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Molecular analysis of the mating type (*MAT1*) locus in strains of the heterothallic ascomycete *Botrytis cinerea*

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Botrytis cinerea shows a heterothallic bipolar mating-type system; homothallism has been occasionally observed. *MAT1* genes and flanking regions in the reference strains SAS56 (*MAT1-1*) and SAS405 (*MAT1-2*) and their monoascosporic progeny were analysed. The two mating types confirmed different sequences of 2513 bp (*MAT1-1*) and 2776 bp (*MAT1-2*), flanked by near identical regions. In all isolates, each idiomorph included two mating-type specific genes: *MAT1-1-1* (1161 bp), encoding an alpha-domain containing protein, and *MAT1-1-5* (1301 bp); or *MAT1-2-1* (1236 bp), encoding a HMG-domain protein, and *MAT1-2-4* (712 bp); the latter genes encode putative proteins of unknown function. Truncated *MAT1-1-1* (670 bp) and *MAT1-2-1* (92 bp) sequences of the opposite mating-type were found in the flanking regions. Idiomorph-specific PCR primer pairs were used to explore the structure of the *MAT1* locus in ascospore progeny and field isolates showing homothallic behaviour, and the locus organization in all of them did not differ from that of heterothallic strains. Constitutive expression of all the four mating-type genes was ascertained by RT-PCR at four different developmental stages (mycelium, sclerotia at two different stages and apothecia). Antisense transcription of the *MAT1-2-1* gene with isoforms from alternative splicing was detected. Comparative analysis of *MAT1* loci in *B. cinerea* and in the closely related homothallic *Sclerotinia sclerotiorum* led to the identification of short nearly identical sequences.

Keywords: antisense transcription, *Botryotinia fuckeliana*, homothallism, mating-type gene expression

Introduction

Botrytis cinerea (anamorph of *Botryotinia fuckeliana*) is the fungus responsible for grey mould, a disease causing severe yield losses on numerous economically important crops, especially grapevine and greenhouse crops, all around the world (Williamson *et al.*, 2007). The fungus is an ascomycete belonging to the family Sclerotiniaceae, along with fungi of the genera *Monilinia* and *Sclerotinia*. The sexual process in Sclerotiniaceae was unknown until Whetzel (1929) suggested that microconidia of *B. fuckeliana* could act as male gametes (spermatia).

Sexual reproduction in fungi is under the control of specialized genomic regions known as 'mating type' (*MAT*) loci. In ascomycetes, a single *MAT1* locus controls sexual compatibility and mating. In heterothallic species, the locus has two alternative forms, nonhomologous sequences having identical chromosomal localization. These sequences, known as idiomorphs, have been characterized in several filamentous ascomycetes, and are variable in size and in number of included genes (Debuchy & Turgeon, 2006). The two idiomorphs contain genes determining mating identity and controlling sexual development, coding for proteins regulating gene expression with typical functional domains: 'alpha-box' and 'HMG' (high mobility group) for the mating types *MAT1-1* and *MAT1-2*, respectively. Hence, although the *MAT1* locus has near identical flanking regions, the *MAT1* genes of the two idiomorphs do not display any homology in nucleotide sequences, although some relations between the functional domains of *MAT1* proteins have been found (Martin *et al.*, 2010).

The mating system in *B. cinerea* was first investigated by Groves & Loveland (1953), who concluded that the fungus is heterothallic. Apothecia were obtained under laboratory conditions (Faretra & Antonacci, 1987), which allowed improved knowledge on the mating system of the fungus, confirming that it is controlled by a single locus with two idiomorphs. The locus has been named *MAT1*; the mating type *MAT1-1* was arbitrarily assigned to the strain SAS56, and *MAT1-2* to the strain SAS405 (Faretra *et al.*, 1988a,b; Faretra & Grindle, 1992). The two mating types have been commonly found on different host plants and in different countries, although MAT1-1 isolates slightly predominated (Faretra *et al.*, 1988b; Beever & Parkes, 1993; Faretra & Pollastro, 1993; van der Vlugt-Bergmans *et al.*, 1993; Delcán & Melgarejo, 2002). This is in agreement with the finding that mating type does not influence the fitness, as shown by comparing near-isogenic strains differing for mating type (Pollastro *et al.*, 1996).

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Some *B. cinerea* isolates fertile with both MAT1-1 and MAT1-2 strains, and often self-fertile (MAT1-1/2), referred to as pseudohomothallic (Faretra & Grindle, 1992) or dual maters (Amselem *et al.*, 2011), were first detected by Lorenz & Eichhorn (1983) in Germany, and then reported in Europe and other countries (Faretra *et al.*, 1988b; Beever & Parkes, 1993; Faretra & Pollastro, 1993; van der Vlugt-Bergmans *et al.*, 1993; Delcán & Melgarejo, 2002). Several mechanisms are responsible for homothallic behaviour in heterothallic fungi, such as heterokaryosis, regular inclusion of nuclei having opposite mating types in single ascospores, coexistence of both idiomorphs in heteroploid isolates, transposition of the *MAT* genes and unisexuality (Roach *et al.*, 2014; Wilson *et al.*, 2015). In *B. cinerea*, heterokaryosis appears to be the main cause of pseudohomothallism of field and monoconidial isolates, as it has been shown that single multinucleate conidia may contain nuclei carrying opposite idiomorphs (Faretra *et al.*, 1988b). The mechanisms causing homothallic behaviour in monoascosporic isolates of the fungus have not yet been clarified. Indeed, cytological observations showed that all the nuclei present in a multinucleate ascospore derive from a single meiotic nucleus though mitotic divisions (Lorenz & Eichhorn, 1983; Faretra & Antonacci, 1987). This has also been supported by tetrad analysis in complete asci (Faretra & Pollastro, 1996).

The structure of the mating type locus (*MAT1*) of *B. cinerea* has previously been reported by Amselem *et al.* (2011) who identified two open reading frames (ORFs) in each idiomorph. The aims of the present study were: (i) to extend characterization of *MAT1* locus and transcript analysis of mating-type genes to other reference strains of the fungus and their progeny, including isolates showing homothallic behaviour; (ii) to assess mating-type gene expression at different vegetative and sexual stages; and (iii) to set up and validate a PCR-based method for assessing mating type of uncharacterized isolates.

Materials and methods

Fungal isolates and media

The strains SAS56 (*MAT1-1*) and SAS405 (*MAT1-2*) were used as references for mating type (Faretra *et al.*, 1988b). The homothallic behaviour was investigated by using monoascosporic strains from three complete asci (SAS25–SAS32, SAS33–SAS40, SAS41–SAS48), from the cross SAS405 × SAS56, which included ascospores expected to be *MAT1-2* but proved fertile with reference strains of opposite mating type (Faretra & Pollastro, 1996). Eighteen homothallic monoascosporic strains and 18 field isolates of different origin were also tested.

The following media were used (ingredients per litre distilled water): water agar (WA, 20 g agar) and malt extract agar (MEA, 20 g Oxoid malt extract and 20 g agar). Agar was technical grade (Oxoid no. 3).

All isolates were routinely grown on MEA at 21 ± 1 °C in the darkness. For long-term storage, suspensions of conidia and mycelium of each isolate were maintained at −80 °C in 10% glycerol and revitalized on MEA just before use.

