolites extraction of wild type *A. westerdijkiae*, *aoΔmsas* and *ao+msas* mutants, 30 mL filtrate sample of the culture medium was acidified with 200 µL of 12 N HCl, mixed with 30 mL chloroform and vigorously shaken for 10 min. The solvent phase was then decanted, dried under vacuum and re-dissolved in 0.5 mL methanol. Twenty microliters of the sample was then further analyzed by HPLC.

2.10. High-performance liquid-chromatography (HPLC) analysis

The HPLC apparatus consisted of a solvent delivery system, with both fluorescence (λ_{ex} = 332 nm; λ_{em} = 466 nm) and UV detectors (BIO-TEK, Milan, Italy). The analytical column used was a 150 × 4.6 mm Uptisphere 5 µm C18 ODB fitted with a guard column of 10 × 4 mm. The column temperature was 30 °C. Kroma 3000 (BIO-TEK) was the data acquisition system. Injections were done with an auto-injector (BIO-TEK, Milan, Italy) and the injection volume was 20 µL. The samples were analyzed by linear gradient elution using 0.2% glacial acetic acid in 99.8% water (v/v) (A) and 100% acetonitrile (HPLC grade) (B). The crude extract was analyzed using a linear elution gradient over 45 min at a flow rate of 1 mL/min, starting from 10% to 50% solvent B over the first 30 min, continued by a linear gradient to 90% of B in 5 min, followed by an isocratic flow of 90% solvent B for 8 min, and a return to initial conditions over the last 2 min of the run.

