Sexual development of *Botrytis* species

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Chapter 1

General introduction and outline of the thesis

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Chapter 1

Introduction

The genus *Botrytis*

The genus name refers to the structure of the macroconidia, which rise and form clusters with the shape of grape bunches: ‘botryose’. *Botrytis* is the asexual stage of *Botryotinia*. The *Botrytis* community has in its recent meeting (Italy, 23-28 June 2013) unanimously recommended the exclusive use of the asexual name *Botrytis* over *Botryotinia*, the name of the sexual stage, since *Botrytis* is historically the oldest name and it is commonly used by plant pathologists, breeders and growers. In line with this recommendation, a list of generic names of fungi for protection under the International Code of Nomenclature has included this genus under the name *Botrytis* and not *Botryotinia* (Kirk et al. 2013). I therefore follow this recommendation in this thesis and will exclusively use the name *Botrytis*.

Species of the genus *Botrytis* infect over 250 host species, including major greenhouse and field crops such as tomato, grape, strawberry, onion and ornamentals such as rose, lily, and tulip (Staats et al. 2005). Most *Botrytis* species are necrotrophic pathogens that are able to kill the host tissue during infection. Interestingly, an endophytic species (*B. deweyae*) has recently been discovered which under appropriate conditions can cause ‘spring sickness’ in ornamental *Hemerocallis* (daylily) hybrids (Grant-Downton et al. 2014). *B. cinerea* is the best-studied species within the genus (Williamson et al. 2007) and was elected as the second most important plant pathogenic fungal species (Dean et al. 2012).

In the asexual state, *Botrytis* produces different morphological structures including mycelia, macroconidia, microconidia, and sclerotia. Macroconidia have an ellipsoidal to ovoid shape and rise from conidiophore branches into botryose clusters. They are pale brown in color and range in size from 9–23 × 8–15 µm. Microconidia are more spherical and much smaller than macroconidia (about 1µm), and function as male spermatia (Groves and Loveland 1953; Faretra et al. 1988; Beever and Parkes 1993; Fukumori et al. 2004). Sclerotia are irregular hemispherical, convex structures which normally have a concave surface. They are black, with sizes ranging between 1 and 10 mm (Whetzel 1945), and function not only as survival structures during the winter but also as maternal parent in the production of apothecia.
During the sexual stage, fruiting bodies are produced which are called apothecia: a cup-shaped ascoma at the top of a stipe that acts as a platform to discharge ascospores from asci. *Botrytis* apothecia vary in size, depending on the species, between 1 and 25 mm in height and 1-6 mm in diameter (Hennebert and Groves 1963; Bergquist and Lorbeer 1972). Apothecia are brown and become dark during maturation (Hennebert and Groves 1963; Bergquist and Lorbeer 1972; Faretra and Antonacci 1987). Multiple apothecia can develop on a single sclerotium. Mature apothecia normally can be observed two months after fertilization (Faretra et al. 1988; Hennebert and Groves 1963; van Der Vlugt-Bergmans et al. 1993).

**Species identification and numbers**

Approximately half of the *Botrytis* species are named after the host that they infect from which they were first isolated and characterized. Table 1 lists the members of the genus *Botrytis* and shows the 16 species that have been reported to be capable of developing apothecia. One hybrid species, *B. allii* which originated from hybridization between *B. byssoides* and *B. aclada* (Nielsen and Yohalem 2001; Martinez et al. 2003) could not be placed in the phylogenetic tree and was therefore omitted from Table 1. The genus *Botrytis* predominantly comprises narrow host range pathogens that infect a single, or a few (often related) host species. There are at least two exceptions to this rule; *B. cinerea* can infect more than 200 host species (Jarvis 1977) and *B. pseudocinerea* has been isolated from several unrelated diseased host species (Walker et al. 2011; Leroch et al. 2013).

Taxonomic classification and nomenclature of *Botrytis* species have rarely been reviewed. Morphological descriptions of most species have been published in the 19th and first half of the 20th century in separate papers, many of which are not easily accessible. The most recent taxonomic compilation of the genus is reported in a monograph by Jarvis (1977), which also lists ~25 excluded or doubtful species, and briefly describes the historical debates between mycologists, exemplifying the confusion in classification of *Botrytis* species. Morphological features were often inadequate to distinguish species and variability often observed among isolates of the same species further complicated the situation (Jarvis 1977). Recent studies have identified *B. cinerea* and *B. pseudocinerea* as species that are very similar in morphology, yet recognized as distinct taxa that diverged several million years ago (Walker
et al. 2011). Even more puzzling, the morphology and narrow host range of *B. fabae* separate this species clearly from *B. cinerea* and *B. pseudocinerea*, but phylogenetic studies revealed it to be a sister species of *B. cinerea* (see below). These examples illustrate the limitations of morphological characters for *Botrytis* species identification.

**Table 1:** List of members in the genus *Botrytis*

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Host</th>
<th>Producing apothecia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botrytis aclada</td>
<td>MUCL8415</td>
<td>Allium spp.</td>
<td>No</td>
</tr>
<tr>
<td>B. byssoida</td>
<td>MUCL94</td>
<td>Allium spp.</td>
<td>Yes</td>
</tr>
<tr>
<td>B. calthae</td>
<td>MUCL1089</td>
<td>Caltha palustris</td>
<td>Yes</td>
</tr>
<tr>
<td>B. cinerea</td>
<td>MUCL87</td>
<td>&gt;200 species</td>
<td>Yes</td>
</tr>
<tr>
<td>B. caroliniana</td>
<td>CB15</td>
<td>Rubus fruticosus</td>
<td>No</td>
</tr>
<tr>
<td>B. convoluta</td>
<td>MUCL11595</td>
<td>Iris spp.</td>
<td>No</td>
</tr>
<tr>
<td>B. croci</td>
<td>MUCL436</td>
<td>Crocus spp.</td>
<td>No</td>
</tr>
<tr>
<td>B. deweyae</td>
<td>CBS 134649</td>
<td>Hemerocallis spp.</td>
<td>No</td>
</tr>
<tr>
<td>B. elliptica</td>
<td>BE9714</td>
<td>Lilium spp.</td>
<td>Yes</td>
</tr>
<tr>
<td>B. fabae</td>
<td>MUCL98</td>
<td>Vicia spp.</td>
<td>Yes</td>
</tr>
<tr>
<td>B. ficariaum</td>
<td>MUCL376</td>
<td>Ficaria verna</td>
<td>Yes</td>
</tr>
<tr>
<td>B. fabiopsis</td>
<td>BC-2</td>
<td>Vicia faba</td>
<td>No</td>
</tr>
<tr>
<td>B. galanthina</td>
<td>MUCL435</td>
<td>Galanthus spp.</td>
<td>No</td>
</tr>
<tr>
<td>B. gladiolorum</td>
<td>MUCL3865</td>
<td>Gladiolus spp.</td>
<td>Yes</td>
</tr>
<tr>
<td>B. globosa</td>
<td>MUCL444</td>
<td>Allium ursinum</td>
<td>Yes</td>
</tr>
<tr>
<td>B. hyacinthi</td>
<td>MUCL442</td>
<td>Hyacinthus spp.</td>
<td>No</td>
</tr>
<tr>
<td>B. narcissicola</td>
<td>MUCL2120</td>
<td>Narcissus spp.</td>
<td>Yes</td>
</tr>
<tr>
<td>B. paeoniae</td>
<td>MUCL16084</td>
<td>Paeonia spp.</td>
<td>No</td>
</tr>
<tr>
<td>B. pelargonii</td>
<td>CBS 497.50</td>
<td>Pelargonium spp.</td>
<td>Yes</td>
</tr>
<tr>
<td>B. polyblastos</td>
<td>CBS287.38</td>
<td>Narcissus spp.</td>
<td>Yes</td>
</tr>
<tr>
<td>B. porri</td>
<td>MUCL3234</td>
<td>Allium spp.</td>
<td>Yes</td>
</tr>
<tr>
<td>B. pseudocinerea</td>
<td>VD110</td>
<td>Vitis vinifera</td>
<td>Yes</td>
</tr>
<tr>
<td>B. ranunculi</td>
<td>CBS178.63</td>
<td>Ranunculus spp.</td>
<td>Yes</td>
</tr>
<tr>
<td>B. sinoallii</td>
<td>HMAS 250008</td>
<td>Allium spp.</td>
<td>No</td>
</tr>
<tr>
<td>B. sphaerosperma</td>
<td>MUCL21481</td>
<td>Allium triquetrum</td>
<td>Yes</td>
</tr>
<tr>
<td>B. squamosa</td>
<td>MUCL1107</td>
<td>Allium cepa</td>
<td>Yes</td>
</tr>
<tr>
<td>B. tulipae</td>
<td>BT9830</td>
<td>Tulipa spp.</td>
<td>No</td>
</tr>
</tbody>
</table>

*No: No record of apothecia in nature and no studies were done in-vitro*

**Molecular phylogeny**

Hoist-Jensen et al. (1998) were the first to use nuclear ribosomal *ITS* sequences to infer a phylogeny of the family *Sclerotiniaceae*, including several members of the genus *Botrytis*. The relationships among *Botrytis* species could not be resolved because of the limited number of informative characters. However the study permitted the conclusion that *Botrytis* constitutes a monophyletic lineage (Hoist-Jensen et al. 1998). The phylogeny of the *Sclerotiniaceae* was further refined by Andrew et al. (2012) using three protein-coding
genes: calmodulin, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and heat shock protein 60 (HSP60).

Staats et al. (2005) performed a comprehensive phylogenetic analysis of the genus Botrytis, at that time comprising 22 recognized species and one hybrid. Using three protein-coding genes (G3PDH, HSP60 and the DNA-dependent RNA polymerase subunit II gene (RPB2), they corroborated the morphological and host plant-based classification of Botrytis species and divided the genus into two (rather widely separated) clades. Clade I contained species that only infect eudicot plants, while clade II contained species that can infect either eudicot or monocot plants. The use of the same three genes facilitated the discovery of several new species: Botrytis sinoallii, a new species infecting Allium species, and its distinction from other Botrytis species infecting the same hosts (Zhang et al. 2010b); B. fabiopsis, a new species infecting broad bean, very distant from B. fabae (Zhang et al. 2010a); and B. caroliniana, a new species infecting blackberry (Li et al. 2012).

Two genes, encoding phytotoxic proteins NEP1 and NEP2, were shown to provide higher resolution in distinguishing species in the genus Botrytis because they seem to be subject to higher evolutionary rates than the housekeeping genes G3PDH, HSP60 and RPB2 (Staats et al. 2007b). The NEP1 and NEP2 genes were shown to have evolved under positive selection which suggested a role of these proteins in the infection process (Staats et al. 2007b). One might therefore infer that such genes cannot serve as neutral phylogenetic markers. Functional analysis in B. cinerea and B. elliptica using targeted knockout mutants failed to reveal a role of NEP genes in virulence of these two species (Staats et al. 2007b; Staats et al. 2007a; Cuesta Arenas et al. 2010), which would lend support to considering these genes as neutral markers and adequate tools in phylogeny.

The studies by Staats et al. (2005) revealed incongruence between the phylogenies of Botrytis species and their hosts. Species infecting the same host clustered in different (sub)clades, e.g. B. aclada, B. squamosa, B. porri, B. byssoidea and B. sinoallii all infecting Allium. Conversely, closely related species can infect very different hosts, e.g. B. elliptica infecting the monocotyledonous host Lilium and B. ficariarum infecting the dicotyledonous host Ficaria (Staats et al. 2005). More recently, similar incongruencies have been reported for
newly described species, e.g. *B. fabiopsis* infecting *Vicia faba* is very distant from *B. fabae* infecting the same host (Zhang et al. 2010a), and *B. caroliniana* infecting blackberries and strawberries is very distant from *B. cinerea* (Li et al. 2012).

Recently, Khan et al. (2013) combined data from *ITS* and *IGS* regions with the *G3PDH* gene, with the aim of improving molecular identification of *Botrytis* species that cause neck rot disease on onion. *ITS* and *IGS* regions were insufficiently informative to distinguish *B. allii* and *B. byssoida*. The sequences of *ITS* and *IGS* for *B. allii* isolates were clearly distinct, some clustering with *B. aclada* and others clustering with *B. byssoida* (Khan et al. 2013), as may be expected for a hybrid species. Sequence analysis of the *G3PDH* and β-tubulin genes amplified from herbarium specimens of *Botrytis* collected from grey mould-infected apple (deposited in 1932) enabled O’Gorman et al. (2008) to corroborate the existence of *B. mali*, a species that had been reported (Ruehle 1931), but by lack of description was considered doubtful.

Figure 1 shows a maximum-likelihood tree of *Botrytis* species, based on concatenated sequences of parts of the *G3PDH*, *HSP60* and *RPB2* genes (Staats et al. 2005; Li et al. 2012; Zhang et al., 2010a,b; Grant-Downton et al. 2014; Walker et al. 2011). Five species described after publication of the phylogeny by Staats et al. (2005), i.e. *B. caroliniana*, *B. deweyae*, *B. fabiopsis*, *B. pseudocinerea* and *B. sinoallii*, clearly cluster within the genus and are genuine *Botrytis* species. *Botrytis mali* could not be included in the tree due to lack of sequence information for the *HSP60* and *RPB2* genes. Based on *G3PDH* and β-tubulin sequences it would cluster with *B. paeoniae* (O’Gorman et al. 2008). As can also be seen in Figure 1, the different *Botrytis* species differ in their capacity to reproduce sexually. In total 11 species in Clade I and 5 species in Clade II are able to produce apothecia. Below I will first discuss the sexual reproduction in Ascomycota in general and then discuss the state of the art of knowledge on the mating system in the genus *Botrytis*. 


Mating systems in Ascomycota

Fungi can display homothallic and heterothallic mating behavior. Homothallic mating refers to the ability of an individual to complete the sexual reproduction cycle in the absence of a mating partner (self-fertile). By contrast, heterothallic mating requires the presence of two genotypes of opposite mating type to undergo sexual reproduction (out-crossing). In the phylum Ascomycota, homothallic and heterothallic mating systems can be found in different species belonging to the same genus, as reported for the genera *Aspergillus*, *Botrytis*, *Neurospora*, and *Sordaria*.
To date only two species in the genus *Botrytis* have been recorded to behave as homothallic, i.e. *B. porri* and *B. globosa* (Buchwald 1953; Elliott 1964). Other species in the genus *Botrytis*, including *B. cinerea*, are reported to have a heterothallic mating behavior (Elliott 1964; Faretra et al. 1988). There are, however, a few records of heterothallic fungi that are also capable of homothallic sexual reproduction. For instance, homothallic behavior of certain *B. cinerea* isolates was first reported by Faretra and Antonacci (1987). They reported that single ascospores collected from apothecia of *B. cinerea* could be grown in culture and self-fertilised to successfully form apothecia; however, no data are available for the segregation of mating type alleles in ascospores retrieved from such apothecia. Other examples of homothallic behavior are reported for *Sordaria brevicollis* (Robertson et al., 1998) and *Ceratocystis coerulescens* (Harrington and McNew 1997), which normally have heterothallic mating systems. Two field strains of *C. coerulescens* of the *MAT1-2* mating type could form fruiting bodies in the absence of a strain of opposite mating type (Harrington and McNew 1997).

**The mating type locus**

The mating system in heterothallic Ascomycota is bipolar, which means that there are two alleles. The MAT alleles in different fungi have historically received different names, for instance *MAT- α* and *MAT- a* (*Saccharomyces cerevisiae*), *MAT- -* and *MAT+* (*Podospora anserina*), or *MAT1-1* and *MAT1-2* (many fungi, including *Botrytis spp.*). The two mating types can be distinguished by the DNA sequences at the mating type (*MAT*) locus and their gene products play a crucial role in regulating sexual reproduction. One peculiarity of the *MAT* locus is the lack of nucleotide sequence homology between opposite mating alleles, which were therefore historically referred to as idiomorphs (Cozijnsen and Howlett 2003; Turgeon et al. 1993; Metzenberg and Glass 1990). The MAT loci of *S. cerevisiae* were the first fungal MAT loci that were identified and sequenced (Astell et al. 1981); the *MAT-α* idiomorph contains two genes *MAT-α1* (α-domain transcription factor) and *MAT-α2* (homeodomain transcription factor) while the *MAT-a* idiomorph contains a single gene *MAT-a1* (homeodomain transcription factor) (Figure 2). In most Ascomycota (but not in *S. cerevisiae*) the *MAT* locus idiomorphs are flanked by two conserved genes, *APN2* and *SLA2* (Heitman et al. 2007; Cozijnsen and Howlett 2003; Metzenberg and Glass 1990; Turgeon et
al. 1993). The conservation of the flanking regions in many Ascomycota suggests that this overall structure represents the ancestral configuration in Ascomycota (Heitman et al. 2007).

The concerted action of \textit{MAT1-1} and \textit{MAT1-2} loci is considered to be essential for activating the expression of genes that are involved in development of the fruiting body, meiosis and formation of asci with ascospores. By convention, the \textit{MAT1-1} idiomorph encodes a transcription factor, containing a DNA-binding domain named alpha domain, while the \textit{MAT1-2} idiomorph encodes a different transcription factor, containing a DNA binding high-mobility group (HMG) domain (Coppin et al. 1997). In most heterothallic Ascomycota fungi the \textit{MAT1-1} and \textit{MAT1-2} loci contain additional genes (Figure 2). For example, in \textit{Neurospora crassa} and \textit{Magnaporthe grisea}, the \textit{MAT1-1} locus contains three genes: \textit{MAT1-1-1} (alpha domain), \textit{MAT1-1-2} and \textit{MAT1-1-3} while the \textit{MAT1-2} locus of \textit{N. crassa} contains two genes \textit{MAT1-2-1} (HMG domain) and \textit{MAT1-2-2} and the \textit{MAT1-2} locus of \textit{M. grisea} contains two genes \textit{MAT1-2-1} (HMG domain) and \textit{MAT1-1-3}.

In most homothallic \textit{Ascomycota} fungi, the \textit{MAT1-1} and \textit{MAT1-2} genes are located in the same location in a single genome, flanked by the \textit{APN2} gene and the \textit{SLA2} gene, just as in heterothallic species. For example, in the homothallic species \textit{Gibberella zeae} the \textit{MAT} locus has four genes at the same chromosomal location; \textit{MAT1-1-1} (alpha domain), \textit{MAT1-1-2}, \textit{MAT1-1-3}, and \textit{MAT1-2-1} (HMG domain) (Figure 2). Also the \textit{Sclerotinia sclerotiorum MAT} locus contains \textit{MAT1-1} and \textit{MAT1-2} genes in a single genomic location (Amselem et al., 2011). By contrast, the homothallic species \textit{Aspergillus nidulans} contains the \textit{MAT1-1} and \textit{MAT1-2} genes in a single genome, yet in different chromosomal locations, i.e. chromosomes 6 and 3 (Paoletti et al., 2007).

Orthologs of the \textit{MAT1-1–2} gene usually can be found in \textit{Sordariomycetes} (Debuchy et al. 2010) and encode a protein with a DNA polymerase processivity factor (DNA\_PPF) domain (Kanematsu et al. 2007; Klix et al. 2010). Orthologs of the \textit{MAT1-1-3} gene encode a protein with a HMG domain (Ferreira et al. 1996; Klix et al. 2010), but their function differs from the \textit{MAT1-2-1} protein, presumably because \textit{MAT1-1-3} lacks a sequence that is conserved at the C-terminal end of the HMG domain of \textit{MAT1-2-1} (Debuchy et al. 2010).
Individual deletion of the MAT1-1-2 or MAT1-1-3 gene in *N. crassa* did not affect sexual reproduction, but the deletion of both these genes resulted in a severely reduced number of asci, although the asci that were present contained eight ascospores (Ferreira et al., 1998). Deletion of the MAT1-1-2 gene in *S. macrospora* and *P. anserina* resulted in sterility and no fruiting bodies were developed (Arnaise et al. 2001; Klix et al. 2010).

The MAT loci in fungal genomes appear to evolve faster than other regions, and therefore the sequences and organization of the MAT locus vary among species (Pöggeler 1999; Turgeon 1998). In spite of their faster evolution, the functions of genes in the MAT locus are well conserved among species (Coppin et al. 1997). For example, the MAT genes in heterothallic Ascomycota *N. crassa*, *Cochliobolus heterostrophus*, *A. nidulans* play the same role in fertilization events (Paoletti et al. 2007; Ferreira et al. 1998). The MAT genes can sometimes be functionally interchangeable between homothallic and heterothallic Ascomycota. For example, a knock out mutant in the MAT1-2-1 gene of the heterothallic species *C. heterostrophus* became self-fertile, when the entire MAT locus from the homothallic species *C. luttrellii* was introduced (Yun et al., 1999). Conversely, replacement of the homothallic MAT locus of *C. luttrellii* by a heterothallic locus of *C. heterostrophus* (either MAT1-1-1 or MAT1-2-1) switched the sexual behavior of *C. luttrellii* to heterothallic (Lu et al. 2011). Remarkably, however, transgenic *C. luttrellii* transformed with a single *C. heterostrophus* MAT allele (either MAT1-1-1 or MAT1-2-1) were able to produce tiny pseudothecia with reduced number of asci, but containing normally viable ascospores (Lu et al. 2011), suggesting the ability of fruiting body development in the absence of a strain of opposite mating type.

In a study by Pöggeler et al. (1997), the MAT genes appeared not to be interchangeable between the closely related species *P. anserina* (heterothallic) and *S. macrospora* (homothallic). Introduction of the entire (homothallic) MAT locus of *S. macrospora* into *P. anserina* MAT-- (MAT1-1) or MAT+ (MAT1-2) strains resulted in transformants that were able to produce a fruiting body structure which, however, completely lacked asci and ascospores (Pöggeler et al. 1997).
Figure 2: MAT loci arrangement in Pezizomycotina and Saccharomycotina adapted from Amselem et al. 2011; Heitman et al., 2007; Klix et al., 2010; Pöggeler and Kück. 2000
Mating system in *Botrytis cinerea*

*B. cinerea* has been classified as a fungus with a heterothallic mating system. Mating occurs between a strain carrying a $MAT1-1$ locus and a strain carrying a $MAT1-2$ locus. In *B. cinerea*, the $MAT1-1$ locus (Figure 3) contains the $MAT1-1-1$ gene, which encodes an alpha domain protein, and a gene named $MAT1-1-5$ of unknown function. The $MAT1-2$ locus (Figure 3) contains the $MAT-1-2-1$ gene, which encodes a HMG-box protein, and the $MAT1-2-4$ gene of unknown function. In the closely related homothallic species *Sclerotinia sclerotiorum*, a single *MAT* locus is present that contains four genes that are orthologous to the genes present in the *B. cinerea* $MAT1-1$ and $MAT1-2$ loci (Amselem et al. 2011). As in most Ascomycota, both *MAT* loci in *B. cinerea* are flanked by the genes $APN2$ (encoding DNA lyase) and $SLA2$ (encoding a cytoskeleton protein) that are convergently transcribed with respect to the *MAT* locus (Amselem et al. 2011).

Dual mater behavior in *B. cinerea*

Faretra et al. (1988), Beever and Parkes (1993), van der Vlugt-Bergmans et al. (1993), and Amselem et al. (2011) discovered unique mating behavior in *B. cinerea*, which was referred to as ‘dual mater’. Approximately 5% of progeny from a cross between two heterothallic strains is a ‘dual mater’, which is able to mate with $MAT1-1$ and $MAT1-2$ reference strains (Amselem et al. 2011). For example a ‘dual mater’ $MAT1-2$ strain is able to mate with another strain with a heterothallic $MAT1-2$ background and undergo sexual development. Similar behavior has also been discovered in strains of $MAT1-1$ background, which are able to mate with other strains of $MAT1-1$ background and undergo sexual development. The mechanisms underlying this unusual behavior in *B. cinerea* were not unraveled. Since the first reports of dual mating in *B. cinerea*, in the 1990’s, some other fungi have been reported to behave in similar ways, and in these cases the phenomenon is referred to as “unisexual reproduction” (Roach et al., 2014; Ni et al., 2013; Lin et al., 2005).
Figure 3: Arrangement of MAT1-1 and MAT1-2 loci in *Botrytis cinerea* (adapted from Amselem et al. 2011)
Chapter 1

Outline of the thesis

The aims of the research described in this thesis were to study the mechanisms involved in apothecium development of *B. cinerea*, and to describe the morphology of *Botrytis* spp and their fruiting bodies.

Chapter 2 describes a genome-wide transcriptome analysis of different stages of apothecium development and a study on the function of *MAT* genes in apothecium development of *B. cinerea*. RNA-seq analysis was performed on sclerotia, apothecia in different stages of development, and ascospores. Transcriptional changes that occurred during developmental transitions were analysed by comparing the transcriptomes of successive developmental stages. Cluster analysis was performed to identify sets of genes with common transcriptional profiles over the developmental stages. Finally, the function of mating type genes was studied by generating knock out mutants, and analyzing their mating behavior.

Chapter 3 describes the functional analysis of three hydrophobin genes in sclerotium and apothecium development of *B. cinerea*. Knockout mutants with single or multiple deletions of hydrophobin genes were used to study the role of hydrophobin genes in asexual and sexual development of *B. cinerea*.

Chapter 4 describes the structure of the *MAT* locus in *B. elliptica* and the morphology of apothecia of *B. elliptica* generated in the laboratory.

Chapter 5 provides a morphological and phylogenetic description of a novel *Botrytis* species, named *Botrytis deweyae*, which is the only species within the genus that behaves as an endophyte. Under certain conditions, *B. deweyae* can cause ‘spring sickness’ disease in hybrid *Hemerocallis* cultivars.

Chapter 6 presents a general discussion of the results presented in Chapter 2-5 and puts them in a broader perspective. In addition experiments aimed at understanding the genetic basis of dual mater behavior are discussed.
References


Buchwald N (1953) *Botryotinia (Sclerotinia) globosa* sp. on *Allium ursinum*, the perfect stage of *Botrytis globosa* Raabe. Phytopathologische Zeitschrift 20 (3):241-254.


Chapter 1


Chapter 2

Transcriptome and functional analysis of fruiting body development in *Botrytis cinerea*

Terhem RB, Rodenburg YA, Stassen JHM, and van Kan JAL
Abstract

Botrytis cinerea is an ascomycetous plant pathogen producing apothecia as sexual fruiting bodies. Not much is known about reprogramming of gene expression during apothecium development. In this study we analysed genome-wide expression profiles of sclerotia, three stages of apothecium development (primordia, full grown stipe, apothecial disk), and ascospores. In addition we studied the role of mating type (MAT) genes in apothecium development. RNA-sequencing was performed on different tissue types, and the reads were mapped on gene models in the B. cinerea genome. The expression profiles were analysed in different ways. First, the transcript levels of B. cinerea orthologs of Aspergillus nidulans and Sordaria macrospora genes known to be involved in sexual development were examined. Secondly, transcriptional changes between successive developmental stages were analysed. Strikingly, more than 5000 genes appeared to be differentially expressed in ascospores as compared to mature apothecial disks. Among the genes that were upregulated in ascospores are several genes encoding virulence factors, which indicates that ascospores are primed for infection of host plants. Thirdly, cluster analysis identified five sets of genes with common transcriptional profiles over the developmental stages. Interestingly, ~60% of the genes that were specifically expressed in mature apothecial disks were of unknown function, were either Botrytis-specific, or had homologs only in closely related taxa.

In order to study the function of mating type genes, single gene deletion mutants were generated in both genes present in the MAT1-1 locus or both genes present in the MAT1-2 locus. All four mutants were crossed with a wild type strain or knockout mutant strain of opposite mating type, in all possible combinations. Knockout mutants in the MAT1-1-1 or MAT1-2-1 gene were sterile. By contrast, mutants in the MAT1-1-5 or MAT1-2-4 gene developed into stipes but never into an apothecial disk. The MAT1-1-5 and MAT1-2-4 mutants showed identical phenotypes, suggesting that both genes control the transition from stipe to apothecial disk, probably by acting as transcriptional regulators. Comparative gene expression profiling between the MAT1-1-5-deficient mutant blocked at the transition from stipes to apothecial disk formation and wild type apothecial disks identified 114 genes that were downregulated in the mutant. These genes might be under (direct or indirect) control of the MAT1-1-5 and MAT1-2-4 genes.
Introduction

*Botrytis cinerea* is a heterothallic Ascomycota classified in the class of Leotiomyces, order Helotiales, family Sclerotiniaceae. As member of the Helotiales, *B. cinerea* develops a fruiting body with an exposed hymenium on top of a stipe, called an apothecium. Apothecia are generally carpogenic from sclerotia, and the sclerotium serves as a source of nutrients during apothecium development (Li et al. 2011). Apothecia function as a platform to discharge ascospores (Pöggeler et al. 2006) which serve as the primary inoculum for plant infection (Jurick et al. 2004), especially in early spring when *B. cinerea* conidia are scarce.