Mating experiments

Isolates were mated to obtain apothecia as described by Faretra *et al.* (1988b). Briefly, isolates grown on MEA in the dark at 21 ± 1 °C for 2 days were incubated at 15 ± 1 °C for 4 weeks to obtain sclerotia, and then at 0 ± 1 °C for a further 4 weeks to induce carpogenesis. After spermatization, sclerotia were exposed to a combination of halogen (Osram, 64543 A ECO) and fluorescent (Osram L40W/20SA) lamps at 11 ± 1 °C to obtain apothecia containing asci and ascospores. Ascospores were spread at low density on WA, then collected singly under a dissecting microscope, with the aid of a micromanipulator for tetrad analysis, and transferred to fresh MEA.

Molecular analysis

Genomic DNA was extracted and purified, basically according to Murray & Thompson (1980), from 2-day-old colonies of *B. cinerea* grown at 21 ± 1 °C on cellophane disks overlaid on MEA. In brief, mycelium was collected by scraping the cellophane, powdered under liquid nitrogen and added to 600 μL CTAB buffer [100 mM Tris-Cl, pH 8.0; 1.4 M NaCl; 20 mM EDTA, pH 8.0; 2% cetyltrimethylethylammonium bromide (w/v); 0.2% β-mercaptoethanol (v/v)]. The samples were frozen and thawed three times using liquid nitrogen and a water bath at 75 °C, and then incubated at 75 °C for 1 h. After chloroform extraction, the clear supernatant was transferred to a new tube and precipitated with isopropanol at −80 °C for 30 min. The tube was centrifuged at 22 000 ***g*** for 15 min, and then the nucleic acid pellet was washed with cold 70% ethanol, air-dried and dissolved in TE (10 mM Tris-Cl; 1 mM EDTA, pH 8.0). The solution was treated with 0.1 μg μL^{-1} DNAase-free pancreatic RNAase (Sigma-Aldrich) for 2 h at 37 °C, and precipitated by the addition of 0.6 vol. 5 M ammonium acetate and 2 vol. cold absolute ethanol. The final DNA pellet, washed with 70% ethanol and air-dried, was dissolved in water and stored at −80 °C until use.

The approach for the sequencing of the mating type locus was the primer-walking method (Sterky & Lundeberg, 2000). All primers were designed using Primer3 software (Rozen & Skaletsky, 2000) or LASERGENE PrimerSelect (v. 8.0.2, DNASTAR Inc.), and synthesized by an external service (Sigma-Aldrich). The primer pairs selected for investigating genomic sequences at the *MAT1* locus and the size of amplified DNA fragments are reported in Table 1. Reaction mixtures (50 μL) contained 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.1 μM each primer, 0.1–0.3 μg of template DNA and 2 U LA *Taq* DNA polymerase (Takara Bio Inc.). Amplifications were carried out in a GeneAmp PCR System 9700 thermal cycler (Perkin-Elmer) set as follows: 95 °C for 5 min; followed by 25 cycles of 1 min denaturation at 95 °C, 1 min annealing at 60 °C and 1 min extension at 72 °C; and a final extension of 7 min at 72 °C. For amplification with primer pairs B1Fw/B1Rev and B2Fw/B2Rev only, the annealing was at 59 °C and extension was for 3 min. Negative controls, in which water was used instead of the target DNA, were run each time to detect potential contaminations.

For the observations on homothallic strains and for the assessment of mating type of fungal isolates, PCR conditions were the same as above but the reaction mixture (25 μL) contained: 0.1 μg of template DNA, 1 × Green GoTaq Flexi buffer, 2 mM $MgCl_2$, 75 μM each nucleotide (dATP, dGTP, dCTP, dTTP), 0.75 U GoTaq DNA polymerase (Promega) and 0.5 μM each primer.

Table 1 Primer pairs used for PCR and sequencing of the *MAT1* locus of *Botrytis cinerea*

Primer	Sequence (5′–3′)	Amplicon size (bp)
MAT1.1	AAGCTTCGATGACCCTTTGA	1295
MAT1.1295	GATCGTGGAGCCGAGATAAT	
A1Fw	GTGAGTGGAATGGGAAGGAA	1294
A1Rev	CCAGCCAATGGGTATGAACT	
A2Fw	TCGTGAAGAGTCGACGAGTT	1273
A2Rev	GGGCATCACAGGTGAAGAAT	
A3Fw	TCAAAGATGAGTCGCATAGCA	1137
A3Rev	TTTGCTGTCGCTTGTAGGTG	
A3bFw	ATGTCGTGTGCATGAAGGAG	617
A3bRev	GGTTTCTGTGTGGCTGGAGT	
A4Fw	AGATGTCCCTTCACCACCAC	1205
A4Rev	GAACTCGGGCTTGAAGATTG	
A5Fw	TTTACTCACGCCTGATGTCG	1116
A5Rev	CAACAGTCCGGAACAACTCA	
HMG3.flank	GTGACCAGGAAACAGCTATGACCGCTCCTTTCCATAAGTCGTAAGTCGTG	1414
HMG5.flank[a]	GTGACTGTAAAACGACGGCCAGTCAAGATCAGACGGAGTGCATTACCTC	
B1Fw	CCGCCATTGAATTCCGCACAG	6460
B1Rev	TCCACCGAATCCTCCATCAACTAT	
B2Fw	AGCGGCGTTTCGAGGACAGT	4344
B2Rev	GGCCGGTTTCATGAGCAAGAG	
B10Fw	CCAGACGGCCCAAGGTAAAC	2350
B14Rev	ATCGCCAACTATCAAGACTCATCA	
B16Fw	ACCATCTCAGCTGCCATACC	840
B16Rev	TAGCTCCATGCTGGAGTGTG	
B18Fw	GTTCGGTCCATGCTTGTGTA	450
B19Rev	AAACGAACCGATCTCTGGTG	

[a]Kindly supplied by J. van Kan (Wageningen University, Netherlands).

Aliquots of DNA (10 μL) were size-separated on 1.5% agarose (Bio-Rad) horizontal gel run in 0.5 × TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0) buffer at 110 V for 110 min. For transcript analysis, PCR amplicons were separated on 8% polyacrylamide gel run in 1 × TBE buffer at 80 V for 180 min.

PCR amplicons were sequenced by external services (Macrogen Europe). DNASTAR v. 8.0.2 (Lasergene) was used for sequence analysis and BLASTX was used for similarity search and multiple sequence alignments with sequences available in the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). The predicted amino acid sequences were analysed by using PROTEAN software (Lasergene).

Transcript analysis

Transcription of the *MAT1* genes in the reference strains SAS56 and SAS405 was verified at the following development stages of the life cycle of *B. cinerea*: (i) mycelium actively growing on MEA at 21 ± 1 °C for 2–3 days; (ii) mature sclerotia differentiated at 15 ± 1 °C for 4 weeks in the darkness; (iii) sclerotia induced to carpogenesis through incubation at 0 ± 1 °C for a further 4 weeks; (iv) fully developed apothecia. Total RNA was extracted from about 130 mg of fungal tissue by using RNeasy Plant Mini kit (QIAGEN) and purified on spin columns with RNase-Free DNase set (QIAGEN), according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA by using iScript cDNA Synthesis kit (Bio-Rad), starting the reaction with a mixture of oligo(dT) and random hexamer primers, or Transcriptor High Fidelity cDNA Synthesis kit (Roche Applied Science) with transcript-specific primers. A 2 μL cDNA aliquot was PCR-amplified as described above using GoTaq DNA polymerase (Promega) and specific primer pairs (Table 2). The RT-PCR product was amplified twice with the same primers following electrophoretic separation and gel elution, and then sequenced.