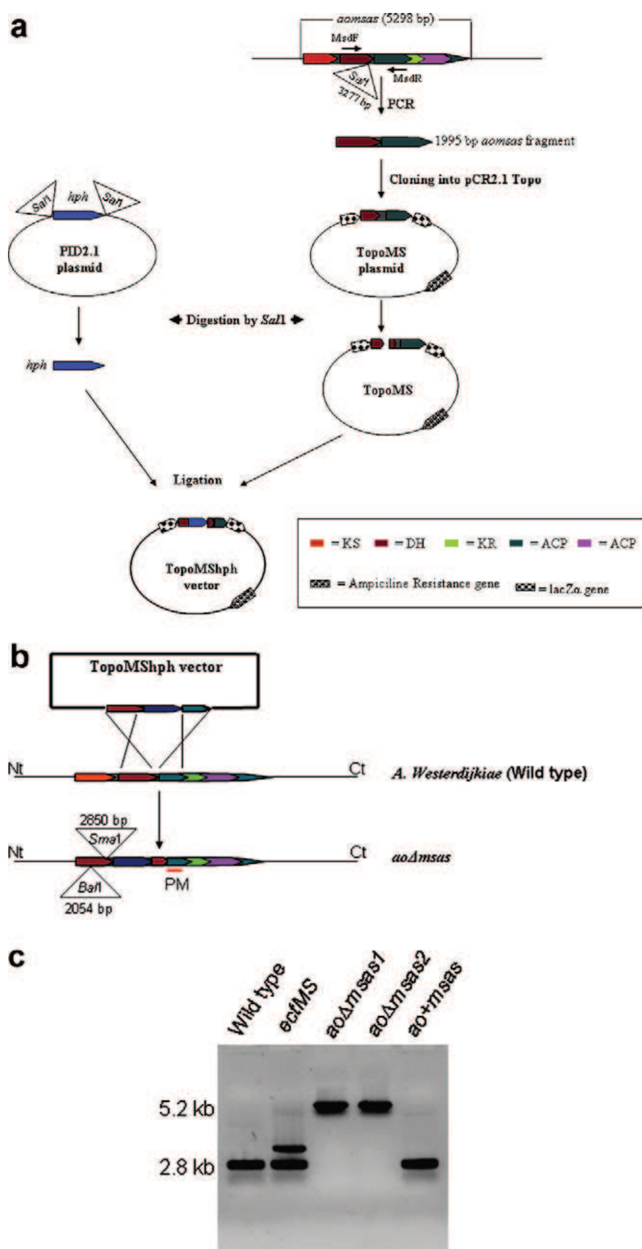

Fig. 4. Schematic representation of *aomsas* gene disruption in *A. westerdijkiae*. (a) Using primer pair MsdF/MsdR (Table 1), 1995 bp *aomsas* fragment containing SalI restriction site (indicated by triangle) was amplified from a 5.298 kb *aomsas* gene. PCR product was cloned into pCR2.1-Topo plasmid to generate plasmid TopoMS. pID2.1 plasmid vector was restricted with SalI (indicated by triangle) to obtain *hph* cassette (2.4 kb). TopoMS was restricted with SalI and ligated with *hph* cassette to genarate TopoMShph transformation vector. Different colors on the *aomsas* gene indicate different functional domains, i.e. β-ketoacyl synthase (KS), acyltransferase (AT), dehydratase (DH), β-ketoacyl reductase (KR), and an acyl carrier protein (ACP). (b) Protoplasts of *A. westerdijkiae* were prepared and *aomsas* gene was disrupted using TopoMShph vector to obtain *aoΔmsas* mutants. The small red line below *aoΔmsas* indicates position of radioactively labeled probe, i.e. PM: *aomsas* gene specific probe amplified by using primer pair AoLC35-2R/AoLC35-2L. Positions of the restriction sites of SmaI and SalI are indicated by triangles. (c) Genomic DNA of wild type *A. westerdijkiae*, *aoΔmsas* and *ao+msas* transformants were digested with SmaI and BalI restriction enzymes and probed with radioactively labeled *aomsas* fragment (PM). In the wild type a 2.8 kb band was detected. In transformant *ectMS* the 2.8 kb band is intacted and an additional band was revealed showing an ectopic insertion of the replacement construct. In the transformants *aoΔmsas1* and *aoΔmsas1* the 2.8 kb band is replaced by the expected 5.2 kb band. In the genetically complemented mutant *ao+msas* the 5.2 kb band is replaced by the wild type 2.8 kb band.

Secondary metabolites of *A. westerdijkiae* were detected by comparing the elution time and maximum absorption of UV with the standard isoasperlactone and asperlactone (Alexis-biochemicals, France) and 6-methylsalicylic acid (ACROS, USA). These standards were used at a concentration of 10 μg/mL. 6-Methylsalicylic acid (maximum absorption λ_{max} = 205, 240, 302 nm) were released at 5 min and isoasperlactone/asperlactone (maximum absorption λ_{max} = 220 nm) were released between 7 min and 9 min.

2.11. Chemical complementation study of aoΔmsas mutants

The *aoΔmsas* mutants were inoculated in 250 mL Erlenmeyer flask containing 100 mL SAM medium and incubated at 25 °C. After 72 h 0.7 mM each of 6-MSA, aspyrone and diepoxide were added independently to separate growing cultures of *aoΔmsas* mutants and again incubated at 25 °C without shaking. After 4 days from precursor addition secondary metabolites were extracted for HPLC analyses.

2.12. Sequence accession number

The sequence obtained was deposited in GenBank under the Accession No. AY540947.

3. Results

3.1. aomsas is a MSAS-type polyketide gene of A. westerdijkiae

With the intent of extending the 700 bp KS domain fragment of a MSAS-type PKS gene identified by Atoui et al. (2006) in *A. westerdijkiae*, we adopted the degenerated primers based gene walking and RACE-PCR techniques. The specific primers MS1, MS3, MS5 and MS6 and degenerated primers MS2 and MS4 (Table 1) allowed the sequencing of a complete 5298 bp *aomsas* gene (Fig. 2). The *aomsas* gene displayed a unique open reading frame (ORF) of 1766 amino acids. Alignment of the amino acid sequence of *aomsas* with other PKS gene displayed an identity of 56% to 6-MSAS gene involved in the biosynthesis 6-MSA in *Byssochlamys nivea*, 54% to 6-MSAS gene involved in the biosynthesis of patulin in *P. patulum* and 50% to a hypothetical 6-MSAS gene in *A. terreus* (Fig. 2). The *aomsas* also displayed a significant identity of 40% with the PKS responsible for orsellinic acid biosynthesis in *Streptomyces viridochromogenes*. Like other 6-MSAS gene, *aomsas* contained characteristic conserved domains of fungal type I PKSs (Fig. 2). These domains were (from N terminus to C terminus) β-ketoacyl synthase, acyltransferase, dehydratase, β-ketoacyl reductase, and an acyl carrier protein. No thioesterase or enoyl-reductase domains were found in *aomsas* gene.