Species within the Sclerotiniaceae can either have heterothallic or homothallic sexual reproduction systems. In a heterothallic species, mating occurs between two isolates with opposite mating types. The opposite *MAT* loci of Ascomycota are generally present at the same chromosomal location and therefore genetically allelic, but they lack sequence similarity and are called idiomorphs rather than alleles (Metzenberg and Glass 1990). Each idiomorph contains at least one gene encoding a transcription factor. By convention, the *MAT1-1* idiomorph encodes a protein with an α domain, while *MAT1-2* encodes a protein with a high mobility group box (HMG-box) (Martin et al. 2011). In *B. cinerea*, the *MAT* loci are designated as *MAT1-1* and *MAT1-2*. The *MAT1-1* locus contains two genes: *MAT1-1-1* encoding the α domain protein (with a length of 354aa), and *MAT1-1-5* of unknown function (377aa). The *MAT1-2* locus also contains two genes: *MAT-1-2-1*, encoding the HMG-box protein (with a length of 376aa), and *MAT1-2-4* of unknown function (197aa). Both *MAT* loci in *B. cinerea* are flanked by the *APN2* gene (an ortholog of the *Saccharomyces cerevisiae* gene encoding DNA lyase) and the *SLA2* gene (an ortholog of the *S. cerevisiae* gene encoding a cytoskeletal protein) which are convergently transcribed toward the *MAT* loci (Amselem et al. 2011). In many Ascomycota with heterothallic reproductive life styles, the *MAT* loci are also surrounded by *APN2* and *SLA2* genes, suggesting that this represents the ancestral configuration in all Ascomycota (Heitman 2007).
Chapter 2

Heterothallic Ascomycota usually carry a single mating type gene in one nucleus, while homothallic Ascomycota carry both mating type genes (MAT1-1 and MAT1-2) in one genome (Glass and Smith 1994; Yun et al. 1999; Yun et al. 2000; Amselem et al. 2011). For example, the homothallic species Sclerotinia sclerotiorum carries four linked MAT genes (MAT1-1-1, MAT1-1-5, MAT1-2-1 and MAT1-2-4), which are equivalent to the heterothallic MAT1-1 and MAT1-2 loci in B. cinerea (Amselem et al., 2011). Another example is the homothallic species Sordaria macrospora which carries four linked MAT genes (MAT1-1-1, MAT1-1-2, MAT1-1-3 and MAT1-2-1), equivalent to both idiomorphs of the heterothallic species Neurospora crassa (Pöggeler and Kück 2000; Pöggeler et al. 1997). Several studies have focussed on unravelling the function(s) of mating type genes in Ascomycota. Table 1 shows the results of functional analyses of MAT genes in the model fungi Neurospora crassa, Podospora anserina and Sordaria macrospora but there are numerous other fungi for which studies of MAT genes have been conducted. So far, the functions of the four genes in the B. cinerea MAT locus in general and more specifically in apothecium development have not been studied. The availability of genome sequences and gene annotations of B. cinerea (Amselem et al., 2011; van Kan et al., unpublished) enabled molecular dissection of apothecium development in B. cinerea, both at the transcriptional and functional level.

Table 1: Role of mating type genes in sexual development of model ascomycetous fungi

<table>
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<tr>
<th>Fungal species</th>
<th>Reproductive system</th>
<th>Gene</th>
<th>Phenotype</th>
<th>Reference</th>
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<td>Sterile</td>
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<td></td>
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<td>Podospora anserina</td>
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<td>Sordaria macrospora</td>
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* phenotype of targeted knockout mutants
Large scale gene expression analyses in fungi have previously been performed using microarrays (Breakspear and Momany 2007; Koch et al. 2005; Kerr et al. 2000). In recent years, transcriptome analyses have shifted towards the use of RNA-sequencing, which provides a higher level of sensitivity and accuracy to quantify expression levels (Soanes et al. 2012; Fu et al. 2009; Marioni et al. 2008) and has become increasingly affordable. Therefore, genome-wide expression profile analysis by RNA-sequencing is being carried out in a rapidly increasing number of filamentous fungi, including Aspergillus niger (Delmas et al. 2012), P. anserina (Bidard et al. 2011), Sclerotinia homoeocarpa (Orshinsky et al. 2012), Trichoderma harzianum (Steindorff et al. 2014), Metarhizium anisopliae (Wang et al. 2014a), Magnaporthe oryzae (Soanes et al. 2012; Kawahara et al. 2012) and B. cinerea (Zhang et al. 2014). The aims of this study were to perform a genome-wide transcriptome analysis during different stages of apothecium development in B. cinerea using RNA-sequencing, and to identify the functions of the B. cinerea MAT genes in apothecium development by targeted deletion.

Materials and Methods

Strains
The wild type strains SAS56 (reference MAT1-1 strain) and SAS405 (reference MAT1-2 strain) were used in this study (Faretra and Antonacci 1987; Faretra et al. 1988), as well as strain B05.10 (MAT1-1), for which the genome sequence was determined (Amselem et al., 2011; van Kan et al., unpublished). Strains were plated on malt extract agar (MEA). For obtaining sclerotia, cultures were grown at 15 °C in darkness. For obtaining sporulation, the cultures were grown at 20°C. Two-to three-day-old plates were exposed to near-UV light to enhance sporulation. Conidia were maintained in 20% glycerol and stored at -80°C.

Sexual crosses
Sexual crosses were performed using three wild type strains (SAS56, SAS405 and B05.10) and four mutants: ΔMAT1-1-1, ΔMAT1-1-5, ΔMAT1-2-1 and ΔMAT1-2-4. Crosses were performed using the described protocols (Faretra et al. 1988; van der Vlugt-Bergmans et al. 1993). Sclerotia (incubated at 0 °C for one month) were sampled from MEA plates and the surfaces were cleaned with a soft toothbrush. Three to five sclerotia of ~1 cm in size were put in
single wells in a six-well microtiter plate. Suspensions of microconidia were prepared by pouring sterile (autoclaved) MilliQ water onto the plate from which sclerotia were removed, followed by gentle rubbing with a plastic spatula. Sclerotia in the microtiter plate were fertilized with 3 ml of this microconidia suspension per well of the microtiter plate that was subsequently sealed with parafilm and incubated at 12°C in candescent light with a 12 h photoperiod.

**DNA extraction**
Freeze-dried mycelium was used for genomic DNA extraction, using a Gentra Puregene DNA purification kit (Qiagen, Venlo, the Netherlands), following the manufacturer’s instructions.

**RNA extraction and RNA-sequencing**
Samples from apothecia and ascospores were freeze-dried and total RNA was isolated using the Nucleospin RNA plant kit (Macherey Nagel, Düren, Germany), according to the manufacturer’s instructions. For isolation of RNA from sclerotia, freeze-dried sclerotia were frozen in liquid nitrogen and ground with a mortar and pestle. Total RNA was isolated with TRIzol Reagent (Life Technologies, Grand Island, NY, USA) in combination with a Nucleospin RNA plant kit (Macherey Nagel, Düren, Germany), used according to the manufacturer’s instructions.

The integrity of RNA was monitored by agarose gel electrophoresis and the concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A total of 20 μg of RNA from each sample was used by Beijing Genome Institute (Hongkong) for library construction (300-700 nt insert sizes) and sequenced using Illumina HiSeq™ 2000. The paired-end RNA-Seq reads with a length of 90 nucleotides were assessed for quality using FastQC software v0.10.1 (Babraham Institute, http://www.bioinformatics.babraham.ac.uk/projects/fastqc). The reads were trimmed to 80, using FASTX-Toolkit v0.0.13.2 (http://hannonlab.cshl.edu/fastx_toolkit). The quality of the RNA libraries was checked by mapping them to the *B. cinerea* reference genome (strain B05.10; van Kan et al., unpublished). The mapping efficiency ranged from 84.2 to 96.9%. The reads mapping on the 11,701 gene models were counted and normalised.
cDNA synthesis and quantitative RT-PCR

First strand cDNA was synthesized from 1 µg total RNA with M-MLV Reverse Transcriptase (Promega, Leiden, The Netherlands) according to the manufacturer’s instructions. Quantitative-Real Time-PCR (qRT-PCR) was performed using an ABI7300 PCR machine in combination with the qPCR SensiMix kit, with primers as listed in Table 2. qRT-PCR conditions were as follows: an initial 95°C denaturation step for 10 min, followed by denaturation for 15 s at 95°C and annealing/extension for 1 min at 60°C, for 40 cycles. The data were analyzed on the 7300 System SDS software and calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The gene expression values were normalized to the constitutively expressed B. cinerea tubulin gene, BctubA (Benito et al. 1998).

Read mapping and differential expression

The trimmed reads were mapped on the genome of B05.10 (van Kan et al., unpublished) using the spliced aligner software TopHat v2.0.8b (Kim et al. 2013). Gene expression values were measured using two different software packages and methods: DESeq2 and Cuffdiff. DESeq2 (Anders et al. 2014; Anders and Huber 2010) was used with default settings for four pairwise statistical differential expression tests between the five stages (2 biological replicates each) in the sexual reproduction of B. cinerea. DESeq2 gives a p-adjusted value (Benjamini and Hochberg 1995), indicating the significant differentially expressed genes, corrected for multiple testing. Differentially expressed genes were considered significant for p-adjusted value <0.05. Cuffdiff (v2.1.1) was used with default parameters (Trapnell et al. 2013). The significant differentially expressed genes were selected on q-value <0.05 for both methods, and for each pairwise test. For each pair, the genes that were tested significant (p-adjusted or q-value <0.05) for differential expression with both methods were considered high confidence differentially expressed genes.

Clustering gene expression patterns

In order to cluster and identify co-expressed genes at different stages the R package Mfuzz was used (Futschik and Carlisle 2005). Prior to clustering, three groups of expression patterns were filtered out: (I) genes which showed expression in only one of the biological replicates, (II) genes that showed <=10 read counts over all 5 conditions and (III) genes that showed >10-fold difference in counts between the biological replicates. To determine gene
expression variation between stages, the coefficient of variance ($c_v$) was calculated for all genes, using the formula $c_v = \sigma \mu^{-1}$.

The 500 genes with the highest $c_v$ were selected (‘Top500’), these displayed the most strongly fluctuating expression patterns (over the five samples), relative to their average expression level. The mean expression of the biological replicates of the Top500 genes was then used for further analyses.

**Enrichment tests**

Fisher’s exact tests were performed to test for enriched GO terms. Four subsets for each differentially expressed gene set (identified both by DEseq2 and Cufddiff), were analyzed for enriched GO terms, using a Fisher’s exact test from the Blast2GO suite.

**Deletion of mating type genes**

The $MAT1-1-1$, $MAT1-1-5$, $MAT1-2-1$ and $MAT1-2-4$ genes were individually targeted for mutagenesis. $MAT1-1-1$ and $MAT1-1-5$ genes were deleted in SAS56, whereas the $MAT1-2-1$ and $MAT1-2-4$ genes were deleted in SAS405. In addition, the $MAT1-1-5$ gene was also deleted in B05.10. The gene knockout strategies for generating *B. cinerea* deletion constructs, *B. cinerea* protoplast transformation and PCR-based screening of transformants were as described by Kars et al. (2005b). Table 2 shows the list of primers that were used for amplification of gene deletion fragments. The hygromycin resistance ($HPH$) cassette, amplified from vector pLOB7 (Zhang et al. 2011) with primer pair Cassette-5/Cassette-3 was used as selection marker. Genomic DNA of transformants was analyzed for the presence/absence of the wild-type target gene by amplifying the target genes $MAT1-1-1$, $MAT1-1-5$, $MAT1-2-1$ and $MAT1-2-4$ using primer combinations $MAT111-5.1/MAT111-3.1$, $MAT115-5.1/MAT115-3.1$, $MAT121-5.1/MAT121-3.1$ and $MAT124-5.1/MAT124-3.1$ respectively.
Transcriptome and functional analysis of fruiting body development in *Botrytis cinerea*

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Chapter 2

Results

Samples representing five different stages of sexual development were used for RNA extraction (Figure 1). The first sample was taken from 4-week-old sclerotia of strain SAS405 (MAT1-2), which had been incubated at 0°C for one month and were ready for fertilization with microconidia of opposite mating type. The second sample was taken from stipe primordia emerging from sclerotia ~30 days after they had been fertilized with microconidia of strain SAS56 (MAT1-1); primordia of stages 1 (size 1-2 mm) and 2 (size 2-4 mm) were combined to form this sample. The third sample was taken from a combination of stipes of stages 3 (>5 mm, stipes elongated but not swollen at the tip) and 4 (stipes fully extended to ~1 cm, swollen at the tip and showing initiation of apothecial disk development). The fourth sample was taken from a combination of apothecial disks of stages 5 (immature disk with diameter <5 mm, partially expanded) and 6 (mature disks fully expanded to ~1 cm, filled with asci containing 8 ascospores). The fifth and last sample was taken from ascospores obtained from mature apothecia by gently crushing the apothecial disks in sterile water and filtering the suspension over glasswool to separate hymenium tissue and large debris from the ascospores (in the flow-through). These five samples were used for RNA extraction and were designated, respectively, as Scl (sclerotia), Apo12 (primordia of stages 1 and 2), Apo34 (stipes of stages 3 and 4), Apo56 (apothecial disks, stages 5 and 6) and Asc (mature ascospores). All samples were processed and prepared for cDNA synthesis and RNA sequencing.

Figure 1: Tissues and stages of apothecium development used in this study. For explanations see text. Scale bars indicate the sizes in mm or µm (ascospores).
Expression of mating type genes during development of apothecia of *B. cinerea*

Figure 2 shows a qRT-PCR analysis of the transcript levels of the $MAT1-1-1$, $MAT1-1-5$, $MAT1-2-1$ and $MAT1-2-4$ genes over the three stages of apothecium development (Apo12, Apo34 and Apo56), relative to the internal, constitutively expressed reference gene $BctubA$ (Benito et al., 1998). Transcript levels of all four genes were lowest in primordia (Apo12), slightly increased in stipes (Apo34) and peaked in apothecial disks (Apo56).

**Figure 2**: Transcript levels of $MAT1-1-1$, $MAT1-1-5$, $MAT1-2-1$ and $MAT1-2-4$ genes in different apothecial developmental stages, relative to the internal standard $BctubA$ (average of two biological replicates).

Whole-genome transcriptome analysis of *B. cinerea* during development of apothecia

RNA-sequencing was performed on the five samples described above, in two biological replicates, except for the sclerotia which were sequenced once because of difficulties in obtaining RNA of sufficient quality due to their high melanin content. Sequencing thus yielded datasets for nine RNA samples. The library size and mapping efficiency of the reads on the *B. cinerea* genome are listed in Table 3. More than 91% of gene models in the *B. cinerea* genome were represented by at least one read; in every sample, at least 75% of all genes had FPKM values $\geq$1. The expression profile of sample Apo56 was the most diverse, with $>90\%$ of gene models displaying an FPKM value $\geq$1.
Table 3: Mapping efficiency of RNA sequencing reads from different samples on the *B. cinerea* genome

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Library size</th>
<th>Mapped reads</th>
<th>% mapped</th>
<th>#genes with FPKM ≥1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sclerotium</td>
<td>12853250</td>
<td>12455209</td>
<td>96.9</td>
<td>8855 (75.6%)</td>
</tr>
<tr>
<td>Apo12A</td>
<td>12853250</td>
<td>12106317</td>
<td>94.2</td>
<td>9698 (82.8%)</td>
</tr>
<tr>
<td>Apo12B</td>
<td>12141566</td>
<td>10281723</td>
<td>84.7</td>
<td></td>
</tr>
<tr>
<td>Apo34A</td>
<td>13416227</td>
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<td>10171 (86.9%)</td>
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<td>Apo34B</td>
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<td>9734272</td>
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<td></td>
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<td>Apo56A</td>
<td>13883171</td>
<td>13075867</td>
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<td>10584 (90.5%)</td>
</tr>
<tr>
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<td>11762996</td>
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<tr>
<td>Asc-A</td>
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<td>8873 (75.8%)</td>
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<tr>
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<td>10953912</td>
<td>91.6</td>
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</tbody>
</table>

Expression of *B. cinerea* genes with orthologs involved in sexual development of model fungi

The transcript levels were examined of *B. cinerea* orthologs of genes in *Aspergillus nidulans* (Dyer and O’Gorman, 2012) and *Sordaria macrospora* (Teichert et al., 2014) known to act as activators or repressors of sexual development. *B. cinerea* orthologs could be identified for 67 of these genes (Supplementary Table 1). Expression for most of these *B. cinerea* genes was detectable in nearly all samples tested, with FPKM values ranging from 1 to 3437 (Supplementary Table 1). Supplementary Figures 1-5 show the abundance in the five RNA samples of transcripts of *B. cinerea* genes orthologous to *A. nidulans* genes involved in perception of environmental signals (Figure S1), mating processes and signal transduction (Figure S2), transcription factors (Figure S3), endogenous physiological processes (Figure S4) and ascospore production and maturation (Figure S5) according to the grouping by Dyer and O’Gorman (2012). Figure 3 shows the expression profiles of a selection of genes in the entire set, which will be discussed in more detail. The *B. cinerea white collar 2* gene (*Bcin05g05530*) and the *VelA* gene (*Bcin15g03390*) both are putative positive regulators of sexual reproduction and their transcript levels increased 2-3 fold in *B. cinerea* primordia and mature stipes, as compared to sclerotia and ascospores. On the other hand, transcripts of the *B. cinerea white collar 1* and *VelB* genes were less abundant and barely changed during sexual development (Figure S1). The *B. cinerea* ortholog to an amino acid sensing bZIP protein (AN3675), considered to be a negative regulator of sexual reproduction in *A. nidulans*, showed a 4-fold reduction in transcript levels in apothecial tissues as compared to
sclerotia and ascospores. Genes encoding components of the \textit{B. cinerea} heterotrimeric G-protein complex (G\(\alpha_1\), G\(\beta\) and G\(\gamma\)) all showed slight increases in transcript levels in apothecial tissues as compared to sclerotia and ascospores. The thioredoxin gene Bcin12g04280 showed an extremely high transcript level (FPKM=3437) in primordia and it was 2.5-fold higher than in sclerotia, mature apothecial discs and ascospores. The transcript level of the gene encoding ATP citrate lyase subunit ACL1 increased 3-fold in the transition from sclerotia to primordial stipes, remained constant in apothecia and further increased up to 9-fold in ascospores. The expression profiles of \textit{B. cinerea} genes orthologous to several \textit{S. macrospora} genes required for sexual development were also examined, and were found to be expressed most abundantly in ascospores (pro4) or in all three stages of apothecial development, but not in sclerotia and ascospores (pro41).

\textbf{Figure 3}: Expression profiles in sclerotia, three stages of apothecial development and ascospores for a subset of \textit{B. cinerea} genes that are orthologous to \textit{Aspergillus nidulans} and \textit{Sordaria macrospora} genes known to act as activators or repressors of sexual development. A: activator of sexual development, R: repressor of sexual development
**Genes differentially expressed between different stages of apothecial development**

Changes in gene expression correlated with transitions between stages of apothecial development (Scl-Apo12; Apo12-Apo34; Apo34-Apo56; Apo56-Asc) were analysed using DESeq2 and Cuffdiff. The sets of differentially expressed genes detected by each of the two methods were compared for each of the four pairwise tested stages of apothecial development. The Venn diagrams in Figure 4 show that DESeq2 and Cuffdiff exhibit different levels of sensitivity and specificity, giving rise to the identification of distinct sets of differentially expressed genes, with a significant overlap between both methods. The genes for which differential expression was detected by both methods were considered as high confidence differentials, and these were further analysed.

**Figure 4:** The numbers of differentially expressed genes between different stages of apothecial development as identified by DESeq2 (blue circle) and Cuffdiff (red circle). Four Venn diagrams are depicted, one for each pairwise comparison (indicated on top of each diagram). The numbers on the left in each Venn diagram represent the number of differentially expressed genes only detected by DESeq2, while the numbers on the right represent the numbers of differentially expressed genes only detected by Cuffdiff. The numbers within the overlapping area represent the number of differentially expressed genes identified by both methods.

Table 4 provides a more detailed overview of the differentially expressed genes detected by both methods. The most pronounced changes in transcript levels were detected between apothecial disks (Apo56) and ascospores (Asc), with over 5000 high confidence differentials, as well as during the transition from sclerotia to primordia (stage Apo12; 2282 high
Transcriptome and functional analysis of fruiting body development in *Botrytis cinerea*

Confidence differentials). By contrast, only 27 genes were differentially expressed between primordia (Apo12) and stipes (Apo34), whereas between stipes (Apo34) and apothecial disks (Apo56), the number of differentially expressed genes was 884. Features of these sets of high confidence differentials are discussed below.

**Table 4**: Differentially expressed genes detected by the DESeq2 and Cuffdiff method

<table>
<thead>
<tr>
<th>Pairwise test</th>
<th>Concordant by DESeq2 and Cuffdiff</th>
<th>Upregulated</th>
<th>Downregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scl – Apo12</td>
<td>2282</td>
<td>1307</td>
<td>975</td>
</tr>
<tr>
<td>Apo12 – Apo34</td>
<td>27</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>Apo34 – Apo56</td>
<td>884</td>
<td>428</td>
<td>456</td>
</tr>
<tr>
<td>Apo56 – Asc</td>
<td>5064</td>
<td>2504</td>
<td>2560</td>
</tr>
</tbody>
</table>

**Differentially expressed genes in the transition from sclerotia to primordia**

The transition from sclerotia to primordia (Apo12) resulted in 1307 upregulated and 975 downregulated genes (Table 4). The upregulated genes showed an overrepresentation of the GO terms “cell division related processes”, “fatty acid biosynthetic process”, “double strand break repair via break-induced replication” and “protein import into peroxisome matrix”, “docking”, “DNA strand elongation involved in DNA replication”, “positive regulation of asexual sporulation resulting in formation of cellular spore”. GO terms that were overrepresented in the down-regulated genes mostly related to the assimilation, dissimilation and transport of carbohydrates, for example “glycolysis” and “gluconeogenesis”.

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Differentially expressed genes in the transition from primordia to stipes

The transition from primordia (Apo12) to stipes (Apo34) resulted in 27 differentially expressed genes that were all down-regulated (Table 4). Among these were genes encoding the oxaloacetate hydrolase *BcOAH1* required for biosynthesis of oxalic acid (Han et al. 2007), an MFS transporter with unknown substrate specificity, as well as several genes in a cluster of secondary metabolite genes including the polyketide synthase gene *BcPKS15*, of which the product is unknown. In view of the small number of differentials, GO enrichment analysis did not detect any significant enrichment.

Differentially expressed genes in the transition from stipes to apothecial disks

The transition from stipes (Apo34) to apothecial disks (Apo56) resulted in 428 upregulated and 456 downregulated genes. The upregulated genes showed an overrepresentation of GO terms related to nutrient transport and cell-wall related processes. “Carbohydrate transport” was the most significantly overrepresented GO term, but this transition also showed an overrepresentation of the GO term “G2/M transition of mitotic cell cycle” and terms related to post-translational modification of proteins. Overrepresented GO terms in the downregulated genes in this set included “peroxisome processes”, many oxidation-reduction processes and the term “negative regulation of sexual sporulation resulting in formation of cellular spore”.

Differentially expressed genes in the transition from apothecial disks to ascospores

Between the apothecial disks (Apo56) and the ascospore sample, >5000 genes were differentially expressed, of which half were upregulated and half were downregulated (Table 4). GO enrichment analysis on the upregulated genes revealed overrepresentation of the terms “isoleucine/valine/leucine biosynthetic process”, “mycelium development” and “ribosome synthesis”. The less related terms “pre-replicative complex assembly involved in nuclear cell cycle DNA replication” and other DNA-replication oriented processes were also overrepresented. Down-regulated genes showed an overrepresentation of GO terms related to “carbohydrate transport”, “hydrogen peroxide transport”, “defense response”, “proteolysis” and “reciprocal meiotic recombination”.
Cluster analysis of expression profiles

A set of genes with the highest (relative) variance in gene expression levels over the five samples was selected and designated as “Top500” genes. Soft clustering of the expression patterns of the “Top500” genes resulted in 6 clusters, containing a minimum of 2 genes and a maximum of 246 genes for clusters 2 and 6, respectively (Figure 5). The clusters represent expression patterns showing almost exclusive peaks at one of the five different stages tested. Clusters 4 and 5 both show transcript peaks in the apothecial disk stage (Apo56), but the genes assigned to cluster 4 showed some rise in transcript levels already in stipes (Apo34), while genes in cluster 5 did not show this early rise.

Figure 5: Soft clustering of the “Top500” genes with relatively most variable gene expression patterns
A hierarchical clustering (Figure 6) ordered the “Top500” genes into five groups, clearly distinguished in the dendrogram by deep splits between branches. The groups represent sets of genes that are predominantly expressed in only one of the five stages of apothecial development, as demonstrated by clear colour boundaries between groups in the heat map (Figure 6). The coloured bar at the right hand side of the gene dendrogram (left of the heat map) shows the correspondence between the hierarchical clustering and the grouping derived from the soft cluster method. The coloured bars are often continuous for multiple genes, indicating they are assigned to the same cluster by both methods, with the exception of the pink and blue bars. These genes were assigned to clusters 4 and 5 by the soft clustering method, but appear to be mingled by the hierarchical clustering. Other than this minor discrepancy, the two methods yielded very concordant results and provide support for the existence of five types of expression profiles within the “Top500” genes. The composition of the clusters will be discussed below.

Figure 6: Heat map created with a k-means hard clustering method. On the vertical axis is a hierarchical tree depicting the clustering of expression patterns of individual genes. The horizontal axis shows a clustering of the samples based on expression patterns of the genes together. The sample labels are shown at the top of the columns. Lines within the plot show the standardized expression levels of genes. The coloured vertical bars indicate to which Mfuzz cluster the gene was assigned. The colour code of these clusters is provided in the left top corner.
Sclerotium-specific expression (Soft Cluster 1)
Cluster 1 (Supplementary Table 2) comprises 167 genes that are expressed predominantly in sclerotia and barely in any of the other four stages tested. Among these are four cytochrome P450 encoding genes, seven MFS transporter encoding genes, four HET domain containing genes, several taurine and carnosine metabolism genes, one mannitol dehydrogenase encoding gene and the polyketide synthase gene BcPKS19. Strikingly, the set of 167 genes contains six clusters of at least three (up to seven) consecutive genes that are all predominantly expressed in sclerotia. However, the function of most of these 26 genes remains unknown because they have no functional annotation.

Primordia-specific expression (Soft Cluster 2)
Cluster 2 (Supplementary Table 3) comprises only two genes that are specifically expressed in stage Apo12. One of these genes (Bc04g02570) encodes a small secreted protein (length of the mature protein 86aa) containing 13 cysteine residues in a pattern reminiscent of hydrophobins. The second member of this cluster is the polyketide synthase gene Bc05g06220 (BcPKS15), which is located in a secondary metabolite cluster on chromosome 5 that also contains genes encoding a methyltransferase, an alcohol dehydrogenase and two transcription factors (one Zn2Cys6 type and one C2H2 type). Closer inspection revealed that these genes show similar expression as BcPKS15, but their absolute transcript levels were too low to be included in the list of “Top500” genes (not shown).

Stipe-specific expression (Soft Cluster 3)
Cluster 3 (Supplementary Table 4) comprises four genes of which three have functional annotations, i.e. a putative ornithine decarboxylase, a secreted tannase and a secreted feruloyl esterase B precursor.

Apothecial disk-specific expression (Soft Clusters 4 and 5)
Clusters 4 and 5 (Supplementary Tables 5 and 6) both contain genes that are predominantly expressed in apothecial disks (Apo56). The distinction between the clusters is that the 65 genes in cluster 4 already show a slight transcript increase in stage Apo34 and peak in stage Apo56, whereas the 16 genes in cluster 5 do not show a similar early increase in Apo34. Of the 65 genes in cluster 4, 45 genes are of unknown function and unique to Botrytis, to
Sclerotiniaceae or to Helotiales, while 5 genes encode proteins of unknown function that are conserved in many filamentous fungi. Of the 15 genes in this cluster with a functional description, there are two genes encoding Zn2Cys6 transcription factors, and other genes encoding an MFS hexose transporter, a putative aquaporin, a homolog of the SNF2 chromatin remodelling protein, a WD-domain containing protein and a CAZY GH76 α-mannanase. Of the 16 genes in cluster 5, six encode secreted proteins (of which one is a predicted glutaminase and one a predicted tannase), one is an MFS transporter encoding gene, and one a calpain family cysteine proteinase encoding gene. Half of the 16 genes have no homologs outside the Helotiales.

**Ascospore-specific expression (Soft Cluster 6)**

Cluster 6 (Supplementary Table 7) is especially rich in transporter genes, with 17 members of the MFS transporter family, including predicted sugar, phosphate and carboxylic acid transporters as well as three ABC transporter genes, including BcatrB and BcatrD which have been implicated in resistance to fungicides and plant defense compounds (Schoonbeek et al. 2003; Schoonbeek et al. 2002; Hayashi et al. 2002; Schoonbeek et al. 2001). Moreover cluster 6 contains a number of genes encoding plant cell wall degrading enzymes, such as BcPG2 (Kars et al. 2005a), BcPME2 (Kars et al. 2005b) and a pectin lyase gene. Furthermore, this cluster comprises two aspartyl proteinase genes, BcAP4 and BcAP14 (ten Have et al. 2010).

**Targeted deletion of the MAT1-1-1, MAT1-1-5, MAT1-2-1 and MAT1-2-4 genes**

In order to study the function of mating type genes MAT1-1-1, MAT1-1-5, MAT1-2-1 and MAT1-2-4 in apothecium development, deletion mutants were generated by replacing the coding region of each gene by the hygromycin phosphotransferase gene (HPH) in wild type strains of B. cinerea, SAS56 and SAS405 (Supplementary Figure 6). In addition, the MAT1-1-5 gene was deleted in the genetic background of strain B05.10 (not shown). Between three and six independent deletion mutants were obtained for each of the MAT genes. The growth rate and morphology of asexual structures (mycelium, sclerotia, macroconidia, microconidia) of all ΔMAT1-1-1, ΔMAT1-1-5, ΔMAT1-2-1 and ΔMAT1-2-4 deletion mutants were indistinguishable from those of the wild type (not shown).
Reciprocal crosses were set up, using two wild type strains, SAS56 and SAS405, and four single gene mutant strains (ΔMAT1-1-1, ΔMAT1-1-5, ΔMAT1-2-1 or ΔMAT1-2-4) in all relevant combinations, the results of which are shown in Table 5. All crosses were carried out with three independent deletion mutants for each gene and results were similar for independent mutants (not shown). In the control cross of wild type sclerotia (maternal parent) fertilized with wild type microconidia (paternal parent), apothecia developed as described by Faretra et al. (1988) and Whetzel (1945). By contrast, no apothecia developed and there was no sign of outgrowth of primordia when crosses were performed between wild type strain SAS405 and mutant ΔMAT1-1-1, or between wild type strain SAS56 and mutant ΔMAT1-2-1 (Figure 7). The lack of development of sexual structures was also observed in reciprocal crosses, indicating that the MAT1-1-1 and MAT1-2-1 genes are essential for the initiation of sexual development, both in maternal and paternal tissues.