Results

Characterization of the *MAT1* locus

The sequence of the supercontig 163 between the two genes encoding the cytochrome C oxidase subunit VIa (*COX13*) (BC1G_15145) and the cytoskeleton assembly control protein (*SLA2*) (BC1G_15149), in the flanking regions of the *MAT1* locus, was selected in the available genome of the *MAT1-1* strain B05.10 of *B. cinerea* (GenBank accession number: GCA_000143535.2). It includes an endonuclease DNA lyase (*APN2*) (BC1G_15146), a predicted protein gene (BC1G_15147), and the *MAT1-1-1* gene (BC1G_14148). Seven primer pairs (MAT1.1/MAT1.1295, A1Fw/A1Rev, A2Fw/A2Rev, A3Fw/A3Rev, A3bFw/A3bRev, A4Fw/A4Rev and A5Fw/A5Rev; Table 1) were designed from the genome sequence included between the *APN2* and *SLA2* genes and used for sequencing, as overlapping contigs, the entire *MAT1-1* idiomorph and flanking regions in the reference strains SAS56 and SAS47. The whole assembled sequence (6433 bp) was identical to that of the B05.10 strain.

The primer pair HMG3.flank/HMG5.flank (Table 1) was used to amplify the *MAT1-2-1* gene of the reference

Table 2 PCR primer pairs for transcript analysis

Gene	Primers	Sequence (5′–3′)	Amplicon size		Intron size (bp)
			cDNA (bp)	gDNA (bp)	
MAT1-1-1 (alpha-domain gene)	Y23Fw	AATGCAGAAGAGCCAACGAT	577	627	50
	Y649Rev	CGGTTCCAACCTTGAACAAT			
	Y2Fw1	ATTTGGACATCATCACCT	470	519	49
	Y2Rev	CTAGAAGTCGGCAAAGA			
	αI33Fw[a]	CACCTACAAGCGACAGCAAA	247	–	–
	αY280Rev[a]	CCGTCAAAATCATGTAGAAGGTACG			
MAT1-2-1 (HMG-domain gene)	A437Fw	AGGTTTCGCCAATCTGTGTCTG	402	457	55
	B893Rev	CTTGGCTTTCTAGGGGCGTATTT			
	I26Fw	GAAAATGGGTACCGCATCAC	422	472	50
	I497Rev	GCCGTTCATCATTCTTCGTT			
	HmgIII268Fw[a]	CTTCATTTTTATCAGCAACACACC	180	–	–
	I497Rev[a]	GCCGTTCATCATTCTTCGTT			
	HmgII250Fw[a]	CCAGAACGTCAAATTTATTGTAGGC	148	–	–
	I456Rev[a]	GACACAGATTGGCGAAACCT			
	HmgI253Fw[a]	GAACGTCAAATTCTATATTGTAGGC	148	–	–
	I456Rev[a]	GACACAGATTGGCGAAACCT			
MAT1-1-5 (predicted protein)	Pp1.156Fw	TTTGGACGCAACACTTATCG	571	620	49
	Pp1.726Rev	GGGCATCACAGGTGAAGAAT			
	Pp1.830Fw	CGAAGGATCACGACCATGTA	101	222	62 + 59
	Pp1.1051Rev	GGAGCAGGAAAATCATCCAA			
MAT1-2-4 (predicted protein)	Pp2.12Fw	GCAGCGTCATTCACAAAGAG	600	697	49 + 48
	Pp2.587Rev	AGCTCAACCCAATGATGTCC			
	Pp2II.31Fw	GTGGAGACATCAACCAGACG	557	921	364
	Pp2.587Rev	AGCTCAACCCAATGATGTCC			
	Pp2.625Fw	CATATTTCAGCGCGAGTTCA	196	244	48
	Pp2.868Rev	TGTCTCCTTTGCCCAAGAGT			

[a]Transcript-specific primer pairs with the forward primer designed on the intron splicing site used in nested RT-PCR to avoid amplification of genomic DNA.

strain SAS405. Two primers (B1Rev, B2Fw) were designed from the obtained sequence and used in pairs with the primers B1Fw and B2Rev designed from the flanking regions of the mating type locus of the strain B05.10 (see above). Subsequently, three primer pairs (B10Fw/B14Rev, B16Fw/B16Rev and B18Fw/B19Rev; Table 1) were designed from the obtained sequences and yielded overlapping contigs encompassing the whole *MAT1-2* idiomorph. The whole assembled sequence (4501 bp) was identical in the two strains tested, SAS405 (MAT1-2) and SAS43 (MAT1-1/2). The 5′ terminus of 514 bp and the 3′ terminus of 753 bp showed 99% homology with B05.10. Hence, it was possible to ascertain that a 2513 bp sequence was unique for the *MAT1-1* idiomorph and that a 2776 bp sequence was unique for the *MAT1-2* idiomorph.

The sequences were analysed with the 'Start/Stop/ORF' procedure of the GeneQuest programme of the Lasergene software package by using the Borodovsky's GeneMark method (http://opal.biology.gatech.edu/GeneMark/) to predict the open reading frames (ORFs) and putative splicing sites. BLASTX and BLASTN analysis suggested the gene structure and the presence of introns and exons (Fig. 1).

The *MAT1-1* idiomorph includes two ORFs. The *MAT1-1-1* gene codes for a protein (XP_001546438) containing the alpha domain (152 aa), with a 75% identity with the homologous protein of *Sclerotinia sclerotiorum* (XP_001594197). The gene contains two introns (50 and 49 bp) and three exons (259, 786 and 17 bp) and extends for 670 bp in the 3′ flanking region of the idiomorph (Fig. 1). The *MAT1-1-5* gene codes for a putative protein with a 77% identity with a putative protein of *S. sclerotiorum* (XP_001594196). The gene contains three introns (49, 62 and 59 bp) and four exons (280, 548, 27 and 276 bp). The 5′ flanking region of the *MAT1-1* idiomorph contains a 92 bp sequence (*dMAT1-2-1*) identical to the 3′-terminal sequence of the *MAT1-2-1* gene (Fig. 1).

The *MAT1-2* idiomorph includes two ORFs. The *MAT1-2-1* gene codes for a putative protein containing the HMG domain (77 aa), with 77% identity with the homologous *S. sclerotiorum* protein (XP_001594199). The gene extends for 92 bp in the 5′ flanking region of the *MAT1-2* idiomorph (Fig. 1) and contains two introns (50 and 55 bp) and three exons (281, 374 and 476 bp). Computational analysis of the structure of the *MAT1-2-4* gene, yielded two alternative predictions: (i) a 712 bp gene containing two introns (49 and 48 bp) and three exons (191, 239 and 185 bp); (ii) a 955 bp gene containing two introns (316 and 48 bp) and three exons (167, 239 and 185 bp). Both translated amino acid sequences