3.2. Asperlactone and isoasperlactone productions and aomsas gene expression in A. westerdijkiae

In liquid synthetic medium (SAM), without shaking at 25 °C, *A. westerdijkiae* presented two exponential growth phases from day 3 to day 5 (early exponential growth phase) and day 6 to day 9 (late exponential growth phase), followed by a stationary phase from day 9 onward (Fig. 3a). Asperlactone and isoasperlactone were simultaneously excreted from day 4 and reach a maximum level at day 9 and day 10, respectively. We have further observed that 6-MSA was transitorily produced in very low quantity between day 6 and day 8 and then disappeared (Fig. 3a).

Tracking the transcriptional signals of *aomsas* gene during different growth stages of *A. westerdijkiae* in SAM medium, we

observed that expression of the gene started from day 3 onwards, stayed constant at maximum level between day 4 and 9 and then stopped after day 11 (Fig. 3b).

3.3. *aomsas gene code for 6-MSA, isoasperlactone and asperlactone in A. westerdijkiae*

Following transformation of *A. westerdijkiae* with TopoMShph vector (Fig. 4b), one hundred and twenty transformants were obtained. Forty transformants were screened by two consecutive PCRs on genomic DNA in order to monitor the integration of *hph* gene into the genome of *A. westerdijkiae*. In only two out of the selected forty transformants the *hph* cassette was detected by the two PCR tests (5% transformation efficiency was obtained). Digested genomic DNA from the wild type *A. westerdijkiae* and the two *aoΔmsas* transformants were subsequently analyzed by southern blotting. In the wild type *A. westerdijkiae* a signal corresponding to 2.8 kb fragment of *aomsas* gene was observed when its digested genomic DNA was probed with PM (Fig. 4c, lane 1). Probing of the *aomsas* disrupted mutants, i.e. *aoΔmsas1* and *aoΔmsas2*, with PM probe resulted into an expected signal of 5.2 kb (2.8 kb correspond to *aomsas* + 2.4 kb correspond to *hph* cassette) (Fig. 4c, lane 3 and 4, respectively). We have also observed one ectopic mutant, i.e. *ectMS*, where the transformation construct was inserted into a non targeted region (Fig. 4c, lane 2).

Wild type *A. westerdijkiae*, *aoΔmsas1*, *aoΔmsas2* and *ectMS*, were grown on solid CYA and liquid SM media, no difference was observed in their colony sizes, growth patterns, visual pigments and conidial counts. Filtrate of the 10 days old culture of wild type *A. westerdijkiae*, *aoΔmsas* and *ectMS* mutants were used to extract secondary metabolites. The HPLC profiles of the extracted secondary metabolites revealed that inactivation of *aomsas* gene by the insertion of *hph* cassette disrupt the production of isoasperlactone, asperlactone (Fig. 5a) and the transitory biosynthesis of 6-MSA. No impact of the ectopic insertion was observed on the biosynthesis of secondary metabolites (data not shown).

To confirm the role of *aomsas* gene, genetic complementation of the two *aoΔmsas* mutants were performed with pAN-MSAS transformation vector (containing *aomsas* gene fragment and phleomycine gene cassette). Filtrate of the 10 days old culture of positively tested complemented mutant *ao+msas* were used to extract secondary metabolites. HPLC profile of *ao+msas* mutant revealed that re-insertion of *aomsas* gene restored the productions of asperlactone, isoasperlactone and 6-MSA (Fig. 5a).

3.4. *6-MSA, diepoxide and aspyrone are intermediates of isoasperlactone and asperlactone*

Chemical complementation was performed by independent incorporation of 6-MSA (0.7 mM), diepoxide (0.7 mM) and aspyrone (0.7 mM) to separate growing cultures of *aoΔmsas* mutants. HPLC profile and UV spectra of the chemically complemented mutants revealed that addition of 6-MSA, diepoxide and aspyrone produced similar effects and restored the biosynthesis of both isoasperlactone and asperlactone (Fig. 5b).