Different results were observed for ΔMAT1-1-5 and ΔMAT1-2-4 deletion mutants. An aberrant development of apothecia was observed in crosses between wild type strain SAS405 and the ΔMAT1-1-5 knockout mutant, as well as in crosses between wild type strain SAS56 and the ΔMAT1-2-4 knockout mutant (Figure 7). The initial stages of primordium emergence and stipe development were similar to those in wild type apothecia, but the stipes remained blocked at the transition to disk development. The stipes did swell at the tip similar to wild type, but failed to expand laterally, even after several weeks of extended incubation. The stipes developed lobed, indented structures at the top (Figure 8), in which asci and ascospores were never observed (not shown).

The failure of ΔMAT1-1-5 and ΔMAT1-2-4 deletion mutants to make the switch from stipes to disks occurred in both reciprocal crosses irrespective whether the mutant was used in a cross as maternal parent (sclerotia) or as paternal parent (microconidia). Crosses between a ΔMAT1-1-5 mutant and a ΔMAT1-2-4 mutant yielded an identical, defective phenotype as crosses between either single mutant and their corresponding wild type mating partner (Figure 7), suggesting that these two genes jointly control the transition from stipe to disk development.
### Table 5: Summary of crosses performed between *B. cinerea* deletion mutants in MAT genes.

<table>
<thead>
<tr>
<th>Type of cross</th>
<th>Sclerotia (maternal parent)</th>
<th>Microconidia (paternal parent)</th>
<th>Fertility</th>
<th>Primordia</th>
<th>Stipe</th>
<th>Apothecial disk</th>
<th>Asci and ascospores</th>
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</thead>
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<td>WT’ x WT</td>
<td>SAS56</td>
<td>SAS405</td>
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<td>Yes</td>
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<td>SAS405</td>
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<td>ΔMAT1-2-1*</td>
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</tbody>
</table>

*mutant created in genetic background of SAS405

*b mutant created in genetic background of SAS56

cSterile MilliQ Water

WT wild type

* Δ mutant
Figure 7: Phenotypes of different crosses between wild type and mutants, as indicated. The maternal parent (sclerotia) is always mentioned first and the paternal parent (microconidia) is mentioned second. For each mutant, results of one deletion mutant are shown; similar results were obtained with two additional, independent deletion mutants.
Figure 8: Close-up of the phenotype resulting from a cross between wild type strain SAS405 (sclerotia) and ΔMAT1-1-5 microconidia. The stipe is blocked in transition to the apothecial disk and forms only a lobed, indented structure at the tip of the stipe.

Gene expression in stipes blocked in transition to the apothecial disk

qRT-PCR was performed on MAT genes (MAT1-1-1, MAT1-1-5, MAT1-2-1 and MAT1-2-4) in the ΔMAT1-1-5 and ΔMAT1-2-4 stipes and compared to wild type stipes at the same stage (Figure 9). In the ΔMAT1-1-5 stipes, the transcript of the MAT1-1-5 gene was undetectable as expected, but also the transcript level of the MAT1-1-1 and MAT1-2-1 genes was lower than in the wild type stipes. In the ΔMAT1-2-4 stipes, the transcript of the MAT1-2-4 gene was undetectable as expected, but also the transcript levels of the other three MAT genes were lower than in the wild type (Figure 9).

Figure 9: Relative expression of MAT1-1-1, MAT1-1-5, MAT1-2-1 and MAT1-2-4 genes in mutant stipes, blocked in transition to apothecial disks, as compared to stipes from a cross between two wild type isolates (competent for apothecial disk development) in the same stage. The left panel shows results for ΔMAT1-1-5 mutant stipes, the middle panel shows results for ΔMAT1-2-4 stipes, and the right panel shows results for wild type stipes. The data presented are the average of two biological replicates, with error bars indicating the standard deviation.
Fully elongated stipes with a swelling at the tip (blocked in transition to apothecial disk development, see Figure 8), were sampled from a cross between the wild type strain SAS405 and the \( \Delta MAT1-1-5 \) deletion mutant, made in the genetic background of strain B05.10 and from a control cross between two wild type strains at the same stage. Several stipes of mutant and wild type apothecia were pooled and RNA was isolated for transcriptome analysis. RNA sequencing reads were mapped on the genome of \( B. \ cinerea \) B05.10 and normalized read counts on gene models were determined.

A total of 6441704 and 6419648 read pairs were obtained from wild type and defective \( \Delta MAT1-1-5 \) stipes (Apo34), respectively, of which 94% (for both samples) could be mapped to the \( B. \ cinerea \) genome. A total of 33 genes with FPKM value >20 were exclusively expressed in wild type stipes (Apo34) (Supplementary Table 8). Another 81 genes with FPKM >20 were at least 50-fold downregulated in the mutant stipes (Supplementary Table 8). Conversely, a single gene with FPKM > 20 was exclusively expressed in the mutant stipes but not detectable in the wild type; moreover, 11 genes showed >20-fold higher transcript level in mutant stipes as compared to wild type (Supplementary Table 8). Most of these 12 genes had no functional annotation, and five of them were physically clustered (Supplementary Table 8).

Among the genes that were downregulated in the \( \Delta MAT1-1-5 \) mutant stipes (Apo34), three encoded RNA binding-domain containing proteins, two encoded transcription factors (Zn2Cys6 and C2H2), one an ortholog of histone H3 acetyltransferase protein GCN5/NGF1, one a FK506 suppressor gene Sfk1, one a high affinity glucose transporter, one a non-ribosomal peptide synthase (NRPS4), as well as dozens of genes that encoded proteins either specific to \( Botrytis \) or to \( Sclerotinia \)ceae.

Among the 114 genes that were downregulated in the \( \Delta MAT1-1-5 \) mutant stipes, 11 were present in the “Top500” clusters 4 and 5 (genes specifically expressed in apothecial disks). Of these only two genes were functionally annotated, as a Zn2Cys6 transcription factor and the FK506 suppressor Sfk1. The remaining 9 genes are unique to \( Botrytis \) or have homologs only in the \( Sclerotiniaceae \). There was also an overlap of 41 genes between the set of 114 genes downregulated in the \( \Delta MAT1-1-5 \) mutant stipes and the genes that are differentially
expressed in wild type apothecia between stages Apo34 and Apo56. These 41 genes encode, among others, a MHYT-domain signalling protein, the FK506 suppressor Sfk1, the putative calcium homeostasis protein regucalcin, the Zn2Cys6 transcription factor mentioned above, the protein kinase CBK1, a glucose transporter, an elongation factor 2 kinase, the non-ribosomal peptide synthase BcNRPS4, the histone acetyltransferase GCN5/NGF1 and a protein tyrosine phosphatase. Other genes in this set of 41 genes have no functional annotation and are mostly unique to Botrytis or have homologs only in the Sclerotiniaceae.

Discussion

B. cinerea apothecium development

Using apothecia as a resource for transcriptome analysis to study sexual development within the Ascomycota is attractive as compared to other ascocarps such as cleistothecia, perithecia and pseudothecia because it is feasible to obtain tissues from distinct developmental stages fairly easily. Apothecia of Sclerotiniaceae germinate carpogenically from sclerotia in an aqueous environment minimizing contamination with hyphal tissue derived from the parents. Once the sclerotia rupture to give rise to the emergence of primordia, the apothecia develop over a period of several weeks. Although different developmental stages can be harvested, >100 primordia (1-3 mm in height) were required to obtain sufficient RNA for sequencing. The samples are clean and distinct, and the use of laser microdissection to obtain pure ascocarp tissues, as reported for Sordaria macrospora (Teichert et al. 2012), was not required for B. cinerea.

We carried out a detailed transcriptome analysis of sexual development of B. cinerea, starting with the sclerotia, through three stages of apothecial development and finally the ascospores that were released from mature apothecia. This study presents, to our knowledge, the first whole genome transcriptional analysis of sexual development within the Helotiales, and the first study on gene expression in ascospores of any filamentous Ascomycota species. Apothecial disks (stages 5-6, Figure 1) contain the greatest diversity of cell types, since they are composed of cell layers of excipulum and hymenium, of dikaryotic cells, diploid cells undergoing meiosis as well as asci and ascospores in different stages of development and maturity. This diversity in tissue types was reflected in the high number of
genes expressed in the apothecial disk sample: more than 98.5% of the gene models in the 
*B. cinerea* genome were represented by at least one read, and >90% of the gene models 
displayed an FPKM value ≥1. By contrast, sclerotia and ascospore samples, which 
represented more homogeneous tissue, displayed FPKM values ≥1 for only 75% of the gene 
models.

**Transcriptome analysis of* B. cinerea* sclerotia, apothecia and ascospores**

The RNA sequencing data were analysed in different ways. Firstly, the transcript levels were 
analysed for *B. cinerea* orthologs of genes from *Aspergillus nidulans* and *Sordaria 
macrospora*, which are known to regulate sexual development. Many *B. cinerea* orthologs 
were expressed to different levels during the consecutive stages of apothecium 
development, no unusual expression patterns could be distinguished. *B. cinerea* genes that, 
based on functions described in *A. nidulans*, likely act as positive regulators of sexual 
development, in several cases showed a high transcript level in early stages of apothecium 
development, which dropped in mature apothecial disks and ascospores. Conversely, some 
*B. cinerea* genes that, based on functions described in *A. nidulans*, likely act as negative 
regulators of sexual development displayed high transcript levels in sclerotia and primordia, 
which dropped in later stages of apothecium development and ascospores. Such patterns 
were, however, not consistently observed for all *B. cinerea* genes that presumably act as 
positive or negative regulators in sexual development, based on functions described in *A. 
nidulans*. In several cases the expression level was fairly constant, or showed a distinct 
pattern (Figures S1-5). It should be kept in mind that the role of any particular gene as a 
positive or negative regulator of sexual development, does not necessarily imply that this 
genome itself is differentially transcribed in distinct stages of sexual development.

The second type of analysis identified genes which displayed significant changes in transcript 
levels between two consecutive stages of sexual development. The biggest changes were 
observed in the transition from sclerotia to primordia (with >2000 differentially expressed 
genes), and even more so in the transition from mature disks to ascospores (with >5000 
differentially expressed genes). Both these transitions reflect a dramatic morphological and 
physiological change. It is thus not surprising that these transitions showed the largest shifts 
in gene expression, but the fact that almost half of the genes in the genome are differentially
Chapter 2

expressed in the transition from apothecial disks to ascospores (with equal proportions up- and down-regulated) was surprising. The transitions from primordia to stipes and from stipes to disks resulted in substantially lower numbers of differentially expressed genes. Especially the primordia and stipes are morphologically similar to each other. There is a continuous tissue elongation, eventually swelling at the tip when the stipe has emerged from the water and reached a height of about 1 cm. It was thus no surprise that only 27 genes were differentially expressed between these stages, however, these genes were all down-regulated. We anticipated that the upregulation of genes required for the swelling and transition to disk development might have been detected, however, no upregulated genes were identified. There were 884 genes differentially expressed between stages Apo34 and Apo56, and GO enrichment analysis showed that some terms related to sexual development were enriched in the dataset. Specifically, the upregulated genes displayed, amongst others, an overrepresentation of the term “G2/M transition of mitotic cell cycle” while the term “negative regulation of sexual sporulation resulting in formation of cellular spore” was overrepresented in the downregulated genes at this transition.

Soft and hierarchical clustering were applied to identify genes with a stage-specific expression pattern. The results of both methods were largely congruent for both methods, with the only difference that soft clustering divided the genes with high expression levels in apothecial disks into two clusters, which differed in the extent to which transcript levels started to increase in stipes. The hierarchical clustering, however, did not support this division. Overall the clustering methods identified five distinct clusters, each typified by genes with a very high transcript level in one stage and extremely low levels in the four other stages.

167 genes were exclusively expressed in sclerotia among which four HET-domain proteins, whose physiological function in B. cinerea remains unknown. Also several genes involved in taurine and carnosine metabolism were highly expressed in sclerotia, possibly because of the function of these compounds as antioxidants. Also mannitol dehydrogenase might function to generate mannitol as a scavenger of reactive oxygen species. Polyketide synthase BcPKS19 was specifically expressed in sclerotia, but its product is unknown yet. The BcPKS19 gene is flanked by a glutathione S-transferase encoding gene, but for the remainder it is
surrounded on both sides by >10 kb repetitive sequences, separated from other genes that are normally present in secondary metabolite gene clusters.

There were very few genes with primordia-specific expression, one of which encodes a small secreted protein, with 13 cysteine residues in a pattern that very much resembles the cysteine spacing present in hydrophobins. The reason for not annotating this protein as a hydrophobin might be the large (and uneven) number of cysteine residues. Also specifically expressed in primordia was a group of five physically clustered genes encoding the polyketide synthase \textit{BcPKS15}, whose product is unknown, an alcohol dehydrogenase, a methyltransferase, and two transcription factors. Stipes showed a specific expression of genes encoding a secreted tannase, a feruloyl esterase and an ornithine decarboxylase, indicating that stipes may produce putrescine.

Among the 81 genes almost exclusively expressed in the apothecial disks, two genes encoded Zn2Cys6-type transcription factors, three encoded putative transporters, and three were single genes encoding a secreted glutaminase, a calpain family cysteine proteinase, a homolog of the SNF2 chromatin remodelling protein, and a WD-domain protein (ortholog of \textit{S. cerevisiae} Cdc20p which is part of the APC complex involved in meiosis; Pan and Chen 2004).

Mature ascospores displayed an expression profile remarkably distinct from apothecial disks. More than 5000 genes were differentially expressed between apothecial disks and the ascospores, representing almost half the genes present in the \textit{B. cinerea} genome. Among the 2500 upregulated genes were many that likely function in plant infection, including genes encoding pectinases (an exo- and endopolygalacturonase, pectin methylsterase), aspartic proteinases, as well as putative sugar, phosphate and carboxylic acid transporters (member of the MFS superfamily) and ABC transporters known to be involved in efflux of plant defence compounds. This observation suggests that ascospores are primed, at least at the transcriptional level, for the production of virulence factors prior to contact with a host plant. Whether the transcripts are actually translated into functional enzymes is uncertain.
The development of an apothecium is a paraphyletic trait

Both the cluster analysis and differential expression analysis between two consecutive developmental stages identified a large number of genes that lack known protein domains and are unique to *B. cinerea*, or restricted to fungi in the family *Sclerotiniaceae*. More than 60% of the genes that were identified by cluster analysis as being expressed in the three stages of apothecium development (primordia, stipes, disks) have no homologues in fungi producing other types of fruiting bodies (perithecia, cleistothecia, pseudothecia). Traeger et al. (2013) reported a similar observation for *Pyrenoma confluens*, a basal Pezizomycete fungus that also produces apothecia and contains in its genome >5700 “orphan” genes, of which >20% were upregulated during sexual development. There was no homology, however, between the *B. cinerea* genes and *P. confluens* genes that are specifically expressed in apothecia. Gargas and Taylor (1995) inferred from SSU rDNA sequences that Ascomycota species that are capable of producing apothecia can be found in four different orders and therefore do not represent a monophyletic lineage. This indicates that the development of an apothecium is a paraphyletic trait. *Blumeria graminis* is the closest relative to *B. cinerea* and *S. sclerotiorum* of which the genome was sequenced (Spanu et al., 2010) and this species produces cleistothecia as fruiting bodies. The apothecium of *Sclerotiniaceae* may be a recent invention that evolved after their divergence from the *Erisyphales*.

Mating type genes MAT1-1-5 and MAT1-2-4 jointly act as dimeric transcriptional regulators

Single gene deletion mutants in the *B. cinerea* MAT1-1 or MAT1-2 locus were strongly compromised in apothecium development. Deletion of the MAT1-1-1 gene or the MAT1-2-1 gene, both of which are highly conserved among Ascomycota, rendered *B. cinerea* entirely sterile, as in other Ascomycota fungi (Ferreira et al. 1998). Even the emergence of primordia was blocked, suggesting that sexual fertility was affected in a very early stage. The precise defect in sexual development was not further investigated since it is unknown when and where in the *B. cinerea* sclerotium the fertilization process occurs during the weeks after adding microconidia of the paternal parent to the sclerotia. In any case, the MAT1-1-1 and MAT1-2-1 genes are essential to initiate apothecium development in *B. cinerea*. 
On the other hand, the MAT1-1-5 and MAT1-2-4 genes are only required in later stages of apothecium development. Deletion of the MAT1-1-5 or the MAT1-2-4 gene still resulted in formation of stipes which were, however, blocked in the transition to an apothecial disk. We never observed asci or ascospores in these mutant stipes. The same phenotype was observed in crosses using ΔMAT1-1-5 or ΔMAT1-2-4 mutants with a wild type strain of opposite mating type, and the defect occurred in reciprocal crosses alike. Also a cross between a ΔMAT1-1-5 and a ΔMAT1-2-4 mutant resulted in the same phenotype, demonstrating that the MAT1-1-5 and MAT1-2-4 genes display a genetic interaction.

The transition from stipe to apothecial disk is typified by the formation of crozier cells in which karyogamy occurs (Malagnac et al., 1997; Coppin et al. 2012) and the resulting diploid nuclei subsequently enter meiosis to eventually form 8 ascospores in an ascus. We propose that MAT1-1-5 and MAT1-2-4 act as dimeric transcriptional regulators that directly or indirectly control the formation of crozier cells, and the absence of either of these proteins results in failure to proceed to karyogamy which blocks further downstream processes such as apothecial disk expansion. It remains to be demonstrated whether MAT1-1-5 and MAT1-2-4 proteins indeed physically interact to perform a complex with DNA binding properties, and to which sequence elements they might bind. To obtain a first glance of genes controlled by the MAT1-1-5 gene, RNA sequencing was performed to reveal which genes were differentially expressed in ΔMAT1-1-5 stipes that were blocked in transition to disks, as compared to wild type apothecia. Among the 114 genes that were entirely switched off or >50-fold down-regulated in the mutant stipes were three genes encoding RNA binding-domain containing proteins, two genes encoding transcription factors (Zn2Cys6-type and C2H2-type), and the ortholog of the histone H3 acetyltransferase protein GCN5. The transcription factors might be important in regulating the expression of downstream genes, whereas the histone H3 acetyltransferase GCN5 might be involved in epigenetic regulation of gene expression, or in facilitating meiotic recombination. Yamada et al. (2004) reported that Schizosaccharomyces pombe double mutants in which the histone acetyltransferase Gcn5 and the Swi2-like chromatin remodeling factor Snf22 were both deleted, showed abolished chromatin remodeling and significant reduction of meiotic recombination in a recombination hotspot.
There is as yet no evidence for epigenetic regulation of sexual development in filamentous Ascomycota, but the expression pattern of the histone acetyltransferase GCN5 and its lack of expression in ΔMAT1-1-5 mutant stipes might suggest that this gene is involved in regulating apothecium disk development. Expression data in the present B. cinerea dataset provide (indirect) hints for epigenetic regulation in certain stages. Several examples were observed of physically clustered genes that are co-regulated during developmental transitions. The most striking example was in the set of genes with a sclerotium-specific expression pattern: of the 167 genes in this set, 26 genes (all with unknown function) are located in clusters of three to seven consecutive genes. Future studies need to resolve whether the co-expression of such physically clustered genes is mediated by common transcription factor binding sites in their promoter regions, or by epigenetic regulation of chromatin architecture.

The present study has revealed that MAT1-1-5 and MAT1-2-4 are important (transcriptional) regulators of B. cinerea apothecium development, and has provided insight into genes and processes that they control. The downstream pathways regulated by MAT1-1-5 and MAT1-2-4 will be subject of future studies.

**Supporting information**

Supplementary Figure 1, Supplementary Figure 2, Supplementary Figure 3, Supplementary Figure 4, Supplementary Figure 5, Supplementary Figure 6, Supplementary Table 1, Supplementary Table 2, Supplementary Table 3, Supplementary Table 4, Supplementary Table 5, Supplementary Table 6, Supplementary Table 7 and Supplementary Table 8.
Transcriptome and functional analysis of fruiting body development in *Botrytis cinerea*

References


Transcriptome and functional analysis of fruiting body development in *Botrytis cinerea*


Supplementary Figure 1
Transcript levels of *B. cinerea* genes orthologous to *A. nidulans* genes involved in perception of environmental signals during sexual development (according to Dyer and O’Gorman, 2012).

Tissue samples analysed are *B. cinerea* sclerotia, apothecial primordia, stipes, disks, as well as ascospores.

Transcript levels are presented as average FPKM values of two independent biological replicates, with the exception of the sclerotia, which represent a single sample. A: activator of sexual development, R: repressor of sexual development.
Supplementary Figure 2

Transcript levels of *B. cinerea* genes orthologous to *A. nidulans* genes involved in mating processes and signal transduction during sexual development (according to Dyer and O’Gorman, 2012).

Tissue samples analysed are *B. cinerea* sclerotia, apothecial primordia, stipes, disks, as well as ascospores.

Transcript levels are presented as average FPKM values of two independent biological replicates, with the exception of the sclerotia, which represent a single sample. A: activator of sexual development, R: repressor of sexual development.
Supplementary Figure 3

Transcript levels of *B. cinerea* genes orthologous to *A. nidulans* genes encoding transcription factors and regulatory proteins involved in sexual development (according to Dyer and O’Gorman, 2012).

Tissue samples analysed are *B. cinerea* sclerotia, apothecial primordia, stipes, disks, as well as ascospores.

Transcript levels are presented as average FPKM values of two independent biological replicates, with the exception of the sclerotia, which represent a single sample. A: activator of sexual development, R: repressor of sexual development.
Supplementary Figure 4

Transcript levels of *B. cinerea* genes orthologous to *A. nidulans* genes involved in endogenous physiological processes during sexual development (according to Dyer and O’Gorman, 2012).

Tissue samples analysed are *B. cinerea* sclerotia, apothecial primordia, stipes, disks, as well as ascospores.

Transcript levels are presented as average FPKM values of two independent biological replicates, with the exception of the sclerotia, which represent a single sample. A: activator of sexual development, R: repressor of sexual development.
Supplementary Figure 5
Transcript levels of *B. cinerea* genes orthologous to *A. nidulans* genes involved in ascospore production and maturation during sexual development (according to Dyer and O’Gorman, 2012).

Tissue samples analysed are *B. cinerea* sclerotia, apothecial primordia, stipes, disks, as well as ascospores.

Transcript levels are presented as average FPKM values of two independent biological replicates, with the exception of the sclerotia, which represent a single sample. A: activator of sexual development, R: repressor of sexual development.
Supplementary Figure 6
Schematic representation of strategy for targeted deletion of MAT genes. Knockout of BcMAT1-1-1, BcMAT1-1-5, BcMAT1-2-1 and BcMAT1-2-4 by targeted gene replacement. Organization of BcMAT1-1-1, BcMAT1-1-5, BcMAT1-2-1 and BcMAT1-2-4 locus before and after homologous recombination. Orientation of the target gene and HPH are indicated by white and grey arrows, respectively. Upstream and downstream flanks of target genes are shown with open boxes. Polymerase chain reaction (PCR) analysis of wild-type strain SAS56, SAS405 and knockout mutant strains. The genomic DNA of each strain was used to verify 5’ and 3’ homologous recombination and absence of targeted genes in corresponding knockout mutants, respectively.
## Supplementary Table 1

Transcript levels of *B. cinerea* genes orthologous to *A. nidulans* genes involved in perception of environmental signals during sexual development (according to Dyer and O’Gorman, 2012).

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Transcriptome and functional analysis of fruiting body development in *Botrytis cinerea*
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Transcript levels of *B. cinerea* genes orthologous to *A. nidulans* genes encoding transcription factors and regulatory proteins involved in sexual development (according to Dyer and O’Gorman, 2012).

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Transcript levels of *B. cinerea* genes orthologous to *A. nidulans* genes involved in endogenous physiological processes during sexual development (according to Dyer and O’Gorman, 2012).

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Transcript levels of *B. cinerea* genes orthologous to *A. nidulans* genes involved in ascospore production and maturation during sexual development (according to Dyer and O’Gorman, 2012).

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## Supplementary Table 2

### Soft cluster 1: genes specifically expressed in sclerotia

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Transcriptome and functional analysis of fruiting body development in *Botrytis cinerea*

- Bc04g03990.1 phosphate permease
- Bc04g04920.1 BcNMT1 thiamine synthase
- Bc05g00100.1 small secreted Botrytis-specific protein
- Bc05g01530.1 conserved fungal protein
- Bc05g05300.1 conserved fungal protein
- Bc05g06500.1 Botrytis/Sclerotinia-specific protein
- Bc06g01480.1 Botrytis-specific protein
- Bc06g01560.1 Botrytis-specific protein
- Bc06g01730.1 conserved fungal protein
- Bc06g03370.1 mitochondrial phosphate carrier protein 2
- Bc06g05580.1 conserved fungal protein
- Bc06g05780.1 Botrytis-specific protein
- Bc06g05790.1 conserved fungal protein
- Bc06g06420.1 MFS transporter
- Bc06g07030.1 Botrytis-specific protein
- Bc06g07590.1 CAZY GH71 family protein alpha-1,3-glucanase
- Bc07g00950.1 short-chain dehydrogenase/reductase
- Bc07g00980.1 MFS transporter
- Bc07g00990.1 O-methyltransferase
- Bc07g01570.1 conserved fungal protein
- Bc07g04230.1 conserved fungal protein
- Bc07g04990.1 BcABA4, short-chain dehydrogenase/reductase
- Bc07g05420.1 conserved fungal protein
- Bc07g05560.1 Botrytis/Sclerotinia-specific protein
- Bc07g06010.1 thiazole biosynthetic enzyme, mitochondrial
- Bc08g00290.1 BcPKS19
- Bc08g00300.1 glutathione S-transferase
- Bc08g00340.1 MFS transporter for polyketide
- Bc08g00970.1 conserved fungal protein
- Bc08g01290.1 secreted protein
- Bc08g01760.1 taurine catabolism dioxygenase TauD
- Bc08g01770.1 putative taurine catabolism dioxygenase protein
- Bc08g02700.1 conserved alpha/beta hydrolase family protein
- Bc08g03080.1 putative helix-loop-helix DNA-binding domain-containing protein
- Bc08g03590.1 cytochrome P450
- Bc08g04060.1 ferric/cupric reductase transmembrane component
- Bc08g04070.1 conserved fungal GPI-anchored protein
- Bc08g05820.1 conserved fungal protein
- Bc08g05870.1 CAZY GH95 family protein, alpha-fucosidase
- Bc08g07130.1 Asp-hemolysin family protein
- Bc08g07150.1 Botrytis-specific protein
- Bc08g07160.1 Botrytis-specific protein
- Bc09g00010.1 Botrytis-specific protein
- Bc09g00500.1 conserved fungal protein
- Bc09g00520.1 conserved fungal protein
- Bc09g01120.1 Botrytis-specific protein
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- Bc09g03310.1 CCCH zinc finger DNA binding protein
- Bc09g03670.1 GABA permease
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- Bc09g04570.1 GMC oxidoreductase, choline or cellubiose dehydrogenase
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Bc10g05620.1 pectate lyase
Bc11g01680.1 putative modin protein
Bc11g02020.1 Botrytis/Sclerotinia-specific protein
Bc11g02170.1 tyrosinase
Bc11g02450.1 ankyrin repeat protein
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Bc11g06520.1 cytochrome P450
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Bc12g01010.1 putative peptidase inhibitor i9
Bc12g02880.1 serine/threonine-protein kinase nrc-2
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Bc12g04300.1 mitochondrial S-adenosylmethionine transporter
Bc12g05860.1 glucose dehydrogenase
Bc12g06750.1 Botrytis-specific protein
Bc13g00040.1 Botrytis-specific protein
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Bc13g03780.1 conserved fungal protein
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Bc13g05320.1 Botrytis-specific protein
Bc13g05330.1 conserved fungal protein
Bc13g05340.1 conserved fungal protein
Bc14g00090.1 thermophilic desulfurizing enzyme family protein
Bc14g00300.1 conserved fungal protein
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Bc14g03670.1 tetrahydrofolylpolyglutamate synthase
Bc14g04030.1 Botrytis-specific protein
Bc14g04530.1 2,3-diketo-5-methylthio-1-phosphopentane phosphatase
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Bc14g05530.1 conserved fungal protein
Bc14g05540.1 putative zinc transporter
Bc14g05560.1 epoxide hydrolase
Bc15g00290.1 conserved fungal protein
Transcriptome and functional analysis of fruiting body development in *Botrytis cinerea*

- **Bc15g00560.1** Botrytis/Sclerotinia-specific protein
- **Bc15g03060.1** conserved fungal protein
- **Bc15g03870.1** Botrytis/Sclerotinia-specific protein
- **Bc15g03940.1** conserved fungal protein
- **Bc15g03960.1** tyrosinase
- **Bc15g03970.1** conserved fungal protein
- **Bc15g03980.1** Botrytis-specific protein
- **Bc15g03990.1** Botrytis-specific protein
- **Bc15g04000.1** unknown protein with poor homology to other fungi
- **Bc15g04010.1** Botrytis-specific protein
- **Bc15g04640.1** Botrytis/Sclerotinia-specific protein
- **Bc15g05670.1** potassium/sodium efflux P-type ATPase
- **Bc16g00780.1** Botrytis/Sclerotinia-specific protein
- **Bc16g01410.1** cytochrome P450
- **Bc16g02740.1** Botrytis-specific protein
- **Bc16g03380.1** mannitol dehydrogenase