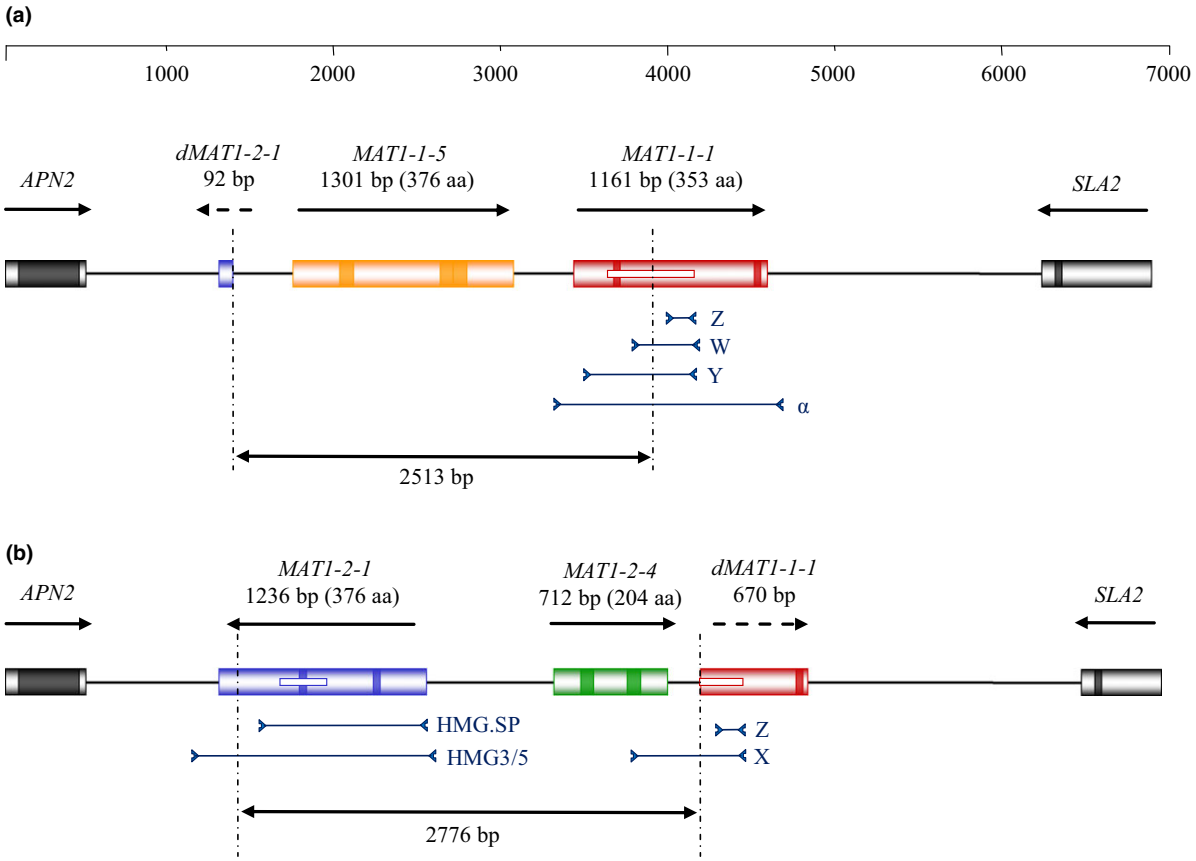

Figure 1 Structures of the mating-type (*MAT1*) locus in the *MAT1-1* (a) and *MAT1-2* (b) idiomorphs of *Botrytis cinerea*. Solid boxes represent the predicted genes interrupted by introns (full coloured boxes). Internal white box in the *MAT1-1-1* (alpha-box) and *MAT1-2-1* (HMG-box) genes indicate the sequences encoding putative conserved domains (DNA binding motifs). Arrows on the top indicate orientation of coding sequences. Sizes of each gene, their encoded proteins, and the unique sequences of each idiomorph (delimited by dotted lines) are noticed. The adjacent genes, labelled *APN2* (encoding DNA lyase) and *SLA2* (encoding a cytoskeletal protein) are represented by solid black boxes in the near-identical flanking sequences of both mating types of *B. cinerea*. Arrows on the bottom indicate primer pairs used to confirm the genetic structure and to amplify selectively DNA sequences common (Z) or specific for the *MAT1-1* (α, Y and W) or *MAT1-2* (HMG.SP, HMG3/5 and X) idiomorphs of *B. cinerea* (see Table 2).

(204 and 196 aa, respectively) showed 61% identity with a predicted protein of *S. sclerotiorum* (XP_001594198). The 3′ flanking region of the *MAT1-2* idiomorph contains a 670 bp sequence (*dMAT1-1-1*) showing high homology (99%) with the 3′ terminal portion of the *MAT1-1-1* gene (Fig. 1).

The assembled sequence of the *MAT1* locus of the strain SAS405 (4501 bp) was compared with the corresponding sequence (bt4_SupSuperContig_246_31_4_1) of the *MAT1-2* strain T4 (genome available at http://urgi.versailles.inra.fr/Species/Botrytis). Overall, 16 SNPs were identified within both coding (4) and noncoding (12) sequences. In detail, two synonymous substitutions (G→A and T→C at positions 948 and 1086) and one transition in an intron (G→A at position 286) were found in the *MAT1-2-1* gene of SAS405, whereas two substitutions (T→G and A→G at positions 410 and 458) in the *MAT1-2-4* gene lead to amino acid replacements (F121V and S137G). Furthermore, a duplication of 12 nucleotides was detected in the coding region of the *MAT1-2-1* gene of the T4 strain (causing a short duplicated sequence of four amino acids, TESS) at position 293–296 in the predicted protein. This amino acid duplication, outside the conserved functional domain, is unlikely to affect gene function because there were no substantial differences in biophysical and structural properties between the protein variants (data not shown).

The sequences of the *MAT1* locus, its gene structure and flanking regions in the *MAT1-1* strain SAS56 and the *MAT1-2* strain SAS405 have been deposited in the GenBank database under accession numbers KF944385.1 and KF944386, respectively.

Transcript analysis

The predicted structure of the genes in the *MAT1-1* and *MAT1-2* idiomorphs was investigated by transcript analysis using the primer pairs reported in Table 2. All

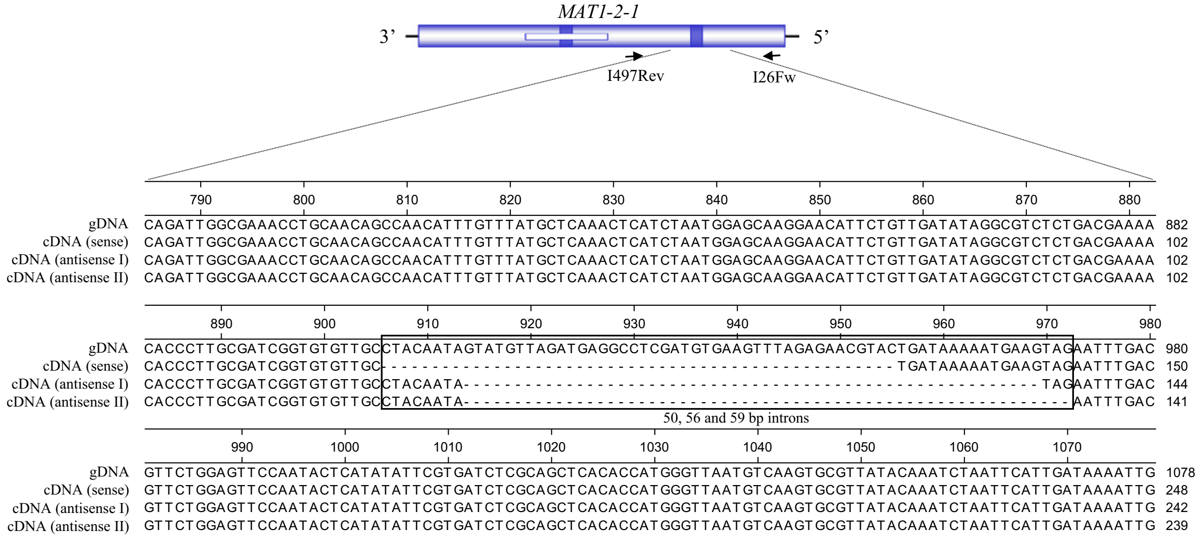

Figure 2 Alignment of partial cDNA sequences of alternative transcripts of the *MAT1-2-1* gene on the genomic DNA (gDNA) of *Botrytis cinerea*. Alternative splicing sites are boxed in solid lines.