4. Discussion

From the analyses of fungal PKS gene sequences, Bingle et al. (1999) suggested that these genes could be divided into two subclasses designated as WA-type and MSAS-type or partially reduced type. They designed two pairs of degenerated primers, i.e. LC1/LC2c and LC3/LC5c for the amplification of fungal PKS genes in each of these subclasses. We have previously utilized LC3/LC5c pair of primer to clone a 700 bp KS domain of *aomsas* gene in *A. westerdijkiae*

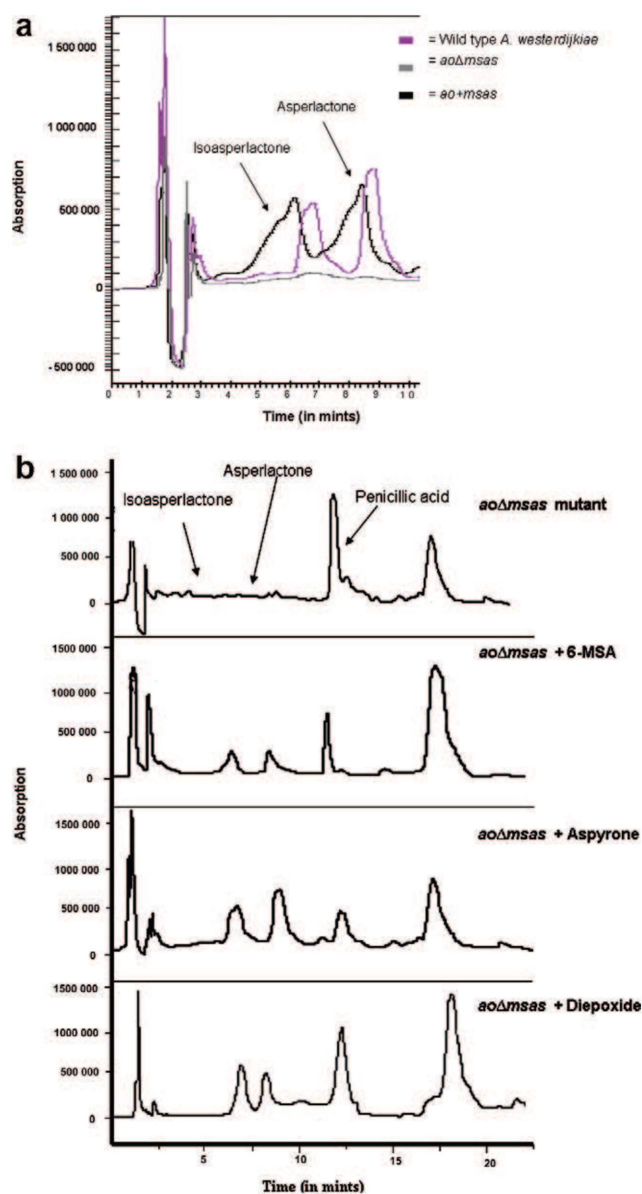

Fig. 5. Secondary metabolites study. (a) Secondary metabolites were extracted from 10 days old cultures of wild type, *aoΔmsas* and *ao+msas* strains of *A. westerdijkiae*. HPLC traces of the lactonic metabolites, i.e. isoasperlactone (eluted at 6 min) and asperlactone (eluted at 9 min) were compared. (b) Separate growing cultures of *aoΔmsas* mutant in synthetic medium at 25 °C were independently complemented with 0.7 mM 6-each of methylsalicylic acid, aspyrone and diepoxide. Secondary metabolites were extracted from 8 days old cultures and analyzed by tracking the HPLC traces.

(Atoui et al., 2006). After prolongation of this fragment by gene walking approach to a complete 5298 bp gene and alignment in database with other PKS genes, we have observed that the amino acid sequence of *aomsas* has high degree similarities to fungal MSAS-type PKS genes (Fig. 2). The different conserved domains identified in *aomsas* further confirmed that this gene belongs to the MSAS-type subclass (Fig. 2). These observations are in accordance to the findings of Bingle et al. (1999), who stated that the degenerated pair of primers LC3/LC5c has the capability to amplify fungal MSAS-type PKS genes.