**Supplementary Table 3**

**Soft cluster 2: genes specifically expressed in primordia (Apo12)**

- Bc05g06220.1 Polyketide synthase BcPKS15
- Bc04g02570.1 small secreted hydrophobin-like protein

**Supplementary Table 4**

**Soft cluster 3: genes specifically expressed in stipes (Apo34)**

- Bc06g02830.1 putative ornithine decarboxylase
- Bc06g07000.1 secreted tannase
- Bc07g00030.1 putative feruloyl esterase B precursor
- Bc10g04740.1 unknown

**Supplementary Table 5**

**Soft cluster 4: genes specifically expressed in stage Apo56 (disks) with some increase in Apo34**

- Bc12g00470.1 = Botrytis/Sclerotinia-specific protein
- Bc12g04400.1 = Botrytis-specific protein
- Bc07g04190.1 = putative transcription factor Zn2Cys6
- Bc01g03280.1 = putative ubiquitin-conjugating enzyme E2
- Bc12g02100.1 = conserved fungal protein
- Bc11g01110.1 = Botrytis-specific protein
- Bc16g01930.1 = putative transcription factor Zn2Cys6
- Bc13g02810.1 = Helotiales-specific protein
- Bc07g05010.1 = Botrytis/Sclerotinia-specific protein
- Bc02g05350.1 = Botrytis/Sclerotinia-specific protein
- Bc06g04430.1 = protein kinase domain-containing protein
- Bc03g07270.1 = Botrytis/Sclerotinia-specific protein
- Bc11g01850.1 = Botrytis-specific protein
Bc14g00450.1 = Botrytis-specific protein
Bc05g06780.1 = Botrytis/Sclerotinia-specific protein
Bc16g02400.1 = secreted Botrytis/Sclerotinia-specific protein
Bc06g06850.1 = Aminoglycoside 3’-phosphotransferase (APH) Choline Kinase (ChoK) family protein
Bc06g02180.1 = Botrytis/Sclerotinia-specific protein
Bc03g09200.1 = conserved fungal protein
Bc03g01600.1 = conserved fungal protein
Bc02g02560.1 = Botrytis/Sclerotinia-specific protein
Bc01g09820.1 = Botrytis/Sclerotinia-specific protein
Bc06g02440.1 = Botrytis/Sclerotinia-specific protein
Bc16g00460.1 = conserved fungal protein
Bc07g05370.1 = Botrytis-specific protein
Bc02g00590.1 = Botrytis/Sclerotinia-specific protein
Bc10g02310.1 = Botrytis/Sclerotinia-specific protein
Bc12g02500.1 = Botrytis-specific protein
Bc10g01370.1 = Botrytis/Sclerotinia-specific protein
Bc14g01470.1 = Botrytis-specific protein
Bc02g03220.1 = aquaporin-like protein (MIP superfamily)
Bc11g02860.1 = Botrytis/Sclerotinia-specific protein
Bc07g02230.1 = Botrytis-specific protein
Bc09g03300.1 = Botrytis/Sclerotinia-specific protein
Bc06g04570.1 = Botrytis-specific protein
Bc04g04620.1 = WD-domain containing protein
Bc14g02040.1 = Botrytis-specific protein
Bc03g01510.1 = Botrytis-specific protein
Bc04g02450.1 = Botrytis-specific protein
Bc02g09080.1 = Botrytis/Sclerotinia-specific protein
Bc09g02120.1 = Botrytis/Sclerotinia-specific protein
Bc02g00370.1 = CAZY GH76 protein, putative alpha-mannanase
Bc02g03720.1 = Botrytis-specific protein
Bc14g03350.1 = Botrytis/Sclerotinia-specific protein
Bc08g06360.1 = Botrytis-specific protein
Bc15g04800.1 = thioesterase-like superfamily protein
Bc05g01070.1 = Botrytis-specific protein
Bc15g04230.1 = Botrytis/Sclerotinia-specific protein
Bc09g02880.1 = Botrytis/Sclerotinia-specific protein
Bc06g06180.1 = MFS hexose transporter
Bc07g03870.1 = Botrytis-specific protein
Bc04g06970.1 = putative cytochrome P450 oxidoreductase
Bc06g01660.1 = Botrytis-specific protein
Bc10g03360.1 = conserved fungal domain-containing protein
Bc05g03480.1 = similar to FK506 suppressor Sfk1
Bc06g00300.1 = Botrytis-specific protein
Bc13g05540.1 = threonine aspartase
Bc03g08580.1 = SNF2 helicase superfamily protein
Bc01g06000.1 = Botrytis/Sclerotinia-specific protein
Bc14g04280.1 = Botrytis/Sclerotinia-specific protein
Bc12g00370.1 = phospholipase/carboxylesterase superfamily protein
Bc10g05300.1 = Helotiales-specific protein
Bc09g03290.1 = Botrytis-specific protein
Bc04g06440.1 = Botrytis-specific protein
Bc16g02350.1 = Botrytis-specific protein
**Supplementary Table 6**

**Soft cluster 5: genes specifically expressed in stage Apo56 (apothecial disks)**

Bc10g02890.1 = secreted Botrytis/Sclerotinia-specific protein  
Bc13g02200.1 = secreted Botrytis-specific protein  
Bc05g00310.1 = secreted glutaminase  
Bc02g09010.1 = conserved fungal protein  
Bc15g04520.1 = CFEM domain containing protein  
Bc14g01760.1 = MFS transporter  
Bc12g00580.1 = secreted protein Botrytis-specific  
Bc08g00940.1 = secreted protein CFEM domain (cysteine-rich)  
Bc05g06950.1 = Helotiales-specific protein  
Bc12g00150.1 = putative serine/threonine kinase  
Bc04g04570.1 = Botrytis-specific protein  
Bc16g03330.1 = predicted protein  
Bc05g02530.1 = secreted tannase/feruloyl esterase family protein  
Bc02g05390.1 = Botrytis/Sclerotinia-specific protein  
Bc10g01910.1 = putative calpain family cysteine protease  
Bc01g10190.1 = Botrytis-specific protein

**Supplementary Table 7**

**Soft cluster 6: genes specifically expressed in ascospores**

Bc01g00160.1: short-chain dehydrogenase, NADB-Rossmann family protein  
Bc01g00790.1: conserved fungal protein  
Bc01g00910.1: Botrytis-specific protein  
Bc01g01400.1: alpha/beta hydrolase superfamily protein  
Bc01g01410.1: aspartate aminotransferase  
Bc01g01430.1: MFS transporter family protein  
Bc01g01500.1: FAD dependent oxidoreductase  
Bc01g01760.1: CAZY GH18 family protein, putative chitinase  
Bc01g02520.1: CAZY GT2 family protein, chitin synthase class III  
Bc01g02530.1: similar to nuclear pore protein  
Bc01g03710.1: siderophore acetyltransferase  
Bc01g04230.1: phosphoadenosine phosphosulfate reductase  
Bc01g04340.1: sulfate adenylyltransferase  
Bc01g05050.1: Botrytis-specific protein  
Bc01g06040.1: methylammonium permease 1 MeaA  
Bc01g06950.1: conserved fungal protein  
Bc01g08010.1: hypoxanthine guanine phosphoribosyltransferase  
Bc01g08220.1: medium chain dehydrogenase/reductase  
Bc01g09270.1: PQ-loop repeat protein  
Bc01g09360.1: ascorbate peroxidase  
Bc01g09710.1: conserved fungal protein  
Bc01g10270.1: CAZY GT31 family protein  
Bc01g10680.1: MFS transporter, putative vitamin B6 transporter BSU1  
Bc01g11030.1: putative 3-hydroxyacyl- NAD binding protein  
Bc01g11480.1: abhydrolase6 domain-contain protein, Botrytis-specific
Bc01g11550.1: putative O-methyltransferase
Bc02g00240.1: cytochrome P450 monoxygenase
Bc02g00720.1: Botrytis-specific protein
Bc02g01180.1: unknown protein
Bc02g01230.1: conserved fungal protein
Bc02g01580.1: acyl-CoA-transferase family III
Bc02g01660.1: putative amino acid transporter
Bc02g03270.1: pyridoxal phosphate (PLP)-dependent aspartate aminotransferase superfamily.
Bc02g03330.1: C4-dicarboxylate transporter/malic acid transporter
Bc02g03560.1: putative MFS phosphate transporter
Bc02g05250.1: Botrytis-specific protein
Bc02g05300.1: Major royal jelly protein
Bc02g05740.1: allantoicase, metallo-dependent hydrolase
Bc02g05800.1: NADB-Rossmann superfamily protein
Bc02g05870.1: NADP-dependent leukotriene B4 12-hydroxydehydrogenase
Bc02g07320.1: RTA1 domain-containing protein
Bc02g07330.1: HeLo domain-containing protein
Bc02g07490.1: conserved fungal protein
Bc02g09380.1: putative lactonohydrolase, gluconolactonase
Bc03g00510.1: Botrytis-specific protein
Bc03g00650.1: allantoicase
Bc03g01840.1: C6 zinc finger protein, fungal-specific TF domain
Bc03g01990.1: 2oxoglutarate-Fe(II) oxygenase family oxidoreductase
Bc03g02760.1: aspartic proteinase BcAP4
Bc03g03630.1: CAZY GH12 protein, endoglucanase, xyloglucan hydrolase
Bc03g03790.1: putative GPI anchored cell wall protein
Bc03g03830.1: pectin methylesterase BcPME2
Bc03g05130.1: pyridine nucleotide-disulphide oxidoreductase AMID-like
Bc03g05980.1: conserved fungal protein
Bc03g06110.1: putative cyclopropane-fatty-acyl-phospholipid synthase, SAM-dependent methyltransferase
Bc03g07360.1: CAZY PL1 family protein, pectin lyase
Bc03g07670.1: putative NAD-specific glutamate dehydrogenase
Bc03g08820.1: ankyrin repeat protein
Bc04g00180.1: aspartate aminotransferase superfamily protein
Bc04g00700.1: conserved fungal protein
Bc04g01520.1: putative branched-chain-amino-acid aminotransferase
Bc04g01690.1: ATP-dependent RNA helicase DBP3
Bc04g02240.1: 2-hydroxyacid dehydrogenase
Bc04g02860.1: Botrytis-specific protein
Bc04g03000.1: putative 3’-5’ exonuclease
Bc04g03100.1: ketol-acid reductoisomerase, mitochondrial precursor
Bc04g03290.1: HSP70-family protein, actin-like ATPase
Bc04g03300.1: conserved fungal protein
Bc04g03360.1: conserved fungal protein
Bc04g04490.1: asparagine synthetase
Bc04g04580.1: MFS transporter
Bc04g06040.1: CAZY GT2 family protein, chitin synthase
Bc04g06510.1: short chain dehydrogenase
Bc04g06890.1: carbonic anhydrase
Bc05g00550.1: no Blast hit
Bc05g00650.1: allantoicase
Transcriptome and functional analysis of fruiting body development in *Botrytis cinerea*

Bc05g01390.1:MFS transporter
Bc05g01660.1:CAZY GH5 family protein
Bc05g01800.1:PhosphatidylEthanolamine-Binding Protein (PEBP)
Bc05g02290.1:glucose-inducible SAM-dependent methyltransferase Rrg1
Bc05g02620.1:isocitrate lyase superfamily protein
Bc05g03370.1:CAZY GT69 family protein, alpha-1,3-mannosyltransferase
Bc05g03800.1:similar to transcription factor Zn, C2H2
Bc05g04170.1:ribosome assembly protein Noc2
Bc05g05450.1:short-chain dehydrogenase/reductase
Bc05g06260.1:Botrytis-specific protein
Bc05g06280.1:medium chain reductase/dehydrogenase
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Bc05g07520.1:MFS sugar transporter
Bc06g00540.1:Botrytis/Sclerotinia-specific protein
Bc06g00660.1:Major Royal Jelly protein
Bc06g00820.1:MFS sugar transporter
Bc06g01140.1:short chain dehydrogenase/reductase
Bc06g01340.1:NAD(P)-binding Rossmann-fold containing protein
Bc06g01350.1:Botrytis/Sclerotinia-specific protein
Bc06g01590.1:C4-dicarboxylate transporter/malic acid transport protein
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Bc06g03100.1:conserved fungal protein
Bc06g05270.1:CAZY GT71 family protein, mannosyltransferase
Bc06g05360.1:Dihydrodipicolinate synthase family
Bc07g00040.1:PI-PLC-like phosphodiesterases superfamily protein
Bc07g00090.1:NAD dependent epimerase/dehydratase family protein
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Bc07g07100.1:MFS sugar transporter
Bc07g07120.1:acyltransferase condensation domain-containing protein
Bc07g07130.1:cytochrome P450 monoxygenase
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Bc09g05210.1: putative bZIP transcription factor protein
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Bc12g03650.1: aldo/keto reductase similar to norsorolinic acid reductase
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Chapter 2

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Bc16g01890.1: Zn2Cys6 fungal-specific transcription factor
Bc16g02000.1: unknown protein
Bc16g02480.1: conserved fungal protein
Bc16g02490.1: conserved fungal protein
Bc16g02770.1: deuterolysin metalloprotease
Bc16g03420.1: succinate/fumarate mitochondrial transporter
Bc16g03500.1: cystathionine beta-synthase
Bc16g03870.1: medium chain dehydrogenase/reductase
Bc16g04050.1: NADP-specific glutamate dehydrogenase
Bc16g05190.1: zinc-binding alcohol dehydrogenase
Supplementary Table 8

Genes downregulated in the ΔMAT1-1-5 stipes

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<td>0.9</td>
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Supplementary Table 8

Genes upregulated in the ΔMAT1-1-5 stipes

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<th>Gene ID</th>
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<th>Ratio wt/mutant</th>
<th>Description</th>
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<td>57</td>
<td>82</td>
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<td>76</td>
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<td>31</td>
<td>NADPH dehydrogenase</td>
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<td>379</td>
<td>20</td>
<td>2,3-diketo-5-methylthio-1-phosphopentane phosphatase</td>
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Chapter 3

Functional analysis of hydrophobin genes in sexual development of *Botrytis cinerea*

This chapter is published as:

Abstract

Hydrophobins are small secreted fungal proteins that play roles in growth and development of filamentous fungi, i.e. in the formation of aerial structures and the attachment of hyphae to hydrophobic surfaces. In *Botrytis cinerea*, three hydrophobin genes have been identified. Studies by Mosbach et al. (2011) showed that hydrophobins are neither involved in conferring surface hydrophobicity to conidia and aerial hyphae of *B. cinerea*, nor are they required for virulence. The present study investigated the role of hydrophobins in sclerotium and apothecium development. Expression analysis revealed high expression of the *Bhp1* gene during different stages of apothecium development. Two *Bhp1* splice variants were detected that differ by an internal stretch of 13 amino acid residues. Seven different mutants in which either a single, two or three hydrophobin genes were knocked out, as well as two wild type strains of opposite mating types, were characterized for sclerotium and apothecium development. No aberrant morphology was observed in sclerotium development when single deletion mutants in hydrophobin genes were analyzed. Sclerotia of double knock out mutant \(\Delta Bhp1/\Delta Bhp3\) and the triple knock out mutant, however, showed easily wettable phenotypes. For analyzing apothecium development, a reciprocal crossing scheme was setup. Morphological aberrations were observed in crosses with two hydrophobin mutants. When the double knock out mutant \(\Delta Bhp1/\Delta Bhp2\) and the triple knock out mutant were used as the maternal parent (sclerotia), and fertilized with wild type microconidia, the resulting apothecia were swollen, dark brown in color and had a blotched surface. After initially growing upwards toward the light source, the apothecia in many cases collapsed due to loss of structural integrity. Aberrant apothecium development was not observed in the reciprocal cross, when these same mutants were used as the paternal parent (microconidia). These results indicate that the presence of hydrophobins in maternal tissue is important for normal development of apothecia of *B. cinerea*. 
Introduction

*Botrytis cinerea* is a haploid necrotrophic pathogen of over 200 plant species including agronomical and ornamental crops of economic importance. *B. cinerea* produces different structures in order to complete its life cycle including macroconidia, microconidia, sclerotia and fruiting bodies called apothecia. Macroconidia and sclerotia are asexual stages while apothecia are the sexual stage. The sclerotium serves as a multicellular resting structure and a source of nutrients during apothecium development (Li and Rollins, 2009). As a heterothallic fungus, *B. cinerea* needs two isolates of opposite mating types to initiate apothecium development. Apothecia of *B. cinerea* have the shape of a cup, are light brown in color, normally 10 mm in height (Whetzel, 1945), and they contain asci with eight ascospores resulting from meiosis. Individual apothecia can produce millions of ascospores that are discharged to the environment to serve as a source of inoculum for disease development, especially in early spring. The macroscopic morphology of apothecia of *B. cinerea* closely resembles that of *Sclerotinia sclerotiorum* and *Sclerotinia minor*. In *S. minor*, the apothecium development was divided into 4 stages (Bullock et al., 1983), whereas in *S. sclerotiorum* 7 stages are distinguished (http://www.sclerotia.org/lifecycle/apothecia).

Hydrophobins are small, surface-active proteins of about 10 kDa with eight cysteine residues in conserved positions. Hydrophobins were originally described by Wessels et al., (1991a) and Wessels et al., (1991b) and have been studied for more than two decades (Zampieri et al., 2010). These proteins can only be found in filamentous Ascomycota and Basidiomycota fungi and they are important in the formation of an amphipathic membrane at a hydrophobic–hydrophilic interface (Sunde et al., 2008; Wösten and de Vocht, 2000). In *Ophiostoma ulmi*, hydrophobins show high expression levels, of about 25% of the total mRNA or 10% of total protein content (Zhang et al., 1994). Hydrophobins can form solid hydrophobic aggregates on the surface by self-assembling activity and thereby coat the surface of conidia, mycelia or fruiting bodies of many fungi (Linder, 2009; Macindoe et al., 2012; Wessels, 1996; Wösten, 2001; Wösten et al., 1993).
Hydrophobins have various functions in supporting fungal growth and development. For example, in *Agaricus bisporus* and *Schizophyllum commune*, secreted hydrophobins help reduce the surface tension and enable the fruiting body to penetrate the water or soil surface (van Wetter et al., 2000; Wessels et al., 1991a; Wösten, 2001; Wösten et al., 1994b). Besides that, hydrophobins were shown to be involved in conidiogenesis or in the triggering of appressorium formation for penetration of host surfaces by *Magnaporthe grisea* (Talbot et al., 1996). They are also believed to be involved in the establishment of lichen symbiosis (Dyer, 2002) and the interaction between the ectomycorrhizal fungus *Tricholoma terreum* and its host trees (Mankel et al., 2002). In addition, hydrophobins are involved in microsclerotia development in *Verticillium dahliae* (Klimes and Dobinson, 2006).

Hydrophobins are divided into two classes based on hydrophobicity, amino acid arrangement and their ability to form layers (Wessels, 1994; Wösten et al., 1994a; Wösten et al., 1994b). Class I hydrophobins are commonly found in Ascomycota and Basidiomycota species, while class II hydrophobins are restricted to Ascomycota. Class I hydrophobins are generally longer than class II hydrophobins, with size ranges of 100–125 residues (class I) versus 50–100 residues (class II), respectively. The class I hydrophobins usually are able to form a stable structure of fibrillar ‘basket-weave’ polymer film, called rodlet layer (Beever et al., 1979; Del Sol et al., 2007), 10–25 nm in width and 35–240 nm in length (Dempsey and Beever, 1979; Gardner et al., 1983; Wu-Yuan and Hashimoto, 1977). Rodlets can be observed on the surface of conidia of *Aspergillus nidulans* (Doss et al., 1997) *Neurospora crassa* (Beever and Dempsey, 1978; Beever et al., 1979; Dempsey and Beever, 1979; Doss et al., 1997) and the fruiting body of *S. commune* (van Wetter et al., 2000). Class I hydrophobins are insoluble in water and can only be dissolved with trifluoroacetic acid (TFA) or formic acid (FA) (Bayry et al., 2012; Hektor and Scholtmeijer, 2005; Linder, 2009; Wösten, 2001; Wösten et al., 1993). Class II hydrophobins do not form rodlet layers but rather form smooth surfaces, and they are soluble in hot sodium dodecyl sulfate (SDS) or 60% ethanol (Hektor and Scholtmeijer, 2005; Linder, 2009; Paananen et al., 2003; Torkkeli et al., 2002; Wösten, 2001). Studies on the surface of *B. cinerea* conidia and hyphae have failed to provide evidence for the production of rodlet layers (Doss et al., 1997; Mosbach et al., 2011).
Various numbers of hydrophobin genes are present in genomes of different fungi, ranging between two and seven genes per species, with *Coprinopsis cinerea* as an exception, containing 33 hydrophobin genes (Bayry et al., 2012; Stajich et al., 2010). In *B. cinerea*, three bona fide hydrophobin genes have been identified which were designated *Botrytis-hydrophobin-1* (*Bhp1*; class I), *Botrytis-hydrophobin-2* (*Bhp2*; class II) and *Botrytis-hydrophobin-3* (*Bhp3*; class II) as well as several genes encoding hydrophobin-like proteins such as *Bhl1* (Mosbach et al., 2011). Previous studies revealed that hydrophobin genes in *B. cinerea* are not required for asexual development or for virulence (Mosbach et al., 2011). The aim of the present study was to investigate the roles of class I and class II hydrophobins in the development of sclerotia and apothecia of *B. cinerea*.

**Materials and methods**

**Strains used**

Seven hydrophobin mutants with a *MAT1-1* background (Mosbach et al., 2011), and the *MAT1-2* reference strain SAS405 (Faretra et al., 1988) were used (Table 1).

**Sclerotium development and sexual crosess**

The wild type and hydrophobin mutant strains were grown on malt extract agar (MEA, Oxoid, Basingstoke, UK) and incubated at 15 °C in darkness to promote sclerotium development (Faretra et al., 1988). After 4 weeks incubation, observations were made of the number of sclerotia per plate, as well as their size, shape and morphology. A protocol described by Faretra et al. (1988) and van der Vlugt-Bergmans et al. (1993) was used to generate apothecia. Crosses were setup using hydrophobin mutants (all carrying the *MAT1-1* locus) and wild type strain SAS405 (carrying the *MAT1-2* locus). All crosses were setup reciprocally, using the mutants either as the maternal parent (sclerotia) or paternal parent (microconidia). As a control, apothecia were generated from a cross between the wild type recipient strain (*MAT1-1*) and SAS405 (*MAT1-2*). Table 2 shows the full scheme of crosses that were performed.
Table 1: Hydrophobin mutants and wild type strains used in this study

<table>
<thead>
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<th>Type</th>
<th>Strain</th>
<th>Hydrophobin class</th>
<th>Mating type</th>
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<tr>
<td>Mutants</td>
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<td></td>
</tr>
<tr>
<td>Single knock out</td>
<td>ΔBhp1</td>
<td>I</td>
<td>MAT1-1</td>
</tr>
<tr>
<td></td>
<td>ΔBhp2</td>
<td>II</td>
<td>MAT1-1</td>
</tr>
<tr>
<td></td>
<td>ΔBhp3</td>
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<td>I and II</td>
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<tr>
<td></td>
<td>ΔBhp1/ΔBhp3</td>
<td>I and II</td>
<td>MAT1-1</td>
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<td>Triple knock out</td>
<td>ΔBhp1/ΔBhp2/ΔBhp3</td>
<td>I and II</td>
<td>MAT1-1</td>
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<td>Wild type</td>
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<td></td>
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Table 2: Sexual crosses performed in this study

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<th>Microconidia</th>
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<th># Experiments&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>4</td>
</tr>
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<td>SAS405</td>
<td>x</td>
<td>3</td>
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<td>3</td>
<td>4</td>
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<td></td>
<td>3</td>
<td>4</td>
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<td>4</td>
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<td>4</td>
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<td>Wild type</td>
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<td>x</td>
<td>3</td>
<td>4</td>
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<td>double mutant</td>
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<td>4</td>
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<td>ΔBhp1/ΔBhp2</td>
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<td>Wild type</td>
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<td>4</td>
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<tr>
<td>ΔBhp1/ΔBhp3</td>
<td>SAS405</td>
<td>x</td>
<td>3</td>
<td>4</td>
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<tr>
<td>ΔBhp1/ΔBhp2/ΔBhp3</td>
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</tr>
<tr>
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<td>Water</td>
<td>control</td>
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<sup>a</sup> number of biological replicates within an experiment  
<sup>b</sup> number of experiments
Apothecium development was monitored 2–3 times per week for up to 90 days after fertilization. *B. cinerea* apothecium development was divided into 6 stages (Figure 1) defined as follows; stage 1: initials 1-2 mm in height; stage 2: stipes 2–4 mm in height; stage 3: stipes >5 mm in height (stipes elongated but not swollen at the tip); stage 4: stipes fully extended to ~1 cm, swollen at the tip and showing initiation of apothecial disk development; stage 5: immature disk with diameter <5 mm, partially expanded; stage 6: mature disks fully expanded to ~1 cm, filled with asci containing 8 ascospores. A preparation of ascospores was obtained from mature apothecia by gently crushing an apothecial disk in water, and filtering the resulting suspension over a plug of glasswool. The flow-through mainly contained a suspension of single ascospores released from asci, with minor contamination of intact asci or fragments of the hymenium.

![Figure 1: Tissues and stages of apothecium development used in this study. Scale bars indicate the sizes in mm or μm](image)

**RNA extraction, quantitative-real time-PCR and reverse transcriptase-PCR**

Nine tissue types were sampled for RNA extraction: 1-week-old mycelia, 2-week-old mycelia, 3-week-old sclerotia, 4-week-old sclerotia grown on MEA at 15°C in darkness; 8-week-old sclerotia grown for 4 weeks at 15°C and then incubated for 4 weeks at 0°C; apothecia harvested in three stages of development: combined stages 1–2, combined stages 3–4, and combined stages 5–6; ascospores collected from mature apothecia. The specimens were freeze-dried and total RNA was isolated using the Nucleospin RNA plant kit (Macherey Nagel, Düren, Germany), according to the manufacturer’s instructions. RNA was isolated from at least two biological replicates for each sample.
Five types of RNA samples were sequenced on an Illumina HiSeq 2000 platform by Beijing Genome Institute (Hongkong, China): 8-week-old sclerotia; apothecia combined stages 1–2; apothecia combined stages 3–4; apothecia combined stages 5–6; ascospores. Two biological replicates were sequenced of each sample, with the exception of the sclerotia. Reads were mapped on the *B. cinerea* B05.10 version 2 genome assembly (Staats and van Kan, 2012) using Tophat v2.0.8b (Kim et al., 2013). This constitutes the RNAseq data.

As template for Reverse Transcriptase PCR (RT-PCR), first strand cDNA was synthesized from 1 μg total RNA with M-MLV Reverse Transcriptase (Promega, Leiden, The Netherlands) according to the manufacturer’s instructions. RT-PCR was performed to assess the existence of splice variants of *Bhp1*. The primer pair Bhp1Fvar1 and Bhp1Rvar1 (Table 3) was used to amplify splice variant 1, while primer pair Bhp1Fvar1 and Bhp1Rvar2 (Table 3) was used to amplify splice variant 2. PCR conditions were as follows: 95 °C for 5 min, then 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, followed by a final extension of 72 °C for 5 min. PCR products were visualized following electrophoresis on a 2.5% agarose gel. All experiments were performed in two biological replicates.

Quantitative-Real Time-PCR (qRT-PCR) was performed using an ABI7300 PCR machine in combination with the qPCR SensiMix kit with primers listed in Table 3. All qRT-PCR reactions were performed in three technical replicates using samples from at least two biological replicates. In order to quantify relative DNA contents of *Bhp1*, *Bhp2*, *Bhp3* and *Hph* genes, primer pairs Bhp1For6683/NewBhp1Rev, Bhp2For4417/NewBhp2Rev, Bhp3For5460/Bhp3Rev5460 and NewHphFor/NewHphRev were used, respectively. The relative DNA content values were normalized to the *Bcpq1* gene (Zhang and Van Kan, 2013). To quantify the levels of *Bhp1*, *Bhp2* and *Bhp3* transcripts, the primer pairs Bhp1For6683/Bhp1Rev6683, Bhp2For4417/Bhp2Rev4417, and Bhp3For5460/Bhp3Rev5460 were used, respectively. Gene expression values were normalized to the expression of the constitutively expressed *BctubA* gene, Genbank accession Z69263.2 (Benito et al., 1998).

qRT-PCR conditions were as follows: an initial 95 °C denaturation step for 10 min followed by denaturation for 15 s at 95 °C and annealing/extension for 1 min at 60 °C for 40 cycles. The data were analyzed on the 7300 System SDS software and calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).
Table 3: List of primers used in this study.

<table>
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<th>Gene</th>
<th>Primer Name</th>
<th>Sequence</th>
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<td>Bhp1</td>
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<td>GGTCGTTGCTATTCCTAC</td>
<td>This study</td>
</tr>
<tr>
<td>Bhp1</td>
<td>Bhp1Rev6683</td>
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<td>This study</td>
</tr>
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<td>NewBhp1Rev</td>
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<td>Bhp1Fvar1</td>
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</tr>
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<td>Bhp1Rvar1</td>
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<td>Bhp1</td>
<td>Bhp1Rvar2</td>
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<td>This study</td>
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<td>NewBhp2Rev</td>
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<td>NewHphFor</td>
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<tr>
<td>BctubA</td>
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<td>GCGTTCGTGCATTGTATGT</td>
<td>Benito et al., (1998)</td>
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<td>Zhang and van Kan (2013)</td>
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<td>Bcpg1</td>
<td>Bcpg1 R</td>
<td>GAACGCAACACCGGTAGATG</td>
<td>Zhang and van Kan (2013)</td>
</tr>
</tbody>
</table>

**Light microscopy**

Transverse sections of apothecia were mounted on slides with water. Observations were made with a Nikon Eclipse 90i (Nikon Instruments, Badhoevedorp, The Netherlands) compound microscope with a Nikon DS-5MC camera attached. Measurements were performed using N.I.S. Elements AR 2.30 software (Nikon Instruments).