primer pairs were designed from sequence regions encompassing single predicted introns in order to distinguish PCR products derived from cDNA or contaminant genomic DNA. Transcript analysis confirmed the predicted gene structure as to number and position of introns of all the genes in the *MAT1* locus (Table 2). For the gene *MAT1-2-4*, two primer pairs (Pp2.12Fw/Pp2.587Rev, Pp2.625Fw/Pp2.868Rev) and one pair (Pp2II.31Fw/Pp2.587Rev) were designed, respectively, for the two gene predictions (see above). The primer pair Pp2II.31Fw/Pp2.587Rev did not yield amplicons, so that transcript analysis corroborated the first gene prediction of the *MAT1-2-4* gene, which contains two introns (49 and 48 bp) and three exons (191, 239 and 185 bp) (Fig. 1).

The primer pair A437Fw/B893Rev confirmed the presence of the intron at the 3′ end of the *MAT1-2-1* gene. Electropherograms obtained by sequencing the PCR amplicon obtained with the primer pair I26Fw/I497Rev from the *MAT1-2-1* transcript showed multiple overlapping traces. Single sequences were compared with the genomic DNA. The overlying peaks started immediately before the predicted splicing site of the intron close to the 5′ end of the gene and corresponded to three transcripts: the predicted one and two different putative sequences originated by transcription of the opposite DNA strand with alternative splice acceptor sites leading to introns of 56 and 59 bp in size (Fig. 2). Three bands, very similar in size (413, 416 and 422 bp), were separated by running RT-PCR products onto polyacrylamide gel. The presence of antisense sequences to the *MAT1-2-1*-coding strand was verified by strand-specific RT-PCR on total RNA, using a single primer (I26Fw) to obtain first-strand cDNA that was PCR-amplified and sequenced in both directions. Furthermore, nested primers in which the forward ones, HmgII250Fw and HmgI253Fw, were specifically designed on the exon-junction site of each of the two antisense sequences were used with the I456Rev primer to prevent amplification of genomic DNA, and confirmed the results.

The primer pairs Y23Fw/Y649Rev, I26Fw/I497Rev, Pp1.156Fw/Pp1.726Rev and Pp2.625Fw/Pp2.868Rev (Table 2) were used to verify the expression of the mating-type genes at different vegetative and sexual stages. RT-PCR was carried out on total RNA extracted from mycelium, sclerotia, either before or following the carpogenesis-inducing cold treatment, and apothecia. The transcripts of the *MAT1-1-5* and the *MAT1-2-4* genes were detected at all the stages (Fig. 3). The *MAT1-1-1* and the *MAT1-2-1* genes were generally not detected and nested RT-PCR was performed. Internal transcript-specific primer pairs (α133Fw/αY280Rev and HmgIII268Fw/I497Rev) were used for the second round of amplification. Forward primers were designed at the exon-junctions to avoid amplification of genomic DNA. The expected transcripts of both the genes were then detected at all analysed stages (Fig. 3).

Observations on the homothallic behaviour

The structure of the *MAT1* locus was investigated in three complete ascospore tetrads previously characterized for mating type through sexual crosses with the reference strains SAS56 (*MAT1-1*) and SAS405 (*MAT1-2*). The selected tetrads contained monoascosporic isolates expected to be *MAT1-2* but proved fertile with both reference strains (MAT1-1/2) (Faretra & Pollastro, 1996). Seven primer pairs were designed to selectively amplify different portions of the *MAT1* locus yielding amplification profiles characteristic for the *MAT1-1* or the *MAT1-2* idiomorphs (Table 3; Fig. 1). All the *MAT1-1* or *MAT1-2* monoascosporic strains yielded PCR amplicons

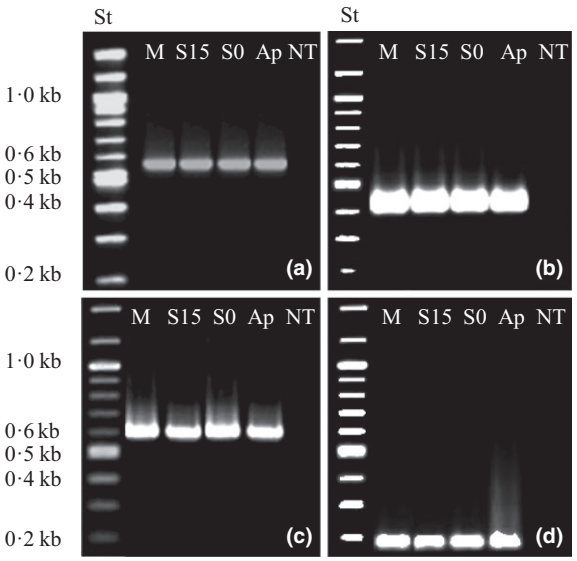

Figure 3 RT-PCR results for the four *Botrytis cinerea* mating-type genes *MAT1-1-1* (a), *MAT1-2-1* (b), *MAT1-1-5* (c) and *MAT1-2-4* (d) in the strains SAS56 (*MAT1-1*) and SAS405 (*MAT1-2*), at different development stages: mycelium (M), sclerotia, before (S15) or following (S0) the cold treatment inducing carpogenesis, and apothecia (Ap). NT = no reverse template control. St = 100 bp DNA ladder (New England Bio-Labs).

of expected sizes. All the MAT1-1/2 monoascosporic strains yielded typical amplicons of the *MAT1-2* idiomorph (Table 4). PCR experiments with the primer pair Z showed that in all the tested strains, irrespective of their mating type, the 133 bp sequence common to the *MAT1-1-1* gene and its truncated form (*dMAT1-1-1*) in the flanking region of the *MAT1-2* idiomorph was always present (Table 4).

Observations were extended to 18 strains from single randomly collected ascospores from seven different crosses and 18 field isolates of different origin (Table 5) showing homothallic behaviour. The majority of isolates showed the typical response of *MAT1-2* (61 and 67%, respectively) and less of *MAT1-1* (39% of monoascoporic strains and 33% of field isolates).

Comparison between *B. cinerea* and *S. sclerotiorum*

The two idiomorphs and their flanking regions of the heterothallic *B. cinerea* were compared to the *MAT1* locus of the homothallic *S. sclerotiorum* (GenBank assembly accession: GCA_000146945.1, as amended by Chitrampalam *et al.*, 2013). The *MAT1* locus of *S. sclerotiorum* contains four genes, the *MAT1-1-1* gene coding for an alpha-domain protein and the *MAT1-2-1* gene coding for the HMG-domain protein and two genes coding for predicted proteins of unknown function.

BLASTN analysis carried out on the *B. cinerea* and the *S. sclerotiorum MAT1* loci showed the following homologous regions: (i) a 250 bp sequence in the *B. cinerea MAT1-1-1* gene and its truncated form in the *MAT1-2* idiomorph with the inverted repeat within the *S. sclerotiorum MAT1* locus (Chitrampalam *et al.*, 2013); (ii) the *B. cinerea MAT1-2-1* gene and in its short truncated portion at the 5′-flanking region of the *MAT1-1* idiomorph shared a 61 bp homologous sequence with the corresponding *S. sclerotiorum* gene; (iii) finally, two short sequences close together in the 3′ flanking region of the *B. cinerea MAT1* locus with two distant sequences of *S. sclerotiorum*, one (40 bp) between the *MAT1-1-1* and the *MAT1-2-4* genes and another (45 bp) downstream from the *MAT1-2-1* gene (Table 6).