To understand the role of *aomsas* gene in *A. westerdijkiae*, we first investigated the kinetic production of secondary metabolites and the expression of the *aomsas* gene. We have observed that both transcriptional signals of *aomsas* and biosynthesis of asper-

lactone, isoasperlactone and 6-MSA occur within the exponential growth phase of *A. westerdijkiae* (Fig. 3). These observations suggest that *aomsas* could be related to the biosynthesis of asperlactone and/or isoasperlactone and/or 6-MSA. It has been reported in the literature that MSAS-type PKS genes are involved in the biosynthesis of several olyketide metabolites varying greatly in their functions. An MSAS-type PKS gene *6msas* has been found to be involved in the biosynthesis myctoxine patuline in *P. patulum*, *P. urticae* and *Byssochlamys fulva* (Beck et al., 1990; Bu'Lock et al., 1968; Puel et al., 2007). Another MSAS gene *AviM* has been found to be involved in the biosynthesis of antibiotic avilamycin in *S. viridochromogenes* (Gaisser et al., 1997). The MSAS gene has also been reported to induce the production plant defense compound salicylic acid in tobacco (Nasser et al., 2001). Although *A. westerdijkiae* is a non-producer of patuline, avilamycin and salicylic acid but this do not preclude the possibility of having an MSAS-type PKS gene in its genome. A precedent of such a situation is provided by Geisen (1996), who used a multiplex PCR method to identify sequences homologous to aflatoxin biosynthesis gene in the non-producing species of *Aspergillus* and *Penicillium* (Geisen, 1996).

Insertional inactivation remained the key technique for functional characterization of various fungal pks genes (Atoui et al., 2006; Bacha et al., 2009; O'Callaghan et al., 2003). So we followed similar technique to produce *aomsas* knockout mutants "*aoΔmsas1*" and "*aoΔmsas2*". These mutants were found deficient in the biosynthesis of two end product lactonic metabolites, i.e. isoasperlactone and asperlactone (Fig. 4a), and one compound of intermediate nature, i.e. 6-MSA. These observations confirmed that *aomsas* played an important role in the biosynthesis of 6-MSA, isoasperlactone and asperlactone. We further proved these results by performing genetic complementation, where the genetically complemented mutant *ao+msas* restored the biosynthesis of all the missing metabolites (Fig. 4a).

It has been previously demonstrated through NMR studies that diepoxide and aspyrone are intermediates in the biosynthetic pathway of isoasperlactone and asperlactone in *A. melleus* (Fig. 1) (James and Andrew, 1991). These authors further stated that asperlactone is directly biosynthesized from the diepoxide, while in the biosynthesis isoasperlactone the diepoxide is first converted to aspyrone and then aspyrone to isoasperlactone. They also suggested an alternate rout for the biosynthesis isoasperlactone without the involvement of aspyrone. Chemical complementation of the asperlactone/isoasperlactone deficient mutant, i.e. *aoΔmsas*, through incorporation of 6-MSA, diepoxide and aspyrone clearly shown that, these products are intermediates in the biosynthetic pathway of asperlactone and isoasperlactone (Fig. 5b). Indeed each of the incorporated metabolite restored simultaneously the biosynthesis of both asperlactone and isoasperlactone. Aspyrone and diepoxide are never observed in the culture medium of wild type *A. westerdijkiae*. This could probably be due to a quick conversion of these compounds to their final products (asperlactone and isoasperlactone), avoiding an intracellular accumulation and extracellular excretion.

Based on our results and the findings of James and Andrew, (1991) we proposed a biosynthetic scheme for the biosynthesis of isoasperlactone and asperlactone (Fig. 1). According to this scheme, during the early biosynthetic steps *aomsas* gene induces the production of 6-MSA, which is then converted to diepoxide. The diepoxide could then follow two routes, one that leads directly to the production of asperlactone while the second leads to the production of isoasperlactone via aspyrone pathway. We have observed during chemical complementation experiment that even incorporation of aspyrone without diepoxide restored the production asperlactone and isoasperlactone in *aoΔmsas* mutant. Previously James and Andrew, (1991) did not proposed this scheme; they proposed aspyrone as intermediate of isoasperlactone but not of asperlactone. Hence we proposed that diepoxide and aspyrone could be enter-convertible compounds which could lead to the production of both asperlactone and isoasperlactone. Our observations are in accordance to the previous findings, suggesting that lactonic metabolites asperlactone and isoasperlactone could be derived from a common biosynthetic precursor, a diepoxy intermediate (Sorensen and Simpson, 1986).

This new *aomsas* gene can be used for further experiments, as a starting material for the identification of asperlactone/isoasperlactone gene cluster.

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