**Scanning electron microscopy**

Three to four sclerotia with sizes of 4–6 mm were collected randomly from MEA plates. Four-week-old sclerotia incubated in darkness at 15°C were used. Apothecia were harvested 60–90 days after fertilization. One to three apothecia per sclerotium were cut off using a scalpel and handled by forceps. The sclerotia and apothecia were fixed in 4% glutaraldehyde in 0.1 M potassium phosphate buffer pH 7.2. The fixation was done under vacuum for 10 min at room temperature, followed by overnight incubation at 4°C. Then, the samples were washed using 0.1 M potassium phosphate buffer pH 7.2 at 4°C (3 x 20 min) followed by post-fixation with 1% OsO4 in 0.1 M potassium phosphate buffer pH 7.2, 1 h at room temperature. The dehydration was done in ethanol (70%, 95% and 100%) for 30 min each
and finally transferred to 100% acetone for 30 min. The apothecia and sclerotia were subsequently critical point dried with carbon dioxide (CPD 030, BalTec, Liechtenstein). The samples were sputter-coated with 15 nm iridium (Leica EM SCD 500) and analyzed at 2 kV at room temperature in a field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, the Netherlands). Digital images were contrast-optimized with Photoshop CS 5.

Results

Gene expression
In a previous study by Mosbach et al. (2011), expression analyses revealed low or undetectable expression of hydrophobin genes $Bhp1$ (class I), $Bhp2$ (class II), $Bhp3$ (class II) in conidia or mycelia. Only $Bhp1$ showed increased expression in sclerotia or apothecia (Mosbach et al., 2011). We performed more detailed analyses to study the expression of hydrophobin genes in sclerotia and during different stages of apothecium development. RNA samples were isolated from sclerotia in different stages of development and from apothecia in stages 1–2, 3–4, and 5–6, as well as from a fraction enriched for ascospores (Figure 1). Transcript levels of $Bhp1$, $Bhp2$ and $Bhp3$ were analyzed by RNA sequencing (five samples, Table 4) and by qRT-PCR (all samples except for the ascospore fraction, Figure 2).

In RNA-seq data, the number of reads derived from $Bhp1$ was by far the highest of the three hydrophobin genes, especially in stages 3–4, while the number of reads derived from $Bhp2$ and $Bhp3$ was extremely low (Table 4). The RNA-seq read mapping revealed a minor splice variant of the $Bhp1$ mRNA (Supplementary Figure S1a), in which an alternative splice acceptor site of the second intron led to an internal insertion of 13 amino acid residues (Supplementary Figure S2). Based on the read coverage, the abundance of the minor splice variant is ~5% of the total. Reverse Transcriptase-PCR was used to confirm the occurrence of both splice variants in all stages of apothecium development (Supplementary Figure S1b).

Figure 2 shows expression profiles of hydrophobin genes as determined by qRT-PCR relative to $\beta$-tubulin. High levels of $Bhp1$ transcript were detected in 3-week-old sclerotia while transcript levels of $Bhp2$ and $Bhp3$ at this time were low. In 4-week-old sclerotia, transcript levels of $Bhp2$ and $Bhp3$ increased while $Bhp1$ decreased. In 8-week-old sclerotia (4 weeks at
15°C and then incubated for 4 weeks at 0°C, Bhp1, Bhp2, and Bhp3 transcripts were undetectable (data not shown). This indicated that the hydrophobin genes were expressed at low levels in early stages of sclerotium development.

During apothecium development, the transcript level of Bhp1 was high in all stages while the transcript levels of Bhp2 and Bhp3 were extremely low in all stages (Figure 2). Overall, transcription patterns of hydrophobin genes were similar for both methods, RNA sequencing and qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sclerotia</th>
<th>Apothecia stages 1-2</th>
<th>Apothecia stages 3-4</th>
<th>Apothecia stages 5-6</th>
<th>Ascospores</th>
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<td>70 (n=1)</td>
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<tr>
<td>Bhp2</td>
<td>13 (n=1)</td>
<td>16 ± 4 (n=2)∗</td>
<td>6 ± 2 (n=2)∗</td>
<td>7 ± 2 (n=2)∗</td>
<td>3 ± 1 (n=2)∗</td>
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<tr>
<td>Bhp3</td>
<td>0 (n=1)</td>
<td>1 ± 0 (n=2)∗</td>
<td>1 ± 1 (n=2)∗</td>
<td>26 ± 6 (n=2)∗</td>
<td>5 ± 4 (n=2)∗</td>
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</table>

*number of reads/sample 10978094 9485388∗ 9757285∗ 9988141∗ 10183343∗

∗, given number of reads is the average for two biological replicates

**Figure 2**: Transcript levels of Bhp1, Bhp2 and Bhp3 mRNA in mycelium and developing sclerotia (grown in MEA in darkness) and in different stages of apothecium development, measured by qRT-PCR. Transcript levels were normalized to the level of β-tubulin mRNA.
Chapter 3

Sclerotium development in hydrophobin mutants
To study the effect of the deletion of hydrophobin genes on sclerotium development, seven hydrophobin mutants and the wild type strain (Table 1) were used. The morphological appearance of sclerotia of the single ΔBhp1 (class I hydrophobin) knock out mutant and two single ΔBhp2 and ΔBhp3 (class II hydrophobins) knock out mutants was similar to the wild type strains. The sclerotia had a silvery, shiny appearance with water droplets remaining on the surface (Figure 3, panels a and d). SEM images revealed the presence of a rough fibrillar layer on the surface of the wild type sclerotia (Figure 3, panels g and j). In double knock out mutants ΔBhp1/ΔBhp2 and ΔBhp2/ΔBhp3, the morphological appearance of the sclerotia was similar to the wild type. By contrast, sclerotia of the double knock out mutant ΔBhp1/ΔBhp3 and the triple knock out mutant were black and showed an easily wettable phenotype, i.e. water droplets applied on the surface easily dissipated (Figure 3, panels b, c, e and f). SEM showed that these mutant sclerotia lack a rough fibrillar layer, but instead have a smooth surface (Figure 3, panels h, i, k and l).

Apothecium development
To study the effect of the deletion of hydrophobin genes on apothecium development, reciprocal crosses were setup between hydrophobin mutants (made in a strain with MAT1-1 identity) with wild type strain SAS405 (of MAT1-2 identity). Sexual crosses of the wild type recipient and SAS405 were used as control. Observations were made 2–3 times a week over a period of at least 12 weeks after fertilization, until apothecia in control crosses were mature. In the crosses using the wild type recipient and the hydrophobin mutants as maternal parent, the primordia ruptured from sclerotia at 21 to 33 days after fertilization. Most of the primordia developed from the center of the sclerotia, scattered on the surface and sometimes at the edge of sclerotia (Figure 4, panel a). Within the first day after emerging, the primordia size was approximately 0.2 mm and growth was up to 1 mm in two days. The primordia continued to grow erected toward the light source and formed stipes (Figure 4, panel c), generally with a cylindrical appearance, common for the genus Botrytis (Whetzel, 1945). The apothecial disk began to expand between 14 and 20 days after emergence of the stipe and formed a cup-shaped structure which became flat and mature 30–60 days post emergence (60–90 days after fertilization) (Figure 4, panel g).
Figure 3: Morphology and wettability of sclerotia. (a and d) Wild type sclerotia had a silvery, shiny appearance with water droplets remaining on the surface; (g) a fibrillar layer is present on the surface of wild type sclerotia; (j) in high magnification the layer appears to have a rough surface; (b, c, e and f) sclerotia of the ΔBhp1/ΔBhp3 double knock out mutant and the triple knock out mutant were black and showed an easily wettable phenotype; (h, i, k, l) in the ΔBhp1/ΔBhp3 double knock out mutant and the triple knock out mutant, sclerotia contain a smooth external layer instead of the rough fibrillar structure.

Figure 4: Aberrant apothecium development in crosses with hydrophobin mutants. Aberrant features are highlighted with a red arrow. (a) Primordia, (b) swollen primordia, (c) stipes, (d) swollen stipes, (e) apothecium developing from SAS405 as the maternal parent, normal morphology, (f) apothecium developing from triple knock out mutant as the maternal parent, collapsed during outgrowth, (g) mature wild type apothecia, (h) swollen cup of apothecia developing from triple knock out mutant, (i) flat apothecium surface, light brown in color, (j) blotched apothecium surface, dark brown in color.
### Table 5: Morphological appearance of apothecium structures in different crosses

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<th>Sclerotia</th>
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<th>Primordia and stipes</th>
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<th>Apothecia surface</th>
<th>Color</th>
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WT, normal morphological appearance; Ab, aberrant appearance.

Apothecia with normal morphological appearance were obtained in all crosses in which sclerotia of wild type strain SAS405 were fertilized with microconidia of hydrophobin mutants, and in most of the crosses in which a hydrophobin mutant was used as the maternal parent (Table 5). Aberrant development was observed in crosses using the double knock out mutant ΔBhp1/ΔBhp2 or the triple knock out mutant as the maternal parent (Figure 4). In these crosses, instead of developing cylindrical stipes, some of the primordia and stipes became swollen (Figure 4, panels b and d). Figure 4 shows two individual mature apothecia developed from the wild type (panel e) and the triple knock out mutant (panel f) as maternal parent. Both stipes had initially developed similarly, however, the apothecium derived from the triple knock out mutant as maternal parent collapsed during disk development in stage 4 (Figure, panel f). Moreover, swelling of the cup occurred before the expansion of the apothecial disk and clearly could be observed in mature apothecia in crosses in which the double knock out mutant ΔBhp1/ΔBhp2 or the triple knock out mutant served as the maternal parent. A typical example of a swollen cup is provided in Figure 4 (panel h). In addition, apothecia developing from double knock out mutant ΔBhp1/ΔBhp2 or the triple knock out mutant as the maternal parent had a blotched surface with dark brown color (Figure 4, panel j), whereas apothecia derived from the reciprocal cross had a flat, light brown surface (Figure 4, panel i).
SEM images revealed structural and morphological differences between apothecia derived from the triple knock out mutant (used as maternal parent), and those derived from two wild type parents, or apothecia derived from the triple knock out mutant used as paternal parent. Apothecia derived from the triple knock out mutant (used as maternal parent) appear to lack a smooth ectal excipulum (Figure 5, panel b). Instead the surface was covered with filamentous hyphae that do not form a continuous layer (Figure 4, panel d). In wild type apothecia (Figure 5, panel a), the cup is covered by a smooth layer and individual hyphae are not visible (Figure 5, panel c).

**Figure 5:** Scanning electron microscopy of cup stage. (a) Lower side of the cup of a wild type apothecium, (b) lower side of the cup of an apothecium developing on sclerotia of the triple knockout mutant, (c) close up of panel a, the tissue is covered with a smooth ectal excipulum, (d) close up of panel b, ectal excipulum is absent, filamentous hyphae do not appear to form a continuous layer.
**Asci and ascospores**

Apothecia from all crosses were dissected and transverse sections were prepared and mounted on slides. No defects or aberrant phenotypes were observed in asci and ascospores of the apothecia produced using the hydrophobin mutants or wild type strain as maternal parent (not shown). The asci were on average 309 ± 36 μm ($n = 50$) in length and 16 ± 2 μm ($n = 50$) in width, while the ascospores were 11.3 ± 1.4 μm ($n = 50$) in length and 4.9 ± 0.1 μm ($n = 50$) in width. Asci are cylindrical and hyaline in color with an apical tip. Ascospores are oblong, hyaline, and there were always eight ascospores present in each ascus. No differences were observed in asci or ascospores from any crosses tested.

**Gene dosage effects in crosses with knockout mutants**

The aberrant apothecium development of mutants lacking both the $Bhp1$ and $Bhp2$ gene appears to be maternally controlled, and occurs even though the paternal mating partner is a wild type strain (SAS405), carrying all three functional hydrophobin genes. It should be considered that a developing apothecium is predominantly composed of maternal tissue, and contains only a limited proportion of dikaryotic cells, in which the paternal nucleus is present (Zickler, 2006). Thus the dosage (and concomitantly the transcript levels) of paternal genes is expected to be substantially lower than that of maternal genes. To examine gene dosage effects, qRT-PCR was performed to compare the DNA and mRNA levels of hydrophobin genes between reciprocal crosses of the double mutants ($\Delta Bhp1/\Delta Bhp2$ or $\Delta Bhp1/\Delta Bhp3$) or the triple knock out mutant with SAS405, using the mutant either as maternal parent (sclerotia) or paternal parent (microconidia). In apothecia from crosses with the triple knock out mutant as maternal parent, the amount of DNA of the three $Bhp$ genes was 100-fold lower (Figure 6a) than in the reciprocal cross in which the same mutant served as paternal parent. In the same stage, the amount of $Bhp1$ mRNA was 60–250 fold lower than in reciprocal crosses in which the mutant served as paternal parent (Figure 6b). For $Bhp2$ and $Bhp3$, transcript levels were too low to calculate a ratio between samples.
Functional analysis of hydrophobin genes in sexual development of *Botrytis cinerea*

Figure 6: Panel a: Relative DNA content of *Bhp* genes and the hygromycin phosphotransferase (*Hph*) gene in apothecia derived from reciprocal crosses between wild type SAS405 and the triple knockout mutant. DNA content was normalized to the *B. cinerea* *Bcpg1* gene. Panel b: Relative expression of *Bhp1* in apothecia derived from reciprocal crosses between wild type SAS405 and double and triple knockout mutants. Transcript levels were normalized to the *B. cinerea* β-tubulin gene *BctubA*.
Hydrophobins are required for normal sclerotium and apothecium development in *B. cinerea*. Based on expression, one would predict that *Bhp1* is the major component required for both structures because *Bhp2* and *Bhp3* are expressed only to low levels in these tissues. However, the single *Bhp1* knock out mutant is fully functional both in sclerotium and apothecium development. A combination of the class I hydrophobin *Bhp1* with one of the two class II hydrophobins is essential for proper development. Specifically, deletion of both *Bhp1* (class I) and *Bhp3* (class II) reduced surface hydrophobicity of sclerotia, and deletion of both *Bhp1* and *Bhp2* affected apothecium development. Aberrant apothecium development was observed only when the double knock out mutant Δ*Bhp1/ΔBhp2* (or the triple knock out mutant) served as the maternal parent but not when wild type strain SAS405 served as the maternal parent.

The maternal control of aberrant apothecium development can be explained by a gene dosage effect: based on DNA measurements, the proportion of dikaryotic cells is only 1% of the total. At this level, nuclei from the paternal parent do not contribute sufficient hydrophobin transcript to produce an adequate amount of protein. Furthermore, the dikaryotic cells containing paternal nuclei are located in the inner part of the developing stipe whereas hydrophobins must function on the surface of the emerging structure. The ectal excipulum of emerging stipes is purely of maternal origin and thus devoid of hydrophobins in the mutants.

The absence of hydrophobins caused the surface of apothecia to be discontinuous, with hyphae exposed to the outer environment without any visible external matrix. The smooth layer covering the apothecial surface in wild type apothecia therefore is proposed to be composed, at least in part, of hydrophobins possibly interacting with other surface proteins or polysaccharides. This is in agreement with the high expression level of hydrophobin *Bhp1* during apothecium development and suggests their function in the formation of a protective coating for fungal structures that are exposed to air.

RNA-seq analysis revealed the occurrence of alternative splicing in *Bhp1* transcripts, which results in an internal insertion of 13 amino acid residues that contains one additional
cysteine residue. Upon aligning the amino acid sequences of the resulting two Bhp1 protein variants with their orthologs in *S. sclerotiorum* and *Sclerotinia borealis* (Supplementary Figure S2), it was striking that the *S. sclerotiorum* ortholog lacks the 13 amino acids stretch insert, whereas *S. borealis* appears to contain the insert. Alignment of the nucleotide sequences of the *B. cinerea*, *S. sclerotiorum* and *S. borealis Bhp1* genes demonstrated that both alternative splice acceptor sites are present in all three genomes (not shown). It is tempting to propose that the alternative splicing event also occurs in *S. sclerotiorum* and *S. borealis* and possibly in other *Sclerotiniaceae*.

The implication of an insert with an additional cysteine residue for the folding of the hydrophobin is unclear. According to structural models of de Vocht et al. (2000), the eight cysteine residues are involved in disulfide bridge formation, with four couples of cysteine residues (always two consecutive residues in the primary sequence) pairing. The short *Bhp1* variant has eight cysteine residues, while the long variant has nine. This raises the question whether in the long variant protein, Cys7 will form a disulfide bond with either Cys8 or Cys9, leaving a free cysteine available for interaction with other proteins. The functional role of producing splice variants of the *Bhp1* protein of *B. cinerea* remains unknown. Alternative splicing appears widespread in fungi, occurring in ~1.5% of genes in *Fusarium graminearum* (Zhao et al., 2013), *Aspergillus flavus* and *M. grisea* (McGuire et al., 2008). Also in *B. cinerea*, alternative splicing occurs on a similar scale (van Kan, unpublished data).

Secreted class I hydrophobins can self-assemble into amphipathic monolayers on fungal surfaces and form a rodlet layer (Wösten, 2001). Previous studies by Doss et al. (1997) and Mosbach et al. (2011) have not provided any evidence for the existence of a rodlet layer on the conidia or hyphae of *B. cinerea*. In this study we investigated the formation of a rodlet layer on the surface of sclerotia and apothecia. Rough fibrillar structures were found to cover the surface of wild type sclerotia, whereas a smooth layer was found to cover the surface of wild type apothecia. The surface layers in *B. cinerea* are distinct from the typical basket-weave rodlet layers produced by fungi such as *A. nidulans* and *N. crassa* (Doss et al., 1997). We conclude that sclerotia and apothecia of *B. cinerea* do not produce a rodlet layer.
This study in *B. cinerea* provides the first example that both class I and class II hydrophobins can be involved in proper fruiting body development. This situation differs from other ascomycota fungi. In *Cryphonectria parasitica*, only class II hydrophobin genes are required for pycnidium and perithecium development (Kazmierczak et al., 2005). It is well established for many Basidiomycota that hydrophobins are required for fruiting bodies development, but Basidiomycota only contain class I hydrophobin genes. It is difficult to envisage how a class I and a class II hydrophobin protein can reciprocally compensate for loss of function. The two classes of hydrophobins differ in their physico-chemical properties, such as solubility. While aggregated class I hydrophobins are insoluble in aqueous solutions and can only be released from aggregates by treatment with pure formic acid or TFA, class II hydrophobins can be readily solubilized in hot SDS or 60% ethanol. It seems plausible that the properties of the *B. cinerea* Bhp1 and Bhp2 proteins are distinct, yet the mutant analysis suggest that the proteins can functionally complement one another. This is especially remarkable because the genes are expressed in such unequal ratio. The levels of Bhp mRNA are approximately 1000-fold lower than those of Bhp1 in all stages of apothecium development (Figure 2), yet the single Bhp1 knockout mutant forms apothecia of normal appearance, for reasons that remain unexplained.

**Supporting information**
Supplementary Figure S1a, Supplementary Figure S1b, Supplementary Figure S2.

**Acknowledgments**
The authors are very grateful to Prof. Matthias Hahn (University of Kaiserslautern, Germany) for providing the mutant strains used in this study, as well as for giving feedback on a draft version of the manuscript, and to Tiny Franssen-Verheijen (Microscopy Centre, Wageningen University) for help in preparing the SEM images.
Functional analysis of hydrophobin genes in sexual development of *Botrytis cinerea*

References


Chapter 3


Supporting information

Supplementary Figure S1a
Differential splicing of the Bhp1 gene. Both splice variant gene models are displayed at the top; below is a plot of the cumulative read coverage across all RNAseq datasets (dark blue); the bottom panel shows reads mapped on the genome sequence (in turquoise) with experimentally validated introns marked by a red box.

Supplementary Figure S1b
Reverse Transciptase PCR validation of splicing variants of Bhp1 transcripts in sclerotia and different stages of apothecium development.

Supplementary Figure S2
Alignment of both Botrytis cinerea Bhp1 protein variants with orthologs in Sclerotinia sclerotiorum (gene ID SS1G_11895, Genbank accession EDN97370) and S. borealis (Genbank accession ESZ95445). The signal peptide is marked with a box. Cysteine residues are highlighted in bold case. Disulfide bridges based on the model of de Vocht et al. (2000) are indicated; alternative options for the 4th disulfide bridge in the longest splice variant are indicated by dotted lines.
Chapter 4

Mating type and sexual fruiting body of *Botrytis elliptica*, the causal agent of fire blight in lily

This chapter is published as:

Abstract

*Botrytis elliptica* is a necrotrophic pathogen that specifically infects *Lilium* species. Previous records show that *B. elliptica* collected in the field can successfully develop apothecia in *vitro*, however, there are no formal descriptions of apothecia of *B. elliptica*. The aim of this study was to analyse the sequence of the mating type loci of *B. elliptica* and produce apothecia in the laboratory in order to describe their morphology. The sequences of both MAT alleles (*MAT1*-1 or *MAT1*-2) of *B. elliptica* were determined and compared to the sister taxa, *Botrytis cinerea* and *Sclerotinia sclerotiorum*. Two strains of each mating type were used in crosses under controlled conditions to produce apothecia. Primordium rupture from sclerotial tissue occurred 74 days after fertilization and a mature apothecium formed within 1 month after rupture. The apothecia are 7 to 12 mm in height with a disk of 3 to 4 mm in diameter and 0.5 to 1 mm in thickness. The apothecial disk is usually umbilicate, depressed to funnel and rounded in shape. The number of apothecia growing on a sclerotium was one to nine. Asci are long, cylindrical with a size of 208 × 14 μm, thin walled and bearing eight ascospores. Ascospores are hyaline in colour, ellipsoidal with rounded ends, usually 18 to 24 μm in length and 6 to 10 μm in width (mean 19.5 × 8 μm). Ascospores were infectious on lily leaves.
Introduction

There are various modern lily cultivars on the market which originate from the genus *Lilium* of the family Liliaceae. The genus *Lilium* comprises about 100 species and was classified into seven sections, of which three comprise important hybrid cultivars for breeding: Asiatic, Oriental and Longiflorum (MacRae 1998; Shahin et al. 2012). Lilies are the second largest flower bulb crop in the Netherlands, and the largest flower bulb product in the world (Kamenetsky and Okubo 2012). Fire blight, also known as leaf blight is a major threat to lily production worldwide. The disease poses a major economic threat and has been recorded in Argentina, China, Korea, Italy, Japan, Netherlands, Taiwan, United Kingdom, and the United States (Bastiaansen et al. 1997; Chiou and Wu 2001; Doss et al. 1988; Furukawa et al. 2005; Hwang 2001; Mansfield and Hutson 1980; Migheli et al. 1990). During the growing season, fire blight may affect numerous lily species such as *Lilium candidum*, *Lilium longiflorum*, *Lilium auratum*, and *Lilium speciosum* (Bastiaansen et al. 1997; Doss et al. 1988; Fang Hsieh et al. 2001; Furukawa et al. 2005). The disease is caused by *Botrytis elliptica* (Berk) Cooke, a necrotrophic pathogen that only infects lilies. Host specificity of *B. elliptica* was proposed to be caused by the production of an as yet unidentified, secreted necrosis-inducing protein that causes programmed cell death (van Baarlen et al. 2004) and therefore presumably serves as a host-specific necrotrophic effector (Vleeshouwers and Oliver 2014). Symptoms such as necrotic spots on leaves and petals are the early signs of infection. The necrotic lesions develop and expand quickly under favourable environmental conditions (Chiou and Wu 2001; Hwang 2001; van Baarlen et al. 2004). Control measures using fungicides for *B. elliptica* are common practice worldwide, however, it has been reported that *B. elliptica* acquires resistance to several fungicides including benzimidazoles, dicarboximides, diethofencarb, and two sterol biosynthesis inhibitors (Chastagner and Riley 1990; Migheli et al. 1990).

Fruiting bodies of species in the genus *Botrytis*, called apothecia, are rarely found in the field. Apothecia are ascomas with an open cup shape, on top of a stipe, and brown in colour (Whetzel 1945). Apothecia develop from sclerotia and act as a platform to discharge ascospores from the asci (Pöggeler et al. 2006). Apothecia of the genus *Botrytis* vary in size depending on the species, ranging from 2 to 25 mm in height and 1 to 6 mm in diameter of the apothecial disk. Several studies have shown that sclerotia must be fertilized with
microconidia in order to develop apothecia (Beever and Parkes 1993; Faretra and Antonacci 1987; Fukumori et al. 2004; Groves and Loveland 1953). Mature *Botrytis* apothecia can normally be observed 2 months after fertilization (Faretra and Antonacci 1987; Hennebert 1973; Terhem and van Kan 2014).

Most members of the genus *Botrytis* are typically designated as having heterothallic mating systems. However, some species in the genus are reported to have homothallic reproductive mating systems, i.e. *Botrytis porri* and *Botrytis globosa* (Buchwald 1953; Elliott 1964). *B. elliptica* is believed to have a heterothallic mating system, requiring a cross between different alleles to undergo sexual development and form apothecia (van den Ende and Pennock-Vos 1996). The mating type locus in the genus *Botrytis* is designated as *MAT*, with two alleles defined as *MAT1*-1 and *MAT1*-2. As is common in Ascomycota, the *MAT* alleles lack sequence similarity but are flanked by identical genes, i.e., *APN2* and *SLA2* (Pöggeler et al. 2006). The presence of both *MAT* alleles will activate sexual development. Two decades ago, efforts were made to generate apothecia from *B. elliptica*, but complete apothecia failed to form. The apothecia failed to expand the apothecial disk which resulted in empty asci and ascospores (Chastagner et al. 1992). Some years later, van den Ende and Pennock-Vos (1996) managed to collect apothecia in a lily production field in Lisse, the Netherlands and successfully performed crosses with *B. elliptica* strains, but a formal description of the apothecia of *B. elliptica* was not provided. Staats et al. (2007) could not detect apothecia of *B. elliptica* during a field survey; however, they performed AFLP analysis to study the genetic diversity in the population. Over 60 % of haplotypes of *B. elliptica* were unique to a single isolate and only 2 % of all pairwise haplotype comparisons differed by less than four markers. Furthermore, haplotypes that were prominent in one growing season were not found again in the next year. These data collectively provided strong evidence for the occurrence of sexual reproduction in the field (Staats et al. 2007). Also field surveys in North America and Taiwan failed to reveal the presence of apothecia of *B. elliptica* in the field (Huang et al. 2001). Currently, there is no documentation or description of apothecia of *B. elliptica* available to the public. The aim of this study was to analyse the sequence of the *MAT* loci of *B. elliptica* and to produce apothecia in the laboratory in order to describe their morphology.
Materials and methods

Strains

Six *B. elliptica* strains (Be9601, Be9605, Be9610, Be9612, Be9623, Be0004) collected in a lily production field in Lisse, the Netherlands in the years 1996 and 2000, were provided by dr. J. van Doorn (PPO Lisse). Strains were plated on malt extract agar (MEA) and incubated at 20 °C with continuous exposure to near-UV light and white light until cultures sporulated.

*B. elliptica* genome sequence

Genomic DNA was isolated from strain Be9601 using a Gentra Puregene DNA purification kit (Qiagen, Venlo, the Netherlands), and used for whole genome sequencing by DNAvision in Liège, Belgium. The library containing inserts with an average length of 157 bp was sequenced on an Illumina HiSeq2000 platform by paired-end sequencing with a read length of 100 bp. A total of 33.2 million reads (yielding a dataset of >5 Gb) were assembled using Velvet 1.2.05 (Zerbino and Birney 2008) with a k-mer value of 43. The Be9601 assembly consisted of 7,860 contigs (N50 = 32,840) and had a total length of 47.6 Mbp.

Identification of mating type alleles

Freeze-dried mycelium was used for genomic DNA extraction using a Gentra Puregene DNA purification kit (Qiagen). The entire MAT locus could be amplified from strains with either of the MAT alleles, using primers annealing in the regions flanking the locus: BOTMAT5 (GTGACTGAAAAACGACGCGCATTCGATTCACATCCATCC) and BOTMAT3 (GTGACCAGAAACAGCTATGACCACATACTCGCATAGTGGAAC). PCR performed on 10–50 ng DNA with Expand polymerase (Roche) and primer pair BOTMAT5/BOTMAT3 in a 25 μl reaction volume yielded a fragment of ~4.3 kb. Amplification conditions were: 95 °C 5 min, then 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 5 min, followed with a final extension of 72 °C for 7 min. The amplicon from *B. elliptica* strain Be9623 was cloned in a TOPO-XL PCR plasmid (Life Technologies) and entirely sequenced by Baseclear (Leiden, The Netherlands) using a primer walking strategy. To identify the MAT alleles of strains or single ascospore progeny cultures by PCR, primer pairs were used that are specific for either of the alleles: MAT1-1 F (TGGTGTAAAAAGATTCCGTATCCG) and MAT1-1R (CACCATATGCATTCTGAGTGGAAG); or MAT1-2 F (ATCAGGGCACTGTCCTCGAAAC) and MAT1-
2R (GAAGAGGTCCAGACACAGATTG). PCRs were performed with GoTaq polymerase (Promega, Leiden, The Netherlands), according to the manufacturer’s instructions. Amplification conditions were: 95 °C 5 min, then 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 2 min, followed with a final extension of 72 °C for 5 min.

**Sexual crosses**

To obtain sclerotia, strains were plated on MEA and incubated in complete darkness at 15 °C for 4 weeks, followed by incubation at 0 °C for 4 weeks. Sexual crosses were set up in a reciprocal way using a standard protocol for crosses in *B. cinerea* (Faretra et al. 1988; van der Vlugt-Bergmans et al. 1993). In total, 24 sclerotia were used in one microtiter plate of six wells. Every well contained four sclerotia (maternal parent) that were fertilized with 3 ml of microconidia (paternal parent) of the mating partner. The microtiter plate was then sealed and incubated at 12 °C under neon light with a 12-h photoperiod. Non-spermatized sclerotia in sterile MilliQ water were used as a negative control. All crosses were set up with two strains of opposite mating types.