Molecular assay for mating type

The primer pairs α or Y and HMG3/5 or HMG.SP yielded the best results in recognizing the *MAT1-1* and the *MAT1-2* idiomorphs, respectively (Fig. 4). The results of mating-type molecular recognition were validated by testing the reference strains SAS56 (*MAT1-1*) and SAS405 (*MAT1-2*) and 40 field isolates collected from different crops (citrus trees, cucurbits, gerbera,

Table 3 PCR primer pairs used to assess genomic structure of the *MAT1* locus in *Botrytis cinerea* isolates

Primer pair	Primers	Sequence (5′–3′)	Amplicon size (bp)
α	MAT1.1	AAGCTTCGATGACCCTTTGA	1295
	MAT1.1295	GATCGTGGAGCCGAGATAAT	
Y	alpha.Y.23	AATGCAGAAGAGCCAACGAT	627
	alpha.Y.649	CGGTTCCAACCTTGAACAAT	
W	alpha.W.42	CCTTTTCACGCCAAATGGT	353
	alpha.W.394	ATGTTGGCCATTGGATGC	
Z	alpha.Z.51	CACCAGAGATCGGTTCGTTT	133
	alpha.Z.183	CGGTTCCAACCTTGAACAAT	
X	alpha.X.6	GTTCGGTCCATGCTTGTGTA	637
	alpha.X.642	CGGTTCCAACCTTGAACAAT	
HMG3/5	hmg.3	GCTCCTTTCCATAAGTCGTAAGTCGTG	1414
	hmg.5	CAAGATCAGACGGAGTGCATTACCTC	
HMG.SP	hmg.sp.162	GTGGAGATGGTGGTGGAGTT	958
	hmg.sp.1119	GAAAATGGGTACCGCATCAC	

Table 4 PCR analysis of *Botrytis cinerea* ascospore strains from complete asci (cross SAS405 × SAS56) including strains showing a homothallic behaviour (MAT1-1/2) using primers amplifying DNA sequences common (Z) or specific for the *MAT1-1* (α, Y and W) or *MAT1-2* (X, HMG.SP and HMG3/5) idiomorphs (see Fig. 1)

			Primer pairs[a]						
			MAT1-1			*MAT1-2*			*MAT1-1-1* and *dMAT1-1-1*
Isolate	Ascus	Mating type	α	Y	W	X	HMG.SP	HMG3/5	Z
SAS25	A37	1/2	–	–	–	+	+	+	+
SAS26		1/2	–	–	–	+	+	+	+
SAS27		2	–	–	–	+	+	+	+
SAS28		2	–	–	–	+	+	+	+
SAS29		1	+	+	+	–	–	–	+
SAS30		1	+	+	+	–	–	–	+
SAS31		1	+	+	+	–	–	–	+
SAS32		1	+	+	+	–	–	–	+
SAS33	A45	1	+	+	+	–	–	–	+
SAS34		1	+	+	+	–	–	–	+
SAS35		1	+	+	+	–	–	–	+
SAS36		1	+	+	+	–	–	–	+
SAS37		1/2	–	–	–	+	+	+	+
SAS38		2	–	–	–	+	+	+	+
SAS39		1/2	–	–	–	+	+	+	+
SAS40		2	–	–	–	+	+	+	+
SAS41	A48	1/2	–	–	–	+	+	+	+
SAS42		2	–	–	–	+	+	+	+
SAS43		1/2	–	–	–	+	+	+	+
SAS44		2	–	–	–	+	+	+	+
SAS45		1	+	+	+	–	–	–	+
SAS46		1	+	+	+	–	–	–	+
SAS47		1	+	+	+	–	–	–	+
SAS48		1	+	+	+	–	–	–	+

[a]+, amplicon production; –, no amplicon production.

grapevine, lettuce, strawberry, rose, tomato, etc.) collected in several countries (Albania, Belgium, Croatia, France, Greece, Italy, Japan, Lebanon, Serbia, Switzerland) whose mating type was assessed through crosses with reference strains under laboratory conditions. All tested isolates were heterothallic, being fertile in crosses with only one of the reference strains. Seventeen field isolates were *MAT1-1* and 23 were *MAT1-2*. The two mating types were equally distributed on host plant and countries. The PCR assessment of mating type yielded results always in agreement with those from sexual crosses.

Discussion

The structure of the mating type locus (*MAT1*) of *B. cinerea* has previously been reported by Amselem *et al.* (2011) who identified two genes in each idiomorph. The present study investigated the structural organization of the *MAT1* locus in additional strains of opposite mating type and in isolates of the fungus able to yield apothecia and viable ascospores in crosses with reference strains of opposite mating types. The molecular characterization through a primer-walking approach yielded the whole locus sequence in the *MAT1-1* strains SAS56 and SAS47, in the *MAT1-2* strain SAS405 and in the MAT1-1/2 isolate SAS43. The *MAT1-1* and *MAT1-2* idiomorphs in *B. cinerea* displayed unique sequences of 2513 and 2776 bp, respectively, and the flanking sequences of the *MAT1* locus were almost identical in isolates of opposite mating type. Conventionally, the *MAT1-1* and the *MAT1-2* idiomorphs are characterized, respectively, by the alpha domain and the HMG domain. BLASTX analysis identified the conserved functional domains in proteins coded by the mating type genes *MAT1-1-1* and *MAT1-2-1* and the casual attribution of the *MAT1-1* mating type to the SAS56 strain and of the *MAT1-2* mating type to the SAS405 strain, as a result of previous study on the sexual behaviour of the fungus (Faretra *et al.*, 1988b), was proved to be correct. The two genes *MAT1-1-1* and *MAT1-2-1* showed high homology with the corresponding genes in the *MAT1* locus of the closely related homothallic species *S. sclerotiorum*. Two introns were detected in the conserved regions of alpha box and HMG box domains in the *B. cinerea MAT1-1-1* and *MAT1-2-1* genes at the same relative position of homologous genes in other ascomycetes (Debuchy & Turgeon, 2006). As reported by Amselem *et al.* (2011), each idiomorph also included an additional gene, *MAT1-1-5* or *MAT1-2-4*, both having corresponding sequences in the *MAT1* locus of *S. sclerotiorum*, but no apparent homologues in other fungal

Table 5 PCR analysis of *Botrytis cinerea* strains from single random ascospores and field isolates displaying homothallic behaviour (MAT1-1/2) using primers amplifying DNA sequences common (Z) or specific for the *MAT1-1* (α, Y and W) or *MAT1-2* (X and HMG.SP) idiomorphs (see Fig. 1)

		Primer pairs[a]					
Isolate	Origin	α	Y	W	Z	X	HMG.SP
Strains from random ascospores							
SAR181	WS55 × WS131	–	–	–	+	+	+
SAR183		+	+	+	+	–	–
SAR307	WS138 × WS158	–	–	–	+	+	+
SAR2628	SAS405 × SAS56	–	–	–	+	+	+
SAR2639		–	–	–	+	+	+
SAR2656		+	+	+	+	–	–
SAR2673		–	–	–	+	+	+
SAR10990	SAS56 × SAR10905	–	–	–	+	+	+
SAR11102	SAS405 × SAR1093	+	+	+	+	–	–
SAR11104		+	+	+	+	–	–
SAR11105		–	–	–	+	+	+
SAR11106		+	+	+	+	–	–
SAR11121	SAS56 × SAR10938	–	–	–	+	+	+
SAR11123		+	+	+	+	–	–
SAR11130		–	–	–	+	+	+
SAR11132		–	–	–	+	+	+
SAR11133		–	–	–	+	+	+
SAR11198	SAS56 × SAR10959	+	+	+	+	–	–
Field isolates							
B5	Carnation	+	+	+	+	–	–
VB31	Cucumber	+	+	+	+	–	–
WS220	Freesia	–	–	–	+	+	+
B7	Gerbera	–	–	–	+	+	+
WS230	Gladiolus	+	+	+	+	–	–
WS61	Grapevine	–	–	–	+	+	+
WS92		–	–	–	+	+	+
WS180		–	–	–	+	+	+
WS184		–	–	–	+	+	+
WS232	Lettuce	–	–	–	+	+	+
WS197	Rose	+	+	+	+	–	–
WS211		–	–	–	+	+	+
WS231		–	–	–	+	+	+
B1		+	+	+	+	–	–
VB29		–	–	–	+	+	+
WS90	Pear	–	–	–	+	+	+
WS221	Tomato	–	–	–	+	+	+
WS189	Tulip	+	+	+	+	–	–

[a]+, amplicon production; –, no amplicon production.

species. Additional *MAT1* genes may have an essential role during sexual reproduction, although only the *MAT1* genes encoding transcription factors containing the alpha- and HMG-domains seem essential for mating functions in several species. Terhem (2015) generated knockout mutants of *B. cinerea* and used them in appropriate sexual crosses. *MAT1-1-1* and *MAT1-2-1* mutants proved sterile, thus confirming the essential role of the alpha-domain protein and the HMG-box protein in the mating process. Moreover, *MAT1-1-5* and *MAT1-2-4* mutants were able to produce stipes but failed to develop apothecial disks, showing that these two genes jointly play a key role in disk development.

The genomic sequence from the *MAT1-1* strain SAS56, including the *MAT1* locus and wide portions of its flanking regions, was identical to the homologous sequence of the reference strain B05.10. With regard to the *MAT1-2* idiomorph, the comparison between the strain SAS405 and the reference strain T4 revealed small differences in nucleotide sequences including a short duplication of 12 bp in the coding region of the T4 *MAT1-2-1* gene and two expressed SNPs in the *MAT1-2-4* gene. The amino acid duplication (TESS) is outside the conserved functional domain of the protein and hence is unlikely to affect its function. Similarly, Chitrampalam *et al.* (2013) reported SNPs, short insertions and deletions in coding and non-coding regions at the *MAT1* locus in *S. sclerotiorum* isolates.

The presence of a truncated gene sequence of the opposite mating type, *dMAT1-1-1* or *dMAT1-2-1*, in the flanking regions of the *MAT1* locus was confirmed in all tested *B. cinerea* isolates. According to the genome annotations of the two reference strains B05.10 and T4, both the truncated gene sequences are in transcribed but untranslated regions (UTR): *dMAT1-1-1* in the 3′ UTR of the *MAT1-2-4* gene and *dMAT1-2-1* in the 5′ UTR of the *MAT1-1-5* gene. Although UTRs are noncoding portions of genomes of eukaryotic organisms, they are involved in many post-transcriptional regulatory pathways. For example, in *Cochliobolus heterostrophus*, the UTRs of the *MAT1-1-1* and *MAT1-2-1* genes extend in the common flanking regions; the 5′ UTR contains alternative transcription start sites and intron splicing sites generating multiple transcripts (Leubner-Metzger *et al.*, 1997) and the 3′ UTR contains a 160 bp sequence essential for the fertility of the fungus (Wirsel *et al.*, 1998).

Expression analysis by RT-PCR or nested RT-PCR showed that all the mating-type genes were transcriptionally active in different vegetative stages (mycelium and sclerotia) as well as during the sexual process (carpogenesis-induced sclerotia and fully developed apothecia). In particular, transcripts of the *MAT1-1-1* and *MAT1-2-1* genes, encoding DNA-binding proteins, were at a lower level than those of the genes *MAT1-1-5* and *MAT1-2-4*, coding for unknown putative proteins. However, transcripts of all the *MAT* genes were detected in RNA samples from both vegetative and sexual stages, although their low abundance hampered their quantification. Constitutive expression of the mating-type genes has been recorded in several unrelated fungal species, suggesting their possible role in processes other than mating. For example, in *Neurospora crassa*, the *mtA-2* and *mtA-3* genes are transcribed at low level during both vegetative growth and sexual reproduction (Ferreira *et al.*, 1996). In *Podospora anserina*, the *FPR1* and *FMR1* genes, present in the *mat+* and *mat−* idiomorphs, respectively, are expressed during either vegetative or sexual stages, whereas the *SMR1* and the *SMR2* genes specific for the *mat−* idiomorphs are expressed only during perithecia development of the fungus (Bidard *et al.*, 2011).

Sequence data of RT-PCR products of the *B. cinerea* HMG-encoding gene (*MAT1-2-1*), including the

Table 6 Comparison of nucleotide sequences of the *MAT1* locus of *Sclerotinia sclerotiorum* (JQ815883.1; Chitrampalam *et al.*, 2013) and the *MAT1-1* (KF944385.1) and *MAT1-2* (KF944386.1) idiomorphs of *Botrytis cinerea*

	S. sclerotiorum		*B. cinerea*					
	44Ba1		SAS56 (*MAT1-1*)			SAS405 (*MAT1-2*)		
DNA sequence (bp)	Nucleotide position	Orientation	Nucleotide position	Orientation	Identity (%)	Nucleotide position	Orientation	Identity (%)
250	5517–5766	5′–3′	3688–3937	5′–3′	81	3235–3484	5′–3′	78
	9372–9621	3′–5′						
61	9294–9354	5′–3′	1170–1230	5′–3′	87	454–514	5′–3′	89
40	6512–6551	5′–3′	4749–4788	5′–3′	93	4296–4335	5′–3′	93
45	9671–9715	5′–3′	5061–5105	5′–3′	89	–	–	–

intron splicing site close to the starting codon, suggested the existence of co-expressed transcript isoforms, resulting from antisense transcriptions, in addition to the transcript expected by gene prediction. The antisense RNA is typically encoded in *cis* and transcribed from the same gene but on the opposite DNA strand. The *in silico* sequence analysis led to the identification of two putative isoforms of *MAT1-2-1*-antisense RNA both overlapping the transcription unit and resulting from alternative splicing. Putative antisense transcripts have been recently reported for several eukaryotic genes resulting from alternative polyadenylation or different transcription start sites. For instance, antisense transcription yielding several mRNA isoforms caused by alternative splicing has been described for the transcription factor *Foxl2*, involved in the development of ovary and eyelid in mammals (Cocquet *et al.*, 2005). In *MATa/α* diploid cells of *Saccharomyces cerevisiae* the switch from mitotic to meiotic division is controlled by an antisense-mediated regulation of the *IME4* (Initiator of Meiosis 4) gene encoding a putative RNA methyltransferase (Hongay *et al.*, 2006). Transcript isoforms of the *MAT1-2-1* and *MAT1-1-1* genes have also recently been observed in *S. sclerotiorum* strains (Chitrampalam *et al.*, 2013). Further transcriptional characterization of the *MAT1* genes in *B. cinerea* is needed to better understand their role in gene regulation during its life cycle.