**Sampling of ascospores and production of single ascospore progeny**

To obtain ascospores, an apothecium was gently grinded with a mortar and pestle, followed by the addition of sterile MilliQ water, and mixed. The homogenate containing complete asci, ascospores and hymenium debris was filtered over glasswool fibre in a 5 ml pipette tip. The flowthrough contained predominantly ascospores, with little contamination by asci and hymenium tissue. Ascospores were counted using a haemocytometer. To obtain single ascospore progeny cultures, ascospores were plated on MEA at low density, up to 30 ascospores per plate, and incubated at 20 °C. After 2 days of incubation, germlings emerging from single ascospores were transferred to fresh MEA plates. At least 15 single ascospore cultures were collected from each individual apothecium. Genomic DNA was extracted from single ascospore cultures after 4 days of growth on MEA. PCR was performed as described above to identify their *MAT* allele. A chi-square test for the segregation of *MAT* alleles in the progeny was performed with a confidence level $P = 0.05$. 
Light microscopy

Transverse sections of apothecia were mounted on a slide with filter-sterile MilliQ water. Observations were made with a Nikon Eclipse 90i (Nikon Instruments, Badhoevedorp, the Netherlands) compound microscope attached with a Nikon DS-5MC camera. Measurements were performed using N.I.S-Elements AR 2.30 software (Nikon Instruments, Badhoevedorp, the Netherlands). At least 40 ascospores, asci, hymenia, paraphyses, ectal excipulum, and medullary excipulum were measured. The mean, standard deviation, and 95% confidence intervals were calculated.

Infection assay

Ascospores were collected from a mixture of 10 apothecia using the method described above. The ascospore suspension was adjusted to $1 \times 10^6$ ml$^{-1}$ in potato dextrose broth (12 g/l). 6–12 droplets of 3 μl of ascospore suspension were inoculated on the lower sides of lily leaves freshly cut from mature plants. Inoculated leaves were stored in a box with high relative humidity, > 95%, and placed at room temperature. The infection assay was performed with 3–5 leaves per treatment and was repeated three times.

Results

The mating type locus of Botrytis elliptica

As in most Ascomycota, the mating type (MAT) locus in Sclerotiniaceae is flanked by two highly conserved genes, APN2 (a homologue of the Saccharomyces cerevisiae APN2 gene encoding a DNA lyase) and SLA2 (a homologue of the S. cerevisiae SLA2 gene encoding a cytoskeleton assembly protein) (Amselem et al. 2011). The draft genome sequence of B. elliptica isolate Be9601 was determined (van Kan and Staats, unpublished). Nucleotide alignment was performed to identify the region that is homologous to the MAT locus of B. cinerea (Amselem et al. 2011). The MAT locus of Be9601 (Figure 1) appeared to contain the MAT1-1 allele, which includes the MAT1-1-1 gene (encoding the MAT-alpha domain) and the MAT1-1-5 gene, in the same configuration as in B. cinerea strain B05.10 (Amselem et al. 2011). Using primers BOTMAT5 and BOTMAT3 on DNA of B. elliptica Be9623, a fragment was amplified that contains the MAT1-2 allele, which includes the MAT1-2-1 gene (encoding
a high mobility group domain protein) and the MAT1-2-4 gene (Figure 1), in the same configuration as in B. cinerea strain T4 (Amselem et al. 2011). The four MAT genes in B. elliptica were >90% similar to their orthologs in B. cinerea and >75% similar to their orthologs in Sclerotinia sclerotiorum (Table 1; Supplemental Figure 1). Alike in the MAT loci in B. cinerea, there was a truncated fragment of the MAT1-2-1 gene in the MAT1-1 locus and a truncated fragment of the MAT1-1-1 gene in the MAT1-2 locus (Figure 1). Both gene fragments lack start codons as well as their typical DNA-binding domain and thus appear non-functional. The sequences of both MAT alleles were deposited in Genbank under accession numbers KM219113 (MAT1-1) and KM243314 (MAT1-2).

**Characterization of strains**

Four different B. elliptica strains were analyzed by PCR for their MAT allele. Two strains carried the MAT1-1 allele and another two strains carried the MAT1-2 allele. To analyse the production of sclerotia, these strains were plated on MEA plates and incubated in a 15°C incubator in darkness. All strains formed sclerotia, except for Be9610 (MAT1-1). Approximately 30 sclerotia were produced in one plate. Fully developed sclerotia were observed in the MEA plate after 30 days. Table 2 shows the characterization of strains.

**Sexual crosses**

To analyse the ability of B. elliptica to develop apothecia, the MAT1-1 strains were crossed with the MAT1-2 strains in all possible combinations. Because of the failure of Be9610 to produce sclerotia, this strain could only be used as paternal parent (microconidia). Of the nine crosses that were set up, four crosses were fertile and resulted in the formation of apothecia. The crosses that produced apothecia were all set up from MAT1-2 strains as the maternal parent (sclerotia). No apothecia formed from crosses that used MAT1-1 strain Be9612 as the maternal parent. As a control, sclerotia from the MAT1-1 strain or the MAT1-2 strain that were incubated in sterile MilliQ water did not produce apothecia. Table 3 displays the results of the sexual crosses. The experiment was performed three times with identical results.
Mating type and sexual fruiting body of *Botrytis elliptica*, the causal agent of fire blight in lily

**Figure 1**: Structure of the MAT locus in *B. elliptica*. Top panel: MAT1-1 allele of strain Be9601; bottom panel: MAT1-2 allele of strain Be9623. MAT alleles are non-homologous in DNA sequence, but are flanked by identical genes, APN2 and SLA2. Introns in the MAT genes are indicated by lines connecting the exons (indicated by boxes). The boxes marked with dotted circles represent truncated fragments of the MAT1-2-1 gene (in the MAT1-1 locus in the top panel) and the MAT1-1-1 gene (in the MAT1-2 locus in the bottom panel), respectively. The direction of transcription is indicated by arrows.

**Table 1**: Homology of *B. elliptica* MAT genes to orthologs in other Sclerotiniaceae

<table>
<thead>
<tr>
<th>B. elliptica gene</th>
<th>Protein length</th>
<th>Homology to <em>B. cinerea</em> ortholog</th>
<th>Homology to <em>S. sclerotiorum</em> ortholog</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAT1-1–1</td>
<td>353 aa</td>
<td>Identity 96, Similarity 97</td>
<td>Identity 81, Similarity 84</td>
</tr>
<tr>
<td>MAT1-1–5</td>
<td>376 aa</td>
<td>Identity 86, Similarity 92</td>
<td>Identity 75, Similarity 87</td>
</tr>
<tr>
<td>MAT1-2–1</td>
<td>376 aa</td>
<td>Identity 94, Similarity 96</td>
<td>Identity 78, Similarity 86</td>
</tr>
<tr>
<td>MAT1-2–4</td>
<td>279 aa</td>
<td>Identity 82, Similarity 90</td>
<td>Identity 63, Similarity 76</td>
</tr>
</tbody>
</table>

**Table 2**: Morphological and molecular characterization of *B. elliptica* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Microconidia</th>
<th>Macroconidia</th>
<th>Sclerotia</th>
<th>Mating type allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>Be9610</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>MAT1-1</td>
</tr>
<tr>
<td>Be9612</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>MAT1-1</td>
</tr>
<tr>
<td>Be9605</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>MAT1-2</td>
</tr>
<tr>
<td>Be0004</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>MAT1-2</td>
</tr>
</tbody>
</table>
Table 3: Results of sexual crosses

<table>
<thead>
<tr>
<th>Cross</th>
<th>Sclerotia</th>
<th>Microconidia</th>
<th>Replicates</th>
<th># Repeats</th>
<th>Apothecia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Be9612</td>
<td>Be0004</td>
<td>3</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>Be0004</td>
<td>Be9612</td>
<td>3</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Be9612</td>
<td>Be9605</td>
<td>3</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>Be9605</td>
<td>Be9612</td>
<td>3</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Be0004</td>
<td>Be9610</td>
<td>3</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Be9605</td>
<td>Be9610</td>
<td>3</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Be9612</td>
<td>Sterile MilliQ water</td>
<td>3</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>Be0004</td>
<td>Sterile MilliQ Water</td>
<td>3</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>Be9605</td>
<td>Sterile MilliQ Water</td>
<td>3</td>
<td>3</td>
<td>–</td>
</tr>
</tbody>
</table>

a number of biological replicates within an experiment
b number of repeated experiments

Apothecia were sampled from two different fertile crosses, and at least 15 single ascospore progeny were cultured from three independent apothecia of each. To identify the MAT alleles in the progeny, amplification of genomic DNA was performed by PCR with MAT-allele-specific primers. Table 4 shows that the MAT alleles segregated almost 1:1 in progeny. In one of the three experiments, the segregation was skewed, with the MAT1-1 allele being dominant for reasons that remain unexplained. A chi-square test indicated that, over the entire set of three biological repeats, the segregation ratio between the MAT alleles did not significantly deviate from 1:1 (P < 0.05, Table 4).

Table 4: Segregation of MAT alleles in the progeny

<table>
<thead>
<tr>
<th>Cross</th>
<th>Sclerotia</th>
<th>Microconidia</th>
<th>Apothecium (biological replicates)</th>
<th>Mating types</th>
<th>Total no. of ascospores</th>
<th>Chi square value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MAT1-1</td>
<td>MAT1-2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Be0004</td>
<td>Be9612</td>
<td>1</td>
<td>11</td>
<td>13</td>
<td>24</td>
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<td></td>
<td></td>
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<td>2</td>
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<td>3</td>
<td>18</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td>36</td>
<td>23</td>
<td>59</td>
</tr>
<tr>
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<td>Be9605</td>
<td>Be9612</td>
<td>1</td>
<td>11</td>
<td>10</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>7</td>
<td>9</td>
<td>16</td>
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<td></td>
<td>3</td>
<td>16</td>
<td>2</td>
<td>18</td>
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<td></td>
<td></td>
<td></td>
<td>Total</td>
<td>34</td>
<td>31</td>
<td>55</td>
</tr>
</tbody>
</table>

* no significant deviation from 1:1 segregation in the progeny (P < 0.05)
Morphological description of sclerotia and apothecia

Morphological structures of apothecia of *B. elliptica* were described based on the general descriptions by Whetzel (1945). Sclerotia are plano-convex, black, with irregular shape, and sometimes firmly attached to each other. Sclerotia range in size from 3 to 14 mm, and are frequently concave or hollow on the inward. Optimal conditions for development of sclerotia are in malt extract agar in darkness, in a temperature range between 15 °C and 20 °C. From 74 days after fertilization onwards, primordia ruptured from the surface of sclerotia and sometimes from the bottom (Figure 2a). The primordia develop into a stipe in 7 to 10 days after emergence. The stipe is cylindrical and erected (Figure 2b, c). The stipe surface is pubescent, scabrous, light brown in colour, 7 to 12 mm in height, and 1.5 to 2 mm in width. One to nine (mean = four) apothecia emerged on a sclerotium. The apothecial disk is umbilicate, depressed to funnel and round in shape, usually 3 to 4 mm in diameter and 0.5 to 1 mm thick. It is slightly shallow, cup-shaped and becomes flat when mature; light brown in colour, becoming dark brown at maturity (Figure 2d, e). Apothecia are mature 94 to 104 days after fertilization. At first the margin of the disk is entire, becoming serrate to irregular (not shown). Asci formed in the apothecial disk were long and cylindrical in shape with an average dimension of 200 × 14 μm (*n* = 40) (Figure 2f). An apical pore was not clearly observed in the ascus tip. The ascus has a thin wall and bears eight ascospores (Figure 2f). The ascospores are hyaline, ellipsoidal with rounded ends, usually 18.5 - 24 μm in length (mean 19.5 ± 1.3 μm, *n* = 40) and 6 - 10 μm in width (8.1 ± 0.8 μm, *n* = 40) (Figure 2g). The medullary excipulum ranges in shape between textura globosa and textura angularis, with a thickness 240 - 344 μm (*n* = 40) (Figure 2h). The underside of the apothecial disk is pubescent. Ectal excipulum is in the shape of textura globosa with a thickness of 59 to 87 μm (*n* = 40) (Figure 2i). The hymenium ranges from 87 to 106 μm in height (*n* = 40) and the subhymenium from 100 to 124 μm (*n* = 40). The size of paraphyses ranges from 197 μm to 291 μm (*n* = 40).

Infectivity of ascospores

Droplets of ascospore suspensions were inoculated on the lower side of lily leaf surfaces. As a control, suspensions of asexual conidia were inoculated at the same density. Symptom development became visible at 20–24 h post inoculation (hpi) and was indistinguishable between the ascospores and conidia (not shown). The infected area turned brown in colour.
and necrotic spots developed at the infection site. At 24 hpi, the lesion was approximately 1 mm in diameter, equivalent to the size of the inoculation droplet. Hyphae were growing on the infected area and could be examined under the light microscope. The lesions expanded to 2–4 mm in diameter at 36 hpi and lesions were circular to oval in shape (Figure 3). At 48 hpi, the first botryose clusters of spores could be observed in some infected areas. At 4 weeks post inoculation, sclerotia formed on lily leaves and had a typical appearance of *B. elliptica* sclerotia (not shown).

**Figure 2**: Morphology of sexual structures of *B. elliptica*. a,b primordia. c, stipe. d,e mature apothecia. f, ascospores in asci. g, ascospores. h, mendullary excipulum. i, ectal excipulum.

**Figure 3**: Disease symptoms of *B. elliptica* on lily leaves, 36 hpi. a. inoculation with ascospores, b. inoculation with conidia.
Mating type and sexual fruiting body of *Botrytis elliptica*, the causal agent of fire blight in lily

**Discussion**

In this study we confirmed that *B. elliptica* has a heterothallic mating system with two alleles: MAT1-1 and MAT1-2. Apothecia of *B. elliptica* can be produced in the laboratory after sexual crosses of two strains of opposite mating types, using a standard protocol developed for *B. cinerea* (Faretra et al. 1988; van der Vlugt-Bergmans et al. 1993) although *B. elliptica* apothecia took significantly longer to develop than those of *B. cinerea*.

The MAT1-1-1 and MAT1-2-1 genes in *B. elliptica* are orthologous to the alpha-domain protein and the HMG-domain protein, respectively, which are conserved in all Ascomycota studied thus far. These two proteins jointly act as master regulators of the initiation of sexual fruiting body development, when expressed together in a dikaryotic cell (Pöggeler et al. 2006). The MAT1-1-5 and MAT1-2-4 genes in *B. elliptica* are orthologous to the MAT1-1-5 and MAT1-2-4 genes in *B. cinerea* and have no homologues outside the family Sclerotiniaceae (Amselem et al. 2011). Deletion of either the *B. cinerea* MAT1-1-5 gene or the MAT1-2-4 gene results in a defect in sexual reproduction, specifically in the developmental transition from stipe to apothecial disk (Chapter 2). Whether the *B. elliptica* MAT1-1-5 and MAT1-2-4 genes have a similar function in apothecial disk development remains to be established.

Alike in *B. cinerea*, the MAT alleles of *B. elliptica* both contain a truncated MAT gene fragment derived from the opposite allele (i.e., a non-functional fragment of the MAT1-2-1 gene in the MAT1-1 locus and a non-functional fragment of the MAT1-1-1 gene in the MAT1-2 locus). The lengths and positions of the non-functional MAT gene fragments are nearly identical between *B. cinerea* and *B. elliptica* (van Kan, unpublished observations). This configuration suggests that contemporary MAT loci of *B. cinerea* and *B. elliptica* have both evolved from an ancestral, homothallic locus, by two separate deletion events. Amselem et al. (2011) proposed an evolutionary scenario for the *B. cinerea* MAT locus, in which deletion of the MAT1-1-5 gene as well as a large part of the MAT1-1-1 gene resulted in the emergence of a heterothallic MAT1-2 allele, whereas deletion of the MAT1-2-4 gene and the major part of the MAT1-2-1 gene yielded the heterothallic MAT1-1 allele. The configuration of the *B. elliptica* MAT alleles is in full agreement with the occurrence of such a scenario in a common ancestor of *B. cinerea* and *B. elliptica*. 

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The morphology of *B. elliptica* apothecia is in agreement with the general description of apothecia in the genus *Botrytis* (Whetzel 1945). The apothecia of *B. elliptica* range between 7 and 12 mm in height and the apothecial disks range from 3 to 4 mm in diameter and 0.5 to 1 mm in thickness. *B. elliptica* apothecia are similar in size to those of *Botrytis ranunculi*, but smaller than apothecia of *B. cinerea*, which can reach up to 25 mm in height (Bergquist and Lorbeer 1972; Faretra and Antonacci 1987). The ascospores of *B. elliptica* (23 × 10 μm) are substantially larger than those of *B. cinerea* (13 × 8 μm). The earliest primordia ruptured from the sclerotium in *B. elliptica* 74 days after fertilization; which is more than 7 weeks later than in *B. cinerea* (Faretra and Antonaci, 1987; Terhem and van Kan 2014). Whether the slow development of *B. elliptica* apothecia is due to conditions employed here, or is intrinsically slower than in *B. cinerea* remains unknown. *B. elliptica* apothecia are brown in colour and become darker when mature, alike typical characteristics of apothecia of the genus *Botrytis* (Whetzel 1945).

When comparing *B. elliptica* apothecia with *B. cinerea* side by side, we noticed that *B. elliptica* apothecia in a young stage appear slightly more yellow-greyish as compared to the yellow-brown appearance of *B. cinerea*. The apothecia of *B. elliptica* are thicker and more deeply cup-shaped than those of *B. cinerea*. Furthermore, the stipes of *B. elliptica* are uniform in width, whereas stipes of *B. cinerea* are tapered to a narrow base (not shown).

Apothecia only developed in crosses with *MAT1-2* strains acting as the maternal parent, but not in crosses of *MAT1-1* strain Be9612 as the maternal parent, in spite of several attempts in multiple replicates. The reasons for the unsuccessful development of apothecia in the crosses with Be9612 as the maternal parent is unclear, but a similar feature was reported for certain *B. cinerea* strains (van der Vlugt-Bergmans et al. 1993). The observation that apothecia were never produced when sclerotia were incubated in sterile water indicates that the *B. elliptica* strains tested are not homothallic. Individual ascospores collected from *B. elliptica* apothecia were analysed for the segregation of mating type alleles. The analysis of segregation showed that the MAT loci segregate nearly 1:1 in two independent experiments (Table 4). In a third experiment, a skewed segregation was observed, the reason of which remains unexplained. Nevertheless, in the total dataset, the segregation ratio did not significantly deviate from 1:1.
The four strains of *B. elliptica* used in these crosses were obtained from a field in which there was apparently a potential for sexual reproduction, as indicated by the presence of opposite mating types at the same location. Van den Ende and Pennock-Vos (1996) collected apothecia of *B. elliptica* on plant debris in the same fields as the strains used in this study, and they were the first to succeed in inducing production of apothecia of *B. elliptica* in vitro. They also tested the ascospores produced from apothecia by inoculating them on lily leaves. In the present study, inoculation of ascospores of *B. elliptica* on lily leaves caused similar symptoms as described by van den Ende and Pennock-Vos (1996). Disease development and lesion outgrowth was indistinguishable between inoculation with ascospores or conidia of *B. elliptica*. In view of the detection of *B. elliptica* apothecia in plant debris at the end of the winter (van den Ende and Pennock-Vos 1996), a high genetic diversity in the *B. elliptica* population that is indicative of extensive sexual reproduction (Staats et al. 2007) and our observation that ascospores are equally infectious as conidia, we propose that ascospores make an important contribution in the epidemiology of *B. elliptica*, by infecting young lily shoots emerging in early spring.

**Supporting information**

Supplementary Figure S1

**Acknowledgements**

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References


Beever, R. E., Buchwald, N. (1953). The connection between Botrytis cinerea and Botrytis tulipae (Berkeley) Cooke.


Buchwald, N. (1953). The connection between Botrytis cinerea and Botrytis tulipae (Berkeley) Cooke.


Mating type and sexual fruiting body of *Botrytis elliptica*, the causal agent of fire blight in lily


Supporting information

Supplementary Figure S1
Alignment of *B.elliptica* MAT genes to their orthologs in *B.cinerea* and *S.sclerotiorum*

**BeMAT1-2-1**
MLSSLRLGKWPHHTVLETPLANVPYPAVDTPPEAYMPGKLTLKIYNAABMFINELD 60
**BeMAT1-2-2**
MLSSLRLGKWPHHTVLETPLANVPYPAVDTPPEAYMPGKLTLKIYNAABMFINELD 60
**BeMAT1-2-4**
MLSSLRLGKWPHHTVLETPLANVPYPAVDTPPEAYMPGKLTLKIYNAABMFINELD 60
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**BcMAT1-2-1**
LYNALDIGNWCLQGDYEMSTGPERGILLHYQHRTDRKGVFVRDAYINRMFLAPLDEF 120
**BcMAT1-2-2**
LYNALDIGNWCLQGDYEMSTGPERGILLHYQHRTDRKGVFVRDAYINRMFLAPLDEF 120
**BcMAT1-2-4**
LYNALDIGNWCLQGDYEMSTGPERGILLHYQHRTDRKGVFVRDAYSFLAPLDEF 120
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**SsMAT1-2-1**
EHKMLAVAGFTNLCEPLQIEQRMNSWDEGQLQPEHYRLSYDSDEAMGMNFAPTE 180
**SsMAT1-2-2**
EHKMLAVAGFTNLCEPLQIEQRMNSWDEGQLQPEHYRLSYDSDEAMGMNFAPTE 180
**SsMAT1-2-4**
EHKMLAVAGFTNLCEPLQIEQRMNSWDEGQLQPEHYRLSYDSDEAMGMNFAPTE 180
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**BeMAT1-2-1**
PAAAKPPMNLYGDLQLANLQNFNETTSTESSESS---S 291
**BeMAT1-2-2**
PAAAKPPMNLYGDLQLANLQNFNETTSTESSESS---S 291
**BeMAT1-2-4**
PAAAKPPMNLYGDLQLANLQNFNETTSTESSESS---S 291
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**BcMAT1-2-1**
AWQQTVQQQDLYTFFSPQQQY 380
**BcMAT1-2-2**
AWQQTVQQQDLYTFFSPQQQY 380
**BcMAT1-2-4**
AWQQTVQQQDLYTFFSPQQQY 380
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**SsMAT1-2-1**
SWQMVQQQDLYFFSPRQITLGEATAVQICQGSSSDMCWKRTDI 394
Mating type and sexual fruiting body of *Botrytis elliptica*, the causal agent of fire blight in lily

**BeMAT1-1-1**
MTAPFKTMKTHKQRQRKLTTQYRSVPSKPAKTCKRRAANDAMQLTYSAPFISEAEGMQ 60

**BcMAT1-1-1**
MTAPFKTMKTHKQRQRKLTTQYRSVPSKPAKTCKRRAANDAMQLTYSAPFISEAEGMQ 60

**SsMAT1-1-1**
MTTPFKTMKTHKQRQRKLTTQYRSVPSKPAKTCKRRAANDAMQLTYSAPFISEAEGMQ 60

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**BeMAT1-1-1**
HVAMERNQTAWVEAPRRAVSNXYLRTFTYMTILTVNQYKASIALSVLWGRDPFHKAW 120

**BcMAT1-1-1**
HVAMERNQTAWVEAPRRAVSNXYLRTFTYMTILTVNQYKASIALSVLWGRDPFHKAW 120

**SsMAT1-1-1**
HVAMERNQTAWVEAPRRAVSNXYLRTFTYMTILTVNQYKASIALSVLWGRDPFHKAW 120

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**BeMAT1-1-1**
SIIARATLMRDAGVRSVEFSEALICVPICHIGILTVYYLSDLWDFTNEMEETVCLRTQTS 180

**BcMAT1-1-1**
SIIARATLMRDAGVRSVEFSEALICVPICHIGILTVYYLSDLWDFTNEMEETVCLRTQTS 180

**SsMAT1-1-1**
SIIARATLMRDAGVRSVEFSEALICVPICHIGILTVYYLSDLWDFTNEMEETVCLRTQTS 180

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**BeMAT1-1-1**
PPEIGSFPHFIARTYLTDLIDITFCGAQQLPAATASIVQGWNRFMANMAIQGTLPV 240

**BcMAT1-1-1**
PPEIGSFPHFIARTYLTDLIDITFCGAQQLPAATASIVQGWNRFMANMAIQGTLPV 240

**SsMAT1-1-1**
PPEIGSFPHFIARTYLTDLIDITFCGAQQLPAATASIVQGWNRFMANMAIQGTLPV 240

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**BeMAT1-1-1**
TGIASTQNYGAEMPSGVIVAPPPKPAFDQPLAYTPPALIAGTSGVTLSSLPFFPPPS 300

**BcMAT1-1-1**
TGIASTQNYGAEMPSGVIVAPPPKPAFDQPLAYTPPALIAGTSGVTLSSLPFFPPPS 300

**SsMAT1-1-1**
TGIASTQNYGAEMPSGVIVAPPPKPAFDQPLAYTPPALIAGTSGVTLSSLPFFPPPS 300

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**BeMAT1-1-1**
TGMMAGYNGSDTGLSAIDTLDFQEFQNTSGLADAGLYMPEDVPSPPLFADF 353

**BcMAT1-1-1**
TGMMAGYNGSDTGLSAIDTLDFQEFQNTSGLADAGLYMPEDVPSPPLFADF 353

**SsMAT1-1-1**
TGMMAGYNGSDTGLSAIDTLDFQEFQNTSGLADAGLYMPEDVPSPPLFADF 353

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**BeMAT1-1-1**
MTTPFKTTMKHTMKRQQRKLTTQYRVSKPATKCRRANDAMQSLTYHAPFISGEEIQGQ 60

**BcMAT1-1-1**
MTTPFKTTMKHTMKRQQRKLTTQYRVSKPATKCRRANDAMQSLTYHAPFISGEEIQGQ 60

**SsMAT1-1-1**
MTTPFKTTMKHTMKRQQRKLTTQYRVSKPATKCRRANDAMQSLTYHAPFISGEEIQGQ 60

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**BeMAT1-1-1**
EEVS 376

**BcMAT1-1-1**
EEVS 376

**SsMAT1-1-1**
EEVS 376

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**BeMAT1-1-1**
MKVNTAQMTREAFRLRKYLGKLSARYRPAFLDALVTDCCVLIWE 120

**BcMAT1-1-1**
MKVNTAQMTREAFRLRKYLGKLSARYRPAFLDALVTDCCVLIWE 120

**SsMAT1-1-1**
MKVNTAQMTREAFRLRKYLGKLSARYRPAFLDALVTDCCVLIWE 120

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**BeMAT1-1-1**
AAALGKRELLTQMGKMSGGVSLSYSNYPVEATANPFFCDPFDMLALVTDCCVLIWE 120

**BcMAT1-1-1**
AAALGKRELLTQMGKMSGGVSLSYSNYPVEATANPFFCDPFDMLALVTDCCVLIWE 120

**SsMAT1-1-1**
AAALGKRELLTQMGKMSGGVSLSYSNYPVEATANPFFCDPFDMLALVTDCCVLIWE 120

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**BeMAT1-1-1**
FSLLRHTDNSMHDLPMTKYADIFHDNLTVFGQCRRLKSPELFSTLAQERQFKAEMR 180

**BcMAT1-1-1**
FSLLRHTDNSMHDLPMTKYADIFHDNLTVFGQCRRLKSPELFSTLAQERQFKAEMR 180

**SsMAT1-1-1**
FSLLRHTDNSMHDLPMTKYADIFHDNLTVFGQCRRLKSPELFSTLAQERQFKAEMR 180

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**BeMAT1-1-1**
IAEVIDLHRLVEVRLNKDVNQKMQNREKEELARLDPFYFPRADGKHPWTRWLENSPSV 240

**BcMAT1-1-1**
IAEVIDLHRLVEVRLNKDVNQKMQNREKEELARLDPFYFPRADGKHPWTRWLENSPSV 240

**SsMAT1-1-1**
IAEVIDLHRLVEVRLNKDVNQKMQNREKEELARLDPFYFPRADGKHPWTRWLENSPSV 240

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**BeMAT1-1-1**
MPPGWGQIQKPQIPHGDNIIRRMMRDLFEYRQFIVKLIIDSKVNDLDDPFAPGEAFFE 300

**BcMAT1-1-1**
MPPGWGQIQKPQIPHGDNIIRRMMRDLFEYRQFIVKLIIDSKVNDLDDPFAPGEAFFE 300

**SsMAT1-1-1**
MPPGWGQIQKPQIPHGDNIIRRMMRDLFEYRQFIVKLIIDSKVNDLDDPFAPGEAFFE 300

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**BeMAT1-1-1**
MNYSRFSTLAVNYQYRTQGRDPATDKNGMLRVEKLEEKLRTSVEQSSQVSNFLCNTWPNV 360

**BcMAT1-1-1**
MNYSRFSTLAVNYQYRTQGRDPATDKNGMLRVEKLEEKLRTSVEQSSQVSNFLCNTWPNV 360

**SsMAT1-1-1**
MNYSRFSTLAVNYQYRTQGRDPATDKNGMLRVEKLEEKLRTSVEQSSQVSNFLCNTWPNV 360

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**BeMAT1-1-1**
RFMGMYKVEVLNEEV 376

**BcMAT1-1-1**
RFMGMYKVEVLNEEV 376

**SsMAT1-1-1**
RFMGMYKVEVLNEEV 376
A novel *Botrytis* species is associated with a newly emergent foliar disease in cultivated *Hemerocallis*

This chapter is adapted from:

Abstract
Foliar tissue samples of cultivated daylilies (Hemerocallis hybrids) showing the symptoms of a newly emergent foliar disease known as ‘spring sickness’ were investigated for associated fungi. We isolated repeatedly a fungal species which proved to be member of the genus Botrytis, based on immunological tests. DNA sequence analysis of these isolates, using several phylogenetically informative genes, indicated that they represent a new Botrytis species, most closely related to B. elliptica (fire blight) which is a major pathogen of cultivated Lilium. The distinction of the isolates was confirmed by morphological analysis of asexual sporulating cultures. Pathogenicity tests on Hemerocallis demonstrated that this new species was able to induce lesions and rapid tissue necrosis. We infer that this new species, described as B. deweyae, is an important contributor to the development of ‘spring sickness’ symptoms. The emergence of this disease is suggested to have been triggered by breeding-related changes in cultivated hybrids, particularly the erosion of genetic diversity.
A novel *Botrytis* species is associated with a newly emergent foliar disease in cultivated *Hemerocallis*

**Introduction**

The emergence of new fungal pathogens in cultivated and wild plants is a major cause for concern (Fisher et al., 2012) and many factors influence such patterns of emergence and establishment. Interspecific hybridisation between different fungal species may be a major contributor to the evolution of novel diversity (Schardl and Carven, 2003). In plant pathogens, it has been demonstrated that rapid genome evolution and host shifts may occur after hybridisation events. For example, establishment of a new species of *Zymoseptoria*, *Z. pseudotritici*, exhibiting an expanded host range on grasses took place within just a few hundred generations of a hybridisation event between *Z. tritici* and an unidentified species (Stukenbrock et al., 2012). There is also considerable evidence that the commensal endophytic state and a parasitic, pathogenic state can be highly plastic (Schardl and Craven, 2003; Schulz, 2005). Indeed, there is confirmation that several fungal diseases of plants can exist as a symptomless, endophytic infection – as, for instance, in the necrotrophic generalist *Botrytis cinerea* (grey mould) (Barnes and Shaw, 2003; Sowley at al., 2010) and obligate biotrophic *Albugo* species (Ploch and Thines 2011). Anthropogenic change to natural environments is also likely to be a major factor in promoting the emergence of new pathogens (Fisher et al., 2012). Important issues such as climate change, degradation of natural environments (such as forest clearance and loss of genetic diversity in natural plant populations), dispersal of plants and their associated fungi to new areas and dependence on agricultural monoculture systems are likely to enhance the probability of new diseases emerging, establishing and spreading. The initial emergence events are likely to go unreported not only because of their low frequency, but also because the damage resulting from a new pathogen could be attributed to other microbial species or to abiotic stress-induced damage.