The structural organization of the *MAT1* locus was investigated in *B. cinerea* strains that showed homothallic behaviour (MAT1-1/2) in sexual crosses. No differences were found comparing the *MAT1* locus and its flanking sequences in the MAT1-2 strain SAS405 and the MAT1-1/2 strain SAS43. Idiomorph-specific primers were used to explore the structure of the *MAT1* locus in a large number of MAT1-1/2 ascospore progenies and field isolates. Their organization of the *MAT1* locus did not differ from heterothallic strains, and this corroborates previous findings from the analysis of a few isolates (Amselem *et al.*, 2011). In yeasts, homothallism is due to a mating type 'switching' mechanism based on the substitution of the mating type sequences with sequences of the opposite mating type present in different genomic sites (Arcangioli & de Lahondès, 2000) and submitted to gene silencing (Fraser & Heitman, 2003). The data presented here show that homothallic behaviour in *B. cinerea* occurs predominantly, but not exclusively, in *MAT1-2* strains, but no sequences of the opposite idiomorph could be detected in homothallic isolates, so that a yeast 'switching'-like mechanism cannot be responsible for the homothallic behaviour in *B. cinerea*.

The presence of a truncated gene sequence of the opposite mating type, *dMAT1-1-1* or *dMAT1-2-1*, in the flanking regions of the *MAT1* locus suggests the hypothesis that unequal recombination events between highly homologous sequences at the *MAT1* locus on paired chromosomes could lead to complex structural changes, possibly unstable or transient. Indeed, on the grounds of results obtained, monoascosporic isolates that showed

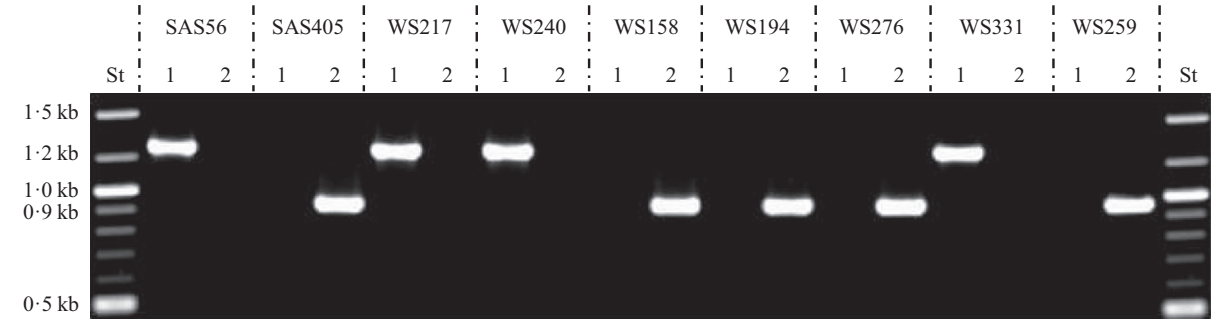

Figure 4 Example of PCR-assay for mating type assessment using the idiomorph-specific primer pairs α (*MAT1-1*) (columns 1) or HMG.SP (*MAT1-2*) (columns 2) and genomic DNA of SAS56 and SAS405 reference strains and seven field isolates of *Botrytis cinerea*. St = 100 bp DNA Ladder (New England Bio-Labs). The 1295 bp band is specific for *MAT1-1* and the 958 bp band is specific for *MAT1-2*.

homothallic behaviour were recently crossed twice with SAS56 and SAS405 and all of them lost their homothallism and proved fertile only with the *MAT1-1* reference strain SAS56. Such genomic rearrangements, followed by deletion of partial gene sequence, have been proposed to explain the integration of the *dMAT1-1-1* in the *MAT1-2* idiomorph of *Grosmannia* and *Ophiostoma* species (Tsui *et al.*, 2013). In *S. sclerotiorum* an inversion of a 3.6 kb region of the *MAT1* locus regularly occurs during the sexual cycle (Chitrampalam *et al.*, 2013). The inversion region includes the *MAT1-2-1* and the *MAT1-2-4* genes and a large portion of the *MAT1-1-1* gene and is flanked on either side by a 250 bp inverted repeat, probably involved in the inversion loop formation and crossing-over events (Chitrampalam *et al.*, 2013). In *B. cinerea*, a homologous 250 bp sequence was detected immediately downstream of the mating-type specific sequences in the *MAT1* locus, but its possible biological significance remains to be clarified.

Homothallism has been reported in many species of the genus *Sclerotinia* (Ekins *et al.*, 2006) and in a few *Botryotinia* species, such as *Botryotinia globosa* and *Botryotinia porri* (Lorbeer, 1980). On the grounds of phylogenetic and taxonomic affinity between the heterothallic *B. cinerea* and the homothallic *S. sclerotiorum* and the high homology observed in coding sequences of their *MAT1*-specific genes, the entire sequence of the *MAT1* locus was compared in *MAT1-1* and *MAT1-2* strains of *B. cinerea* and *S. sclerotiorum*.

Amselem *et al.* (2011) explained the configuration of the *MAT1* locus in the heterothallic *B. cinerea* as deriving from a homothallic ancestor carrying a *MAT1*-locus organization similar to *S. sclerotiorum* through inversion and deletion events, and suggested that the presence of truncated gene sequences, *dMAT1-1-1* and *dMAT1-2-1*, derived from the loss of large portions of the complete *MAT1-1-1* and *MAT1-2-1* gene from a homothallic ancestor. Chitrampalam *et al.* (2013) proposed an alternative evolutionary model suggesting that the *MAT1* locus of the homothallic *S. sclerotiorum* derives from a heterothallic ancestor through recombination events resulting essentially in an inversion of the *MAT1-2* idiomorph followed by its fusion with the *MAT1-1* sequence. In this evolutionary scenario, the *dMAT1-1-1* and *dMAT1-2-1* sequences found in *B. cinerea* were probably produced by crossing over between *MAT1-1* and *MAT1-2* idiomorphs after evolutionary separation from the *S. sclerotiorum* lineage. The present data show the presence of short homologous sequences in the *MAT1* idiomorphs of *B. cinerea* and in the *MAT1* locus of *S. sclerotiorum* that might contribute to a better understanding of the evolution of sexual behaviour in the *Sclerotiniaceae* family.

Sequences of the *MAT1-1* and *MAT1-2* loci of *B. cinerea* were used to develop a PCR-based assay with idiomorph-specific primer pairs for an easy and rapid assessment of mating types in isolates of the fungus. The molecular assay was successfully applied on 40 isolates of the fungus whose mating type was assessed by sexual crosses with reference strains, showing a complete agreement between genotypic and phenotypic testing. *MAT1-1* and *MAT1-2* genotypes were detected among isolates from different plants and geographic areas, confirming a balanced distribution of *B. cinerea* mating types in nature, as reported by several authors (Faretra *et al.*, 1988b; Beever & Parkes, 1993; Faretra & Pollastro, 1993; van der Vlugt-Bergmans *et al.*, 1993; Delcán & Melgarejo, 2002). The PCR method is much more rapid than the traditional method, which is based on the judgement of fertility in sexual crosses and requires a very long time (3–4 months). It may be helpful in determining mating type of high numbers of isolates within field populations, as well as in planning sexual crosses for classical genetic analysis of phenotypic traits of interest.

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