The genus *Hemerocallis* (daylily) of the family Hemerocallidaceae in the order Asparagales has been cultivated for thousands of years (Rodriguez-Enriquez and Grant-Downton, 2012). This small genus of petaloid monocotyledons has been valued as a food crop, as a medicinal plant and as an ornamental. The native range extends from eastern Asia, with a centre of diversity in Japan, Korea and China, possibly as far west as central Europe. Widespread cultivation has led to naturalisation of the genus in many parts of the world (Rodriguez-Enriquez and Grant-Downton, 2012). Cultivated *Hemerocallis* in North America, where the
numerous hybrids are popular ornamental garden plants, were recently challenged by the accidental introduction of a genus-specific biotrophic rust pathogen, *Puccinia hemerocallidis* (daylily rust) (Williams-Woodward et al., 2001; Hernández et al., 2002). This pathogen caused a rapidly-spreading epidemic. The lack of resistance amongst hybrid cultivars - with one study showing only a minority of the cultivars tested exhibiting any resistance to even a single *P. hemerocallidis* isolate - demonstrated the susceptibility of cultivated varieties that have been bred outside of their native range in the absence of any pressure from this specific pathogen (Muller et al., 2003). Other emerging threats to cultivated daylily include infection by the *Armillaria* genus (Schnabel et al., 2005; Blaedow et al., 2010). Both in the West and in China, anthracnose diseases caused by *Colletotrichum* species have been identified and a new species of *Colletotrichum, C. hemerocallidis*, was recently described in China (Yang et al., 2012).

However, the etiology of one emerging disease of cultivated *Hemerocallis* in Western gardens has remained elusive. ‘Spring sickness’ describes a group of disease symptoms that primarily affect the emergent post-winter foliar growth of the plant, causing distortion, stunting, chlorosis, ragged leaf edges and necrotic lesions (Bergeron, 2001). These symptoms not only disfigure the foliage but also weaken the plant, resulting in reducing flowering, and -in severe cases - the affected growing point dies. The first reports of this disease come from the U.S.A. in the 1970s (Wooten, 1972) and widespread notification of these symptoms has occurred only over the past 20 years. To date, there has not been a report in peer-reviewed literature of filamentous fungal species being isolated from *Hemerocallis* material showing such symptoms. Nevertheless, there is considerable speculation that fungal pathogens may be responsible or contribute to disease development (Bergeron, 2001; Wooten, 1972; Bergeron and Somers, 2010). Here, we report the isolation of a new species of *Botrytis* from foliar material of *Hemerocallis* from the United Kingdom that showed a range of symptoms that correspond to ‘spring sickness’. We demonstrate that this new species is distinct at the molecular and morphological level from its closest relative, *B. elliptica* (fire blight), and that the two species have diverged in their host range. Our data shows that cryptic fungal species and fungal diseases can be identified from common cultivated plants outside of their natural range, suggesting that there may be a significant reservoir of fungal diversity from which new diseases may emerge.
A novel Botrytis species is associated with a newly emergent foliar disease in cultivated Hemerocallis

**Materials and Methods**

**Isolation from plant material**

Samples were collected from collections of hybrid Hemerocallis growing in gardens in England, from Oxfordshire, Wiltshire and Somerset. Pieces of asymptomatic and symptomatic leaf tissue, each approximately 6 mm², were surface-sterilized by immersing in 30% (v/v) sodium hypochlorite-based domestic bleach containing detergent (Parazone; Jeyes, Cambridge, UK) for 30 sec followed by 4×5 min washes in sterile distilled water. Surface-sterilized leaf pieces were plated out on 2% (w/v) malt extract agar (MEA) (Oxoid, Basingstoke, UK) and grown under natural ambient light. The 6 isolates, labelled B1, B2, B4, B5, B6 and P1, were routinely grown on 2% MEA and stock cultures were maintained at 4°C in the dark. Stocks were also preserved as excised mycelial material in sterile 50% (v/v) glycerol at −80°C.

**Immunological tests of fungal isolates and plant extracts for Botrytis antigens**

Plate cultures of fungal isolates were washed with 5 ml phosphate buffered saline, pH 7.4 (Sigma) plus 0.05% Tween 20 (Sigma) (PBST). 1 ml was removed by suction, centrifuged briefly at 13,000×g in a microcentrifuge and 400 µl of the supernatant tested with Botrytis QuickStixs (EnviroLogix, Portland, Maine, USA) which employs the anti-Botrytis-monoclonal antibody, BC-12.CA4, raised and employed in previous studies (Meyer and Dewey, 2000; Dewey et al., 2008; Dewey et al., 2013). Tests were performed by incubating a QuickStix in the supernatant for 10 minutes, the lower pad was then removed and the intensity of the test line (signal intensity, SI) was determined using an EnviroLogix Quickstix reader (Dewey et al., 2008; Dewey et al., 2013). Extracts of leaf tissues were made by crushing leaf material in an extraction bag (Noegen, UK) with PBST, 1:5 (w/v). 400 µl of the resulting non-particulate extract was tested with EnviroLogix Botrytis QuickStix system as above.

**DNA extraction, PCR amplification and sequencing**

Genomic DNA was extracted from plugs of cultured mycelial material that were frozen and ground in liquid nitrogen. DNA was purified from tissue powder using the DNEasy kit (Qiagen, Manchester, UK) and quantified using a Nanodrop spectrophotometer. PCRs were performed using approximately 4–12 ng genomic DNA at in a 50 µl reaction using the proof-
reading Phusion polymerase kit (Finnzymes, Thermo Scientific, UK) according to the manufacturer’s instructions. Cycling conditions for the amplifications were as follows: 98°C for 1 minute; 10 cycles of 98°C for 10 seconds, primer-specific annealing temperature 1 for 30 seconds, extension at 72°C for 45 seconds; 15 cycles of 98°C for 10 seconds, primer-specific annealing temperature 2 for 30 seconds, extension at 72°C for 45 seconds; final extension at 72°C for 5 minutes.

The 18S rRNA sequence was amplified using ITS1 primers previously described (Staats et al., 2005) (Supplementary Table S1). The primer-specific annealing temperatures 1 and 2 were 60°C and 56°C respectively. The amplification of the G3PDH, HSP60 and RPB2 sequences was achieved using primers for the genus Botrytis described in (Staats et al., 2005) (Supplementary Table S1). Primer-specific annealing temperatures 1 and 2 were 62°C and 60°C respectively for G3PDH and HSP60, and 60°C and 56°C respectively for RPB2. NEP1 was amplified using primers NEP1for and NEP1revB (Staats et al., 2007) (Supplementary Table S1) with primer-specific annealing temperatures 1 and 2 of 62°C and 60°C respectively. NEP2 was amplified using primers NEP2forE and NEP2revE (Staats et al., 2007) (Supplementary Table S1) with primer-specific annealing temperatures 1 and 2 of 62°C and 60°C respectively. PCRs were run on 1.5% (w/v) agarose gels, the bands were cut out with a clean razor and extracted using Qiaex II kit (Qiagen, Manchester, UK). Purified PCR fragments were then cloned into pJET sequencing vector (CloneJET PCR cloning kit; Thermo Scientific, UK) and transformed into E. coli DH5α competent cells. Transformants were selected on LB-ampicillin plates (with 100 µg ampicillin per ml of medium) and colonies with inserts identified by PCR screening. Plasmid DNA was extracted from selected colonies using GeneJET Plasmid Miniprep Kit (Thermo Scientific, UK) and sequenced by Source Biosciences (Nottingham, UK).

Genomic DNA was extracted from leaf material showing symptoms and from leaf material of the same cultivar without symptoms, collected on the same day. Leaf material was derived from the cultivars ‘Gerda Brooker’ and ‘Free Bird’. Small sections of leaf material approximately 2×2 cm were ground to a fine powder in liquid nitrogen. DNA was purified from tissue powder using the DNEasy kit (Qiagen, Manchester, UK) and quantified using Nanodrop spectrophotometer. PCRs were performed using approximately 100 ng genomic
A novel *Botrytis* species is associated with a newly emergent foliar disease in cultivated *Hemerocallis*

DNA as template at in a 50 µl reaction using the proof-reading Phusion polymerase kit (Finnzymes, Thermo Scientific, UK) according to the manufacturer’s instructions. *NEP1* primers that amplify the promoter to 3’ end - NEP1(−207for) and NEP1(+1124rev) - (Staats et al., 2007) (Supplementary Table S1) were used in a primary PCR with cycling as follows.

98°C for 1 minute; 10 cycles of 98°C for 20 seconds, 64°C for 30 seconds, extension at 72°C for 45 seconds; 10 cycles of 98°C for 20 seconds, 60°C for 30 seconds, extension at 72°C for 45 seconds; 15 cycles of 98°C for 20 seconds, 58°C for 30 seconds, extension at 72°C for 45 seconds; final extension at 72°C for 5 minutes. A 2 µl aliquot of primary PCR was then used as a template in a 50 µl reaction with NEP1for and NEP1revB primers (Staats et al., 2007) (Supplementary Table S1) as follows: 98°C for 1 minute; 15 cycles of 98°C for 10 seconds, 64°C for 30 seconds, extension at 72°C for 20 seconds; 15 cycles of 98°C for 10 seconds, 60°C for 30 seconds, extension at 72°C for 20 seconds; final extension at 72°C for 5 minutes. PCRs were run on 1.5% (w/v) agarose gels, the PCR fragments were then removed, purified, cloned and sequenced as described above.

**Phylogenetic analysis**

Sequences of *Botrytis* genes were obtained from GenBank (www.ncbi.nlm.nih.gov/genbank) and aligned with newly sequenced genes from the six isolates using MUSCLE (Edgar, 2004). Phylogenies for single gene and combined datasets were reconstructed using a maximum-likelihood inference conducted with RAxML version 7.2.6 (Stamatakis, 2006) via the raxmlGUI interface (Silvestro and Michalak, 2012). We conducted five independent runs from different starting points to assess convergence within two likelihood units of the best tree, which was consistently selected. The parameters of partition were allowed to vary independently under the GTRGAMMA model of evolution as implemented in RAxML. Maximum-likelihood nodal support was calculated by analysing 1000 bootstrap replicates.

**Induction of sporulation and sclerotia formation**

To determine whether presence of host material stimulated macroconidia production, 1% water agar on which surface-sterilized pieces of greenhouse-grown *Hemerocallis ‘Jurassic Spider’* young leaf tissue had been placed. Plates were also made with 1% water agar with
0.5 ml plant extract added. The plant extract was made by thoroughly crushing young leaf tissue in an extraction bag (Neogen, Ayr, UK) with PBST at 1:5 (w/v). The extract was passed through a 0.25 micron filter (Millipore) attached to a syringe for sterilisation before addition to molten agar. To attempt to stimulate production of macroconidia, MEA plates were incubated without light and under a mixture of day light and near UV light at room temperature (~20°C) for 7 to 10 days. Plates were also incubated in the dark except for a continuous near-UV light source. Isolates were also grown on oatmeal agar (OMA) (Difco, Becton Dickinson BV, Breda, The Netherlands), potato dextrose agar (PDA) (Oxoid, Basingstoke, UK), Czapeks Dox medium (Oxoid, Basingstoke, UK), V8 agar (prepared with 200 ml V8 vegetable juice (Campbell Soup Company, Camden, NJ, USA), 20 g agar, 800 ml water, pH adjusted to 6.0 with NaOH).

**Light microscopy**

Sporulating structures were mounted on slides with filter-sterilised MilliQ water. Observations were made with a Nikon Eclipse 90i (Nikon Instruments, Badhoevedorp, The Netherlands) compound microscope with a Nikon DS-5MC camera attached. Measurements were performed using N.I.S. Elements AR 2.30 software (Nikon Instruments, Badhoevedorp, The Netherlands).

**Electron microscopy**

Spores were removed from the edges of mature cultures of B2 and B4 using a sticky pad mounted in a SEM stub. Samples were directly coated with gold/palladium in a Polaron SC7640 sputter coating unit (Quorum Technologies, Ashford, UK). Spores were also trapped using poly-lysine coated slides and subsequently treated in osmium tetroxide vapour for 3 hours, followed by 4% paraformaldehyde in phosphate buffer for 3 hours, dehydrated then subsequently sputter coating as described. Images were taken using a JEOL JSM-5510 scanning electron microscope unit (JEOL, Welwyn Garden City, UK) operating at 15 kV.

**Pathogenicity assays**

Pathogenicity tests were carried out using detached leaves of the following plants: *Alstroemeria* hybrid; *Tricyrtis formosana*; *Lilium Oriental* hybrid; *Hemerocallis fulva* and *Hemerocallis* ‘Jurassic Spider’. Leaves were placed on 0.3% water agar in large Petri
A novel *Botrytis* species is associated with a newly emergent foliar disease in cultivated *Hemerocallis*. Four replicates were made with the leaf adaxial and abaxial sides upwards. The exposed surfaces were inoculated with a 5 mm mycelial plug from MEA cultures of isolates B1, B2 and B4. The Petri dishes were sealed and incubated at 25°C under 12 hours light and monitored for up to 14 days. As a control, plugs of *B. elliptica* (isolate 9601), grown on MEA agar under identical conditions, were used. A sterile MEA agar control was also performed. Macroconidia of B1, B4 and B5 from sporulating plates were collected in sterile potato dextrose broth (1.2 g/l) and adjusted to concentrations $1 \times 10^6$ ml$^{-1}$. Excised *Hemerocallis* leaves on water agar were inoculated with 3 µl droplets of spore suspensions on their upper sides and three replicates performed for each. The material was sealed in a plastic box with a >90% humidity at 25°C and monitored for up to 10 days after inoculation.

Pathogenicity tests were also carried out on axenic plantlets of *Hemerocallis* ‘Jurassic Spider’ as follows. Previously established cultures of *Hemerocallis* ‘Jurassic Spider’ were maintained at 4°C on a 0.6% agarose growth medium composed of Murashige and Skoog macroelements (Murashige and Skoog, 1962) with Heller’s microelements (Heller, 1953) and 5 ml/litre of 1% ferric ammonium citrate solution, adjusted to pH 5.5 before autoclaving. The clumps of shoots were divided, with the leaves and roots trimmed. The plantlets derived from division were grown in Magenta boxes containing sterilised 100 ml vermiculite plus 80 ml liquid growth medium (as above, but lacking the 0.6% agarose). In each box, 5 divisions were planted and chilled at 4°C in the dark for 2 weeks, then removed in a well-lit growth room at 20°C with 16 hour photoperiod for 2 weeks to allow wound healing and establishment prior to inoculation. Individual plantlets were inoculated with a 5 mm mycelial plug from a plate culture of one of the fungal isolates (B1, B2, B4) and incubated at 25°C under 16 hours direct light in a growth room for up to 10 days. For controls, *B. elliptica* (isolate 9601) and sterile MEA plugs were used. For each fungal isolate and for each control, three replicate Magenta boxes were inoculated.

To confirm *Botrytis* infection of *Hemerocallis* tissue, extracts of plant tissues were made by crushing sections of aerial material in an extraction bag (Neogen, UK) with PBST, 1:5 (w/v). 400 µl of the resulting non-particulate extract was tested with Envirologix *Botrytis* QuickStix system as above. To determine if pathogenicity varied depending on the host cultivar that was challenged, plantlets of a range of cultivars were tested using the same method except
that one plantlet per Magenta box was used. Two replicates were performed for each plant genotype. These pathogenicity assays were all performed with the B1 isolate. The 15 cultivars were as follows: ‘Running Late’, ‘Lavender Curls’, ‘Dark Mosaic’, ‘Heavenly Flight of Angels’, ‘Golden Chimes’, ‘Barbara’, ‘Party Array’, ‘Rococo’, ‘Bo Knows’, ‘Cayenne’, ‘Corky’, ‘Miss Jessie’, ‘flava clone 3’, ‘Persian Pattern’, ‘Jellyfish Jealousy’.

**Identification of mating types and sexual crosses**

Five isolates were analysed (B1, B2, B4, B5 and P1) to identify the mating type alleles. Gentra Puregene DNA purification kit (Qiagen, Venlo, The Netherlands) was used for DNA extraction from freeze-dried mycelia following the manufacturer’s instructions. 10–50 ng genomic DNA was used in 25 µl reaction volume. PCRs were performed with GoTaq polymerase (Promega) according to manufacturer’s instructions. Primers used were MAT1-1 forward/reverse and MAT1-2 forward/reverse (Supplementary Table S1). Amplification conditions were as follows: 95°C 5 minutes, then 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds and 72°C for 2 minutes, followed with a final extension of 72°C for 5 minutes. PCR products were visualised on gel to determine the mating type of each isolate. Crosses were set up between isolates carrying different mating types (Faretra et al., 1998). Isolates B1 and B5 (each of the MAT1-1 mating type) were mated with isolates B2 and B4 (each of the MAT1-2 mating type). To develop sclerotia, strains were plated on oatmeal agar and incubated in darkness for 1 month at 15°C, followed by incubation at 0°C in darkness for 1 month. For mating, sclerotia were sampled from the plates, rinsed in water with a soft toothbrush and placed in a 6-well microtitre plate. Mycelial cultures were flooded with sterile water and a suspension of mycelial fragments and microconidia was obtained by gently rubbing the surface with a spatula. The sclerotia were fertilised by addition of this suspension at 3 ml per well. Reciprocal crosses were set up in this manner, with each partner as a female (sclerotial) or male (microconidial) parent. As a control, sclerotia that were not exposed to microconidia and kept in sterile water were used. The microtitre plates were sealed and incubated at 12°C in normal artificial light with a 12 hour photoperiod.
A novel *Botrytis* species is associated with a newly emergent foliar disease in cultivated *Hemerocallis*

**Results**

**Isolations and preliminary identification**

Over a period of 4 years, leaf samples of *Hemerocallis* cultivars that showed symptoms typical of ‘spring sickness’ such as necrotic patches, necrotic lesions, distortion and chlorosis (Figure 1a,b) were found to be associated with an unidentified filamentous fungus after the material was rigorously surface-sterilised and plated on malt extract agar (MEA). Occasionally, fungal growth was visible on the symptomatic plant material in the form of dense, short mycelia producing microconidia (Figure 1c,d) suggesting that a filamentous fungus triggers symptom development. However, no macroconidia could be observed on the plant material. In total, 6 independent isolates were collected (Table 1). Vegetative growth at 25°C with ambient light and supplemental near-UV light was rapid, but significant macroconidial sporulation did not occur. Although microconidia were readily identified, only a small number of macroconidia could be found on older cultures at the edges of the Petri dish in two isolates (B2 and B4). The overall hyphal morphology and appearance of melanised sclerotia in older cultures (Figure 1e) suggested that the isolates are members of the genus *Botrytis* and prompted further investigations and DNA analyses. The immunological tests of surface washings of cultures of the isolates using the *Botrytis*-specific monoclonal antibody BC-12. CA4 (Meyer and Dewey, 2000; Dewey et al., 2008; Dewey et al., 2013) gave strong positive results (Table 1) indicating that they are all members of the genus *Botrytis*.

![Figure 1](image.png)

**Figure 1**: a,b, Spring foliage of *Hemerocallis* cultivars showing symptoms typical of ‘spring sickness’ disease, c, sporulating fungus on young leaf, d, close up of sporulating fungus *B. deweyae*, e, melanised sclerotia growing on medium
### Table 1: Table of fungal isolates identified in this study

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host name</th>
<th>Host symptoms</th>
<th>Date of isolation</th>
<th>Culture S.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>Hemerocallis ‘Jurassic Spider’</td>
<td>Developing necrosis, distortion and chlorosis of young foliage</td>
<td>30 November 2009</td>
<td>42</td>
</tr>
<tr>
<td>B2</td>
<td>Hemerocallis ‘Lola Branham’</td>
<td>Severe necrosis, distortion and chlorosis of young and maturing foliage</td>
<td>19 February 2010</td>
<td>35</td>
</tr>
<tr>
<td>B4</td>
<td>Hemerocallis unnamed tetraploid</td>
<td>Necrosis of mature outer leaves just above soil level</td>
<td>9 June 2010</td>
<td>11</td>
</tr>
<tr>
<td>B5</td>
<td>Hemerocallis ‘Gerda Brooker’</td>
<td>Severe necrosis of young and mature foliage</td>
<td>15 April 2011</td>
<td>50</td>
</tr>
<tr>
<td>P1</td>
<td>Hemerocallis ‘Free Bird’</td>
<td>Severe necrosis, distortion and chlorosis of aerial parts; eventual death of tissues back to rhizome</td>
<td>15 April 2011</td>
<td>55</td>
</tr>
<tr>
<td>B6</td>
<td>Hemerocallis ‘Ruby Storm’</td>
<td>Severe necrosis and chlorosis of leaves emerging from dormancy</td>
<td>12 March 2012</td>
<td>53</td>
</tr>
</tbody>
</table>

Host name, host symptoms and the date of isolation are described. The post-isolation fungal culture signal intensity (S.I.), from tests of PBST surface washings of fungal cultures, with EnviroLogix Botrytis QuickStix using the anti-Botrytis-monomoclonal antibody BC-12.CA4, is shown.

### Sequence analyses and phylogenetic tree

To determine their identity, all 6 isolates were subjected to DNA sequence analysis. Sequencing of the *ITS* region confirm they all represent *Botrytis* species. Interestingly, all 6 isolates shared a unique polymorphism, an indel, in the highly conserved *ITS* sequence (Figure 2). BLAST analysis of Genbank data showed that no other known member of the genus *Botrytis* possesses this sequence variant.
A novel Botrytis species is associated with a newly emergent foliar disease in cultivated Hemerocallis

EU519207 Botrytis elliptica CCAAAACCCAAATTTTCTATGGTT
EU519208 Botrytis squamosa CCAAAACCCAAATTTTCTATGGTT
EU093077 Botrytis aclada CCAAAACCCAAATTTTCTATGGTT
FJ914712 Botrytis byssoidae CCAAAACCCAAATTTTCTATGGTT
EU519206 Botrytis porri CCAAAACCCAAATTTTCTATGGTT
HM989942 Botrytis cinerea CCAAAACCCAAATTTTCTATGGTT
Botrytis deweyae B1 CCAAAACCCAAA-TTTTCTATGGTT
Botrytis deweyae B2 CCAAAACCCAAA-TTTTCTATGGTT
Botrytis deweyae B4 CCAAAACCCAAA-TTTTCTATGGTT
Botrytis deweyae B5 CCAAAACCCAAA-TTTTCTATGGTT
Botrytis deweyae B6 CCAAAACCCAAA-TTTTCTATGGTT
Botrytis deweyae PI CCAAAACCCAAA-TTTTCTATGGTT

**Figure 2**: Alignment of a region of partial ITS sequence from various Botrytis species. The aligned region shows the indel identified in all 6 examined isolates of B. deweyae.

ITS sequence does not permit sufficient resolution to the species level. To further resolve the relationships of these isolates to other species in the genus Botrytis, analysis of nuclear protein-coding gene sequences that are known to be phylogenetically informative to the species level was undertaken. Five genes previously used to build phylogenies of the genus Botrytis (Staats et al., 2005; Staats et al., 2007) were used. Three were single-copy housekeeping genes - encoding glyceraldehyde-3-phosphate dehydrogenase (G3PDH), a heat-shock protein (HSP60) and a DNA-dependent RNA polymerase subunit II protein (RPB2) (Staats et al., 2005) and two were single-copy genes encoding phytotoxic proteins (NEP1 and NEP2) (Staats et al., 2007).

Combined phylogenetic analysis of these 5 sequences confirmed the distinction between isolate B1 and previously described Botrytis species (Figure 3). The genes encoding phytotoxic proteins NEP1 (Figure 4) and NEP2 (Figure 5) provided higher resolution between B1 and other members of Botrytis.
The \textit{HSP60} sequence was found to be identical to \textit{B. elliptica} and the other sequences were also close to this species, suggesting that its nearest relative was \textit{B. elliptica} (not shown). Phylogenetic analysis confirmed that this isolate was most closely related to \textit{B. elliptica} and formed a monophyletic group with \textit{B. elliptica} and \textit{B. squamosa} (Figure 3). Divergence at synonymous sites between the new isolate and \textit{B. elliptica} and \textit{B. squamosa} was 1.1±0.32%, while divergence at synonymous sites between \textit{B. elliptica} and \textit{B. squamosa} was 1.0±0.31%.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{Figure3}
\caption{Phylogeny of \textit{Botrytis} species based on the combined analysis of 5 different genes. Sequences of \textit{G3PDH, HSP60, RPB2, NEP1} and \textit{NEP2} were used. The phylogenetic position of \textit{B. deweyae} (B1 isolate, type) is underlined. The phylogeny was constructed using the genus \textit{Sclerotinia} as the outgroup.}
\end{figure}

The presence of a polymorphism in the \textit{ITS1} sequence (Figure 2), as well as multiple sequence differences at other genes, strongly suggests that they represent an undescribed species (Schubert et al., 2007). \textit{G3PDH, NEP1} and \textit{ITS} sequences from all six isolates were identical confirming genetic similarity of isolates. Genetic similarity of isolates together with significant divergence between the new isolates and \textit{B. elliptica} and \textit{B. squamosa} strongly suggests that the new isolates represent an undescribed species.
Further supporting our notion that this species may be involved in development of ‘spring sickness’, PCR amplification and sequencing of a NEP1 fragment from DNA extracts of six distinct samples of symptomatic leaf material generated sequences identical to this phylogenetic group (not shown), while PCR amplification on DNA extracts from asymptomatic leaf material did not yield a NEP1 fragment. Sequences from fungal specimens were submitted to the Genbank database with accession numbers HG799518-HG799538.

Morphological analyses

Non-sporulating colonies are smooth to slightly fluffy and occasionally form aerial mycelia; colonies typically whitish to pale brown in colour on malt extract agar (Figure 6 a,b,c,d). The distance between septa ranges from 38.5 µm to 127. Typically, sclerotium development is seen only as cultures mature; formation varies between isolates and depending on environmental conditions (Figure 6e). Growth in darkness promotes sclerotia formation. On oatmeal agar, sclerotia are formed within 4 weeks at 15°C in darkness. Sclerotia are hemispherical convex in shape and with a concave surface, sometimes hollow in the centre. Sclerotia are black with size ranging from 2 to 6 mm (average 3 mm) in diameter.

The initial absence of significant macroconidial development upon isolation indicates that such conditions are sub-optimal for promotion of asexual sporulation. Exclusion of light and exposure to supplemental near-UV light in the presence of daylight individually fails to trigger sporulation. Surface-sterilised host leaf material and sterile, crude extracts of host leaf material also do not stimulate sporulation. Sporulation (Figure 6f) is more reliably initiated after a minimum of 7 days exposure to near-UV light in the absence of other light sources, though this varies with the isolate and the medium (Supplementary Table S2). One isolate, B2, does not appear to sporulate under these conditions. The dependence on specific environmental conditions is a contrast to B. elliptica and B. cinerea which efficiently initiate macroconidia formation even when plates are exposed to daylight. The optimum temperature for sporulation of these isolates is 20°C. Conidiophores are erect, septate, opposite branched and slightly swollen at the top (Figure 7a). Figure 7b shows different shapes of macroconidia and Figure 7c shows a scanning electron micrograph of microconidia.
Figure 4: Phylogeny of *Botrytis* species based on the combined analysis of NEP1 gene. The phylogenetic position of *B. deweyae* (B1 isolate, type) is underlined. The phylogeny was constructed using *Sclerotinia sclerotiorum* as the outgroup.

Figure 5: Phylogeny of *Botrytis* species based on the combined analysis of NEP2 gene. The phylogenetic position of *B. deweyae* (B1 isolate, type) is underlined. The phylogeny was constructed using *Sclerotinia sclerotiorum* as the outgroup.
A novel *Botrytis* species is associated with a newly emergent foliar disease in cultivated *Hemerocallis*.

**Figure 6:** a, b, c, d non sporulating isolates. e, sclerotial formation in OMA medium. f sporulating isolate.

**Figure 7:** a, development of *Botrytis deweyae* showing production of conidiophores and macroconidia. b, macroconidia in different shapes. c, scanning electron micrograph of the typical macroconidia.
Formal description

Based on the molecular, morphological and host specificity data presented herein, this fungal isolate from *Hemerocallis* represents a new species:

**Botrytis deweyae** van Kan, Terhem & Grant-Downton, sp. nov. MycoBank MB804656; Myconame ID 512235.

*Conidiophores* erect, septate, medium brown, smooth, mostly unbranched, with slightly swollen basal and apical cells, 3–4 µm × 10–20 µm. *Conidiogenous cells* predominantly terminal, pale brown, giving rise to botryose clusters of conidia. *Conidia* ellipsoid to ovoid, becoming oblong and 1-septate with age, or irregular and somewhat distorted, hyaline to medium brown, smooth, apex obtuse, base with small flattened abscission scar, 6.5–18 µm × 3.5–11 µm (av. = 12.5×7 µm, n = 50). No sporulation observed on leaf tissue. *Sclerotia* hemispherical, convex, sometimes hollow in the center, with concave surface; black, 2–6 mm (av. 3 mm) diam. Sclerotia develop in oatmeal agar within four weeks of incubation at 15°C in the dark. Sclerotia are scattered; cultures normally develop 25–30 sclerotia per plate.

**Culture characteristics:** Colonies incubated in the dark on MEA, optimal growth at 25°C, minimum at 4°C, maximum at 37°C. Colony growth rate 0.4–15.8 mm/d; surface dirty white to pale brown, reverse ochreous (Rayner 1970), with moderate to abundant aerial mycelium, creating fluffy appearance. Colonies also sporulate on MEA under near-ultraviolet light at 20°C.


**Distribution and host range.**

Known only from cultivated specimens of *Hemerocallis* from the UK but likely to be widespread on *Hemerocallis* in cultivation and possibly in the wild due to the cryptic nature of this species and its broad temperature tolerance.

**Etymology.**

Named after Dr. Molly Dewey in recognition of her outstanding contributions to plant pathology and mycology, in particular relating to the genus *Botrytis*. 
Pathogenicity assays

To determine whether *Botrytis* isolates induce the symptoms of the plant material from which they were originally isolated, basic assays were performed using excised surface-sterilised leaf material of *Hemerocallis* on plates. Inoculation of Hemerocallis leaf material with conidial suspensions did not result in necrosis, though the conidia germinated and grew out into hyphae which after 10 days had covered the leaf surface (Figure 8a). Inoculation of mycelial plugs onto the leaf surfaces did not cause any disease symptoms. Instead after several days of incubation, the fungus formed sclerotia on the leaf surface (Figure 8b). Inoculation of *Hemerocallis* plantlets from *in vitro* culture demonstrated that these isolates had the capacity to cause severe necrosis and death of plantlets. Within 10–14 days of inoculation, plantlets showed chlorosis, necrosis, collapse and death of leaf tissue (Figure 9), although the roots and meristematic region were comparatively intact. Tests on plant material extracts after inoculation with B1, B2 and B4 isolates using the *Botrytis*-specific monoclonal antibody BC-12.CA4 (Meyer and Dewey, 2000; Dewey et al., 2008; Dewey et al., 2013) gave strong positive results with signal intensity values ranging between 34 and 46 whilst the control plant tissue without fungal inoculation gave a signal intensity value of 0. B1 isolates appeared to be the most virulent, rapidly causing complete necrosis and collapse of all inoculated plant material within 14 days (Figure 9). Although microconidia could be found developing on leaf tissue, typical conidiophores producing macroconidia are not produced on the host under these conditions. Plantlets representing a range of different *Hemerocallis* cultivars were tested to determine if there may be easily recognised resistance to infection with isolate B1. All the tested cultivars developed similar symptoms of tissue necrosis, collapse and death within 14 days of inoculation (not shown). Testing the isolates from *Hemerocallis* on the leaves of several other monocots such as *Lilium* did not produce lesions, indicating that the host range may be restricted. However, *B. elliptica* did induce similar lesions on *Lilium* material but not on *Hemerocallis* or any other species tested.
Mating type and sexual crosses

PCR analysis of the mating type locus, *MAT1*, of *Botrytis* revealed that different isolates had different alleles (Table 2). Reciprocal crosses between pairs of strains of opposite mating types, using a method well established for *B. cinerea* (Faretta et al., 1998; van der Vlugt-Bergmans et al., 1993), in all cases failed to result in the development of apothecia. Possibly this was due to the developmental condition of the sclerotia that had developed on plates and were used in the attempted sexual crosses in this study.

**Table 2**: MAT1 alleles in different *Botrytis deweyae* isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>B1</th>
<th>B2</th>
<th>B4</th>
<th>B5</th>
<th>P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAT locus</td>
<td><em>MAT1-1</em></td>
<td><em>MAT1-2</em></td>
<td><em>MAT1-2</em></td>
<td><em>MAT1-1</em></td>
<td><em>MAT1-2</em></td>
</tr>
</tbody>
</table>
Discussion

We report the discovery of a new species of the Ascomycota fungal genus Botrytis from cultivated material of Hemerocallis (commonly known as the daylily) showing a range of symptoms corresponding to those reported for a mysterious disease, ‘spring sickness’. Recently, several new species of Botrytis have been reported, such as B. sinoallii from China which is pathogenic on Allium crops (Zhang et al., 2010a), B. fabiopsis from central China which is pathogenic on broad bean (Zhang et al., 2010b) and B. caroliniana from North America which is pathogenic on blackberry fruits and broad bean leaves (Li et al., 2012). Other as yet undescribed pathogenic Botrytis species are likely to interact with cultivated plants, for example a foliar pathogen of Hosta (also in the order Asparagales) that is closely related to B. tulipae (Laudon, 1978). This new species, B. deweyae, represents the first novel and morphologically distinctive species to be identified in Europe for many years, as compared with recently described B. pseudocinerea that is genetically but not morphologically distinct from B. cinerea (Walker et al., 2011). It is likely that B. deweyae has evaded detection as 1) the critical diagnostic morphological feature of macroconidia do not seem to develop on infected plant material nor are they produced from laboratory cultures except under a specific environmental regime, 2) isolation from infected material has not employed sufficiently stringent surface sterilization to prevent overgrowth from less systemic/pathogenic fungi, 3) the disease symptoms are relatively subtle, have a short temporal manifestation and can easily be attributed to another causative agent, and 4) it is probable that this species has a highly restricted host range.

There have been some reports of investigations of ‘spring sickness’ disease by specialist growers of Hemerocallis (Bergeron, 2001; Bergeron and Somers, 2010), yet the causative agent had not been identified although other fungal species, such as the yeast-like Aureobasidium microstictum (daylily leaf streak) have been implicated (Begeron and Somers, 2010). The rigorous surface-sterilisation procedure we employed in isolations is likely to have been an important factor in finding this new species, as other opportunistic microbial species colonising damaged tissue more superficially would have been removed. In our studies, B. deweyae was repeatedly isolated from diseased material over a 4 year period. Whilst our pathogenicity assays using detached leaf material and plantlets in vitro under laboratory conditions cannot fully replicate mature plants growing in gardens in the colder
months of the year, the rapid development of similar leaf tissue necrosis suggests this species has the capacity to be the major contributor to initiating symptom development. Other microbial species, such as *A. microstictum*, may opportunistically infect as secondary colonisers and aggravate disease development. The rapidity with which plantlet destruction took place was surprising but this is likely to have been enhanced by the absence of any other microbial flora in these cultures. Numerous microbial species have been shown to act antagonistically to *Botrytis* infections of plants (reviewed in Elad and Stewart, 2004). For instance, infection of *Lilium* by *B. elliptica* can be antagonised by specific bacteria (Chiou and Wu, 2001). Our hypothesis that *B. deweyae* is the main agent of ‘spring sickness’ disease development is supported by a report of its closest relative, *B. elliptica*, infecting spring growth of *Hemerocallis fulva* in Korea (Chang et al., 2001). In this case, symptoms were similar with necrosis and death of young leaf tissue, but it was accompanied by significant production of macroconidia. Tests *in vitro* indicated that *B. elliptica* was only capable of infecting *Hemerocallis* tissue after physical damage to the plant (Chang et al., 2001); this suggests that *B. elliptica* lacks the capacity to infect uninjured *Hemerocallis* tissues. There have been no other reports of *B. elliptica* infecting *Hemerocallis*.

The infection of *Hemerocallis* with *B. elliptica* demonstrates that although this species has been most commonly associated with infections of bulbous true lily (*Lilium*, *Liliaceae* *sensu stricta*) - where it is a major disease of cultivated lily bulb crops (Lorbeer et al., 2004) - it is also capable of infecting other distantly related petaloid monocots such as *Hemerocallis* (*Hemerocallidaceae*) and *Tricytris* (*Convallariaceae*) (Chang et al., 2001; Furukawa et al., 2005). There is also evidence that *B. elliptica* can infect dicotyledonous hosts (Tompkin and Hansen, 1950; Harrison and Williamson, 1986), albeit under unnatural conditions. Whether *B. deweyae* is an emergent pathogen that has evolved from a *B. elliptica* population that has undergone a host-shift to *Hemerocallis* is a matter that remains open for further study. The significant morphological and DNA sequence divergences from any described *B. elliptica* strain suggest that this would not have been a recent event. As 2 mating loci (*MAT1*) alleles are present in just 5 tested isolates, recent and rapid sympatric speciation from *B. elliptica* would appear unlikely. To date, only cultivated material from Britain has been examined but it is almost certain that *B. deweyae*, like its closest relative *B. elliptica* (Lorbeer et al., 2004; Huang et al., 2001), is cosmopolitan and will be
A novel Botrytis species is associated with a newly emergent foliar disease in cultivated Hemerocallis detected in cultivated populations of hybrid Hemerocallis elsewhere in the world and perhaps also in native and naturalised Hemerocallis populations in nature.

Given the lack of any report of this species from indigenous Hemerocallis populations, its morphology and the nature of disease development, it is plausible that B. deweyae may be an endophyte that is undergoing the transition to a more aggressive pathogenic state. There was apparent variation in pathogenicity amongst three isolates tested on plantlets, with the isolate B1 being the most pathogenic. Endophytic Botrytis have been identified using sequencing methods from naturalised populations of Centaurea stoebe (Shipunov et al., 2008), clearly demonstrating that outside of their native range plant species can retain or acquire endophytic Botrytis species. Five putative new endophytic species were discovered in this study alone. Intriguingly, there is also evidence that pathogenic B. cinerea (grey mould) can exist as a systemic endophyte without causing pathogenesis in plants (Barnes and Shaw, 2003; Sowley et al., 2010). By some mechanism, B. cinerea must be capable of substantially down-regulating gene expression responsible for aggressive, necrotrophic pathogenesis thereby permitting it to co-exist and grow within plant tissues without damaging them or even triggering defence responses. In the case of B. deweyae the absence of identifiable macroconidia in nature, after growth on the host in vitro and even the absence of sporulation on plates (except under specific conditions) suggests that this development is usually highly suppressed, as might be expected of an endophytic lifestyle (Schulz and Boyle, 2005). In common with other specialised Botrytis species that infect petaloid monocotyledons (Lorbeer et al., 2004), additional studies are required to identify the precise details of the life cycle. Whether, as in B. elliptica (Huang et al., 2001; Staats et al., 2007), sexual reproduction is important in natural populations of B. deweyae requires further investigations.

As this disease has become prevalent only in the past 20 years, one factor that may be precipitating the evolution of pathogenesis is the increasing loss of genetic diversity in the host plant through in-breeding of the cultivated hybrid lines, as demonstrated by an AFLP study (Tomkins et al., 2001). It is known from studies of Colletotrichum that the ability for a fungal isolate to exhibit pathogenic or mutualistic behaviour can depend on the host genotype (Redman et al., 2001). The emergence of in-breeding depression and loss of vigour
amongst *Hemerocallis* hybrids may favour the emergence of strains that are able to effectively take advantage of weakened resistance systems in the plant and hence exploit the host in a more parasitic manner. In hybrid daylily, it is conceivable that the effects of combining divergent genomes followed by multiple generations of in-breeding and selection for novel traits have compromised the effectiveness of mechanisms that regulate pathogen resistance and endophyte activity *in planta*. Such a biotic environment may facilitate the emergence of new diseases, including transitions from endophytes to parasites and host shifts.

Other factors may have assisted the emergence of this disease. The clonal propagation of cultivars by division, which creates large wounds, and the tendency to plant *Hemerocallis* as monocultures or near-monocultures by enthusiasts may assist spread of strains with emerging pathogenic tendencies. A role for bulb mites (for example members of the genus *Rhizoglyphus*) in spreading fungal material such as mycelium fragments to new infection sites appears plausible (Bergeron and Somers, 2007; Gray et al., 1975; Diez et al., 2000). Anecdotal reports suggest that both treatment by both acaricides and fungicides may reduce the incidence of ‘spring sickness’, supporting this view (Bergeron et al., 2001). Young, emerging leaf tissues in late winter and spring may be particularly vulnerable to pathogenesis not only due to injury by fluctuating environments (such as tissue damage by freezing weather) but also due to rapid mobilisation of stored compounds from the tuberous roots to sustain new growth. Both factors may tip the balance in favour of fungal development.

Our discovery suggests that other potentially important pathogenic fungi could be easily overlooked and many are likely to remain unknown to science. The large reservoirs of endophytic fungi residing in plants (Arnold et al., 2000), for example those found in wild grasses (Poaceae) (Schardl et al., 2004), may be a source of important biological diversity for the evolution of new plant pathogens, especially with continued erosion of genetic diversity in cultivated plants and in fragmented natural habitats (Fisher et al., 2012). As threats from emerging pathogens to crop and wild plant resources continue to grow, investment in surveillance and detection systems for new plant diseases should be made a priority. This case in particular demonstrates that diagnostic tools using immunological and DNA
A novel *Botrytis* species is associated with a newly emergent foliar disease in cultivated *Hemerocallis*

sequencing methods, in combination with more conventional morphological and pathology assays, will need to work together in the future if other new plant diseases are to be identified effectively. Widespread ‘cryptic’ diseases of this kind with a small detrimental impact on the host may be important indicators of host and pathogen groups in which more virulent diseases are likely to be emergent.

**Supporting Information**

Supplementary Table S1 and Supplementary Table S2

**Acknowledgments**

The authors wish to thank Prof. Pedro Crous for his assistance with the description of *Botrytis deweyae*, Dr. Martin Bidartondo for providing preliminary ITS sequence data from B1 isolate material and Prof. Hugh Dickinson for preparing SEM samples.
References


Barnes SE, Shaw ME (2003) Infection of commercial hybrid Primula seed by Botrytis cinerea and latent disease spread through the plants. Phytopathology 93: 573–578.


A novel *Botrytis* species is associated with a newly emergent foliar disease in cultivated *Hemerocallis*


## Supporting information

### Supplementary Table S1

Table S1: List of primers employed in PCRs in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3PDH for</td>
<td>ATTGACATCGTCGTGCTCAACGA</td>
</tr>
<tr>
<td>G3PDH rev</td>
<td>ACCCCTCCTTGTCGTACCA</td>
</tr>
<tr>
<td>HSP60 for</td>
<td>CAACAAACGGTTGGTGGGCCACCA</td>
</tr>
<tr>
<td>HSP60 rev</td>
<td>GATGGGATCAGTGTACCCCAGCAT</td>
</tr>
<tr>
<td>ITS for</td>
<td>TCCGTAGGTGAACCTGCGG</td>
</tr>
<tr>
<td>ITS rev</td>
<td>TCCCTCGCTTTATGATAC</td>
</tr>
<tr>
<td>RPB2 for</td>
<td>GATGATCGTGATCTTTTGG</td>
</tr>
<tr>
<td>RPB2 rev</td>
<td>CCCATACCGCTTTACCA</td>
</tr>
<tr>
<td>NEP1(−207)for</td>
<td>CACCTTGTGGGAGATTGGTGGTTGATACAC</td>
</tr>
<tr>
<td>NEP1(+1124)rev</td>
<td>GGTACCTATTGTTGTGGTCATTGC</td>
</tr>
<tr>
<td>NEP1for</td>
<td>CCAACGCAAATACTTTCTCTATCC</td>
</tr>
<tr>
<td>NEP1revB</td>
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</tr>
<tr>
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</tr>
<tr>
<td>MAT 1-1 F</td>
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<tr>
<td>MAT 1-1 R</td>
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<tr>
<td>MAT 1-2 F</td>
<td>GACTAGGAAAATGGGTACCAGACAT</td>
</tr>
<tr>
<td>MAT 1-2 R</td>
<td>GAATGATAGAGATCCTGTGGTGG</td>
</tr>
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</table>

### Supplementary Table S2

Table S2: Production of macroconidia (sporulation) from isolates of *Botrytis deweyae* grown on different media. Colonies were grown at 20°C in darkness except for a UV light source, and examined for sporulation at 6 days and 12 days.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Days of exposure to UV in darkness</th>
<th>B1 isolate</th>
<th>B2 isolate</th>
<th>B4 isolate</th>
<th>B5 isolate</th>
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<tr>
<td>Oatmeal agar</td>
<td>6</td>
<td>No sporulation</td>
<td>No sporulation</td>
<td>Sporulation</td>
<td>No sporulation</td>
</tr>
<tr>
<td>Czapek Dox</td>
<td>6</td>
<td>No sporulation</td>
<td>No sporulation</td>
<td>Sporulation</td>
<td>No sporulation</td>
</tr>
<tr>
<td>V8 juice agar</td>
<td>6</td>
<td>No sporulation</td>
<td>No sporulation</td>
<td>Sporulation</td>
<td>No sporulation</td>
</tr>
<tr>
<td>Oatmeal agar</td>
<td>12</td>
<td>No sporulation</td>
<td>No sporulation</td>
<td>Sporulation</td>
<td>No sporulation</td>
</tr>
<tr>
<td>Czapek Dox</td>
<td>12</td>
<td>No sporulation</td>
<td>No sporulation</td>
<td>Sporulation</td>
<td>No sporulation</td>
</tr>
<tr>
<td>V8 juice agar</td>
<td>12</td>
<td>Sporulation</td>
<td>No sporulation</td>
<td>Sporulation</td>
<td>No sporulation</td>
</tr>
</tbody>
</table>
Chapter 6

General discussion
General discussion

Sex in fungi

Most fungal taxa have both asexual and sexual modes of reproduction. Fungi which lack sexual reproduction, or for which it was never reported, have long been considered to form a separate phylum (Deuteromycota), which became obsolete with the discovery that this phylum was paraphyletic. The loss of the capacity of sexual reproduction has been considered an evolutionary dead-end because of the reduced capacity of asexual organisms to adapt to changing environments. This view is, however, increasingly challenged by the observation of tremendous genetic flexibility and genome dynamics in several asexual fungi including *Fusarium oxysporum* (Rep and Kistler 2010; Ma et al. 2010) and *Verticillium dahliae* (de Jonge et al. 2013; Seidl and Thomma 2014).

Sex in fungi is complex and costly, as it involves many processes that are required to fulfil the different steps at cellular level (plasmogamy, karyogamy and meiosis) as well as the differentiation of the cell types and tissues that make up the fruiting body structure in which the sexual progeny develops (Idnurm 2011; Nelson and Metzenberg 1992). Yet, the advantages conferred by the capacity of sexual reproduction (maintenance of genetic diversity in the population, facilitating adaptation to adverse conditions) makes the costs affordable. Fungi have been attractive model systems for studies on sexual development because of the ease of cultivation, short generation times and large population sizes.

Fungi have evolved two sexual reproduction systems: heterothallism and homothallism. Fungi with a heterothallic sexual reproduction system require two partners of opposite mating type for mating to occur, whereas fungi with a homothallic reproduction system are able to self-fertilize, although they also remain capable of outcrossing (Ni et al. 2011). Whether a species is heterothallic or homothallic is determined by genes in the mating type (*MAT*) locus. In heterothallic fungi, isolates of opposite mating types have distinct, non-homologous alleles of the *MAT* locus, i.e. *MAT1-1* and *MAT1-2*, and the presence of both alleles in a single (dikaryotic) cell is required for sexual development to occur. By contrast, most homothallic fungi have a *MAT* locus in which all genes required for sexual development are present in a gene cluster, and it is the environment that determines whether or not the
fungus will start sexual development. Homothallic sexual reproduction is in fact similar to clonal reproduction which does not result in recombinant progeny. The advantage of such a reproduction system, however, might be the formation of ascospores which serve as survival structures or propagules for dispersal and infection of plants.

**The mating type locus and its functions**

The first description of the organization of the *MAT* locus in the genus *Botrytis* was from *B. cinerea* (Amselem et al. 2011). The two alleles of the *B. cinerea* *MAT* locus are designated as either *MAT1-1* or *MAT1-2*, and each is flanked by the *APN2* gene on the 5’-end and the *SLA2* gene on the 3’-end. The *MAT1-1* locus contains two genes: the *MAT1-1-1* gene encoding a protein with an alpha domain and the *MAT1-1-5* gene. The *MAT1-2* locus also contains two genes: the *MAT1-2-1* gene encoding a protein with a high mobility group domain and the *MAT1-2-4* gene. **Chapter 4** describes the organization and sequence of the *MAT* locus of *B. elliptica*. Both alleles of the *MAT* locus in *B. elliptica* are also flanked by the *APN2* gene and the *SLA2* gene, similar to *B. cinerea*. In addition the *MAT1-1* and *MAT1-2* loci of *B. elliptica* each contain the same two genes, orthologous to their counterparts in *B. cinerea*, in identical organization. *B. cinerea* and *B. elliptica* are distant taxa within the genus, clustering in different clades (Staats et al., 2005). If the organization of the *MAT* locus in these two species is representative for their respective clades, it suggests that the organization of the *MAT* loci is conserved in the genus. Amselem et al. (2011) suggested a scenario for the evolution of the heterothallic *MAT* locus in *B. cinerea* from an ancestral, homothallic locus by two inversion events and a deletion. This scenario might have occurred in the common ancestor of the genus and the locus organization was therefore retained in all *Botrytis* species.

In **Chapter 2**, functional analysis by targeted deletion showed that the genes in the *MAT* locus play different roles in sexual development of *B. cinerea*. The *MAT1-1-1* (alpha domain) and *MAT1-2-1* (HMG domain) genes are the key regulatory genes that control the initiation of sexual development in *B. cinerea*, while the *MAT1-1-5* and *MAT1-2-4* genes are essential to control the development of an apothecial disk. I propose that the orthologous genes in mating type loci of other *Botrytis* species have an identical function.
Chapter 5 describes a new Botrytis species, B. deweyae which appeared to be a sister taxon of B. elliptica. The MAT1-1 and MAT1-2 mating type alleles are present in the different strains of B. deweyae that were available. However, B. deweyae apothecia were never observed even though several trials of sexual crosses were performed. It appears that all B. deweyae strains used in this study were infertile under the tested conditions (Chapter 5), which are routinely conducive for sexual reproduction in both B. cinerea and B. elliptica (Chapters 2, 3 and 4). Infertile strains are not only observed in B. deweyae, but also in B. elliptica (Chapter 4) and in B. cinerea (Van Der Vlugt-Bergmans et al. 1993). Different experimental approaches might be needed to produce apothecia and ascospores in B. deweyae. Firstly, more strains need to be tested; this would require more extensive sampling from Hemerocallis hybrids showing typical symptoms of ‘spring sickness’. In fact, in this study only five strains were available of which one produced sclerotia, which, however, looked distinct from those of other Botrytis species. B. deweyae sclerotia had a lighter color, they did not dehydrate and shrink during long term cold storage, as is usually observed for sclerotia of other Botrytis species when they mature. As a consequence, the B. deweyae sclerotia were fragile and broke easily during manipulation in sexual crosses. It is important to obtain sclerotia that mature in a similar way as those of other species. In addition to testing more strains, different media and temperature regimes need to be tested.

Hydrophobins and the rodlet layer

Hydrophobins are small secreted proteins present in almost all Ascomycota and Basidiomycota. Hydrophobins can be divided into Class I and Class II based on their properties (Wösten and Scholtmeijer 2015; Wösten 2001; Wessels 1994). Hydrophobins play different roles in fungi, either during development or the infection of host plants. Several studies have shown that hydrophobins are present in so-called rodlet layers (Zhang et al. 2011; Wösten 2001; Van Wetter et al. 2000; Talbot et al., 1996; Stringer and Timberlake 1995). In Chapter 3 we demonstrate that B. cinerea hydrophobins are expressed mainly during sclerotium and apothecium development. In Aspergillus nidulans, a mutant lacking the hydrophobin gene rodA did not produce rodlet layers while a mutant lacking the dewA gene was not affected in rodlet layer formation (Stringer and Timberlake, 1994). Rodlet layer formation in B. cinerea has also been investigated in macroscopic structures of wild type and mutant strains in which one or several hydrophobin genes were deleted; a rodlet layer was
never observed, neither in wild type nor in any of the mutant strains. These findings are in agreement with earlier observations by Mosbach et al. (2011) and Doss et al. (1997). **Chapter 3** demonstrated that *B. cinerea* expresses hydrophobin genes to very high transcript levels during sclerotium and apothecium development. The aberrant surface morphology of hydrophobin mutants clearly demonstrated that the *B. cinerea* hydrophobins are important in conferring surface integrity in the wild type, apparently without the need of being deposited in a rodlet layer. We also provided the first evidence in Ascomycota fungi for the existence of a splice variant in a hydrophobin gene transcript. The *Bhp1* gene produced two types of transcripts that differed by the presence of an additional stretch of 13 amino acid residues (including one cysteine residue) resulting from intron retention. Further study is required to investigate the biological functions of both hydrophobin variants in *B. cinerea*.

**Transcriptome analysis of apothecium development**

Fungi in the phylum Ascomycota produce different types of fruiting bodies: cleistothecia, perithecia, apothecia or pseudothecia. Until now, several studies have focused on investigating the mechanisms involved in ascocarp development, mainly in model species, such as *Neurospora crassa* (Nowrousian 2009), *Aspergillus nidulans* (Dyer and O’Gorman 2012) and *Sordaria macrospora* (Teichert et al. 2012). Apothecia produced by *B. cinerea* are better suited for transcriptome analysis in ascocarp development than Ascomycota species forming perithecia, cleistothecia or pseudothecia. This is due to the fact that different developmental stages of apothecia can be sampled more easily without being contaminated by mycelium or other somatic tissue than those of other ascocarps. In addition, the genome of *B. cinerea* is well assembled without gaps (van Kan et al., unpublished) which is not the case for many other fungi.

In earlier studies on gene expression in fungal ascocarp development, EST sequencing and microarray hybridization were used. Both methods have their limitations; the EST analysis generally has a low sequencing depth, whereas microarrays often provide low signals and only provide information about the genes present on the array. The quality of the data in earlier studies was also influenced by contamination of the ascocarp tissue with mycelium or other somatic tissue, especially in the young stages of development. Cleistothecia, perithecia and pseudothecia are small (< 500 μm for the mature ascocarp), and it is difficult to sample
clean ascocarp tissue (Teichert et al. 2012). In many studies such as those by Nelson et al. 1997; Qi et al. 2006; Hallen et al. 2007, ascocarps were sampled by removing or scraping developing structures from a solid agar plate. Teichert et al. (2012) used laser microdissection to excise *Sordaria macrospora* ascocarp tissue with minimal mycelial contamination, and used these samples for RNAseq analysis. They analyzed young perithecia (protoperithecia) that were grown on special membrane slides covered with a thin layer of agar. However, even by using laser microdissection, the ascocarps still contained small quantities of mycelium that grew across the fruiting bodies, as concluded from the observation that the data showed overlap of reads with data obtained from mycelia. In spite of the difficulties in obtaining clean RNAseq data from young perithecia by laser microdissection, the data obtained by RNAseq analysis were much better than those obtained by EST sequencing and microarray hybridization (Teichert et al., 2012).

**Figure 1:** Schematic overview of processes occurring during apothecium development and the role of *MAT* genes. Red arrow indicates down-regulated genes, green arrows indicate up-regulated genes.

**Chapter 2** describes the use of RNA sequencing to study the transcriptome during apothecium development of *B. cinerea*. The data were analysed in different ways. Differential expression analysis was used to examine the changes in gene expression
between two consecutive stages of apothecium development, while cluster analysis was used to identify the genes that were co-expressed at specific stages. The data provide detailed information on the transcriptional changes during apothecium development and on the requirement of MAT genes for this process. Figure 1 illustrates different stages of B. cinerea apothecium development and some of the physiological processes that occur based on transcriptional changes during these transitions. Firstly the initiation of sexual development requires plasmogamy between microconidia of the paternal parent and receptive cells within the sclerotium of the maternal parent, through a fertilization process that is poorly studied. Plasmogamy gives rise to dikaryotic cells that express both the MAT1-1-1 gene (alpha domain) and MAT1-2-1 gene (HMG domain). These MAT proteins likely dimerize to act as a transcriptional regulator that enables initiation of sexual development. Once the primordium of a stipe ruptures from the sclerotium, the hydrophobin gene Bhp1 is very highly expressed and the secreted Bhp1 protein assembles at the outer surface during stipe elongation. The hydrophobin is continuously synthesized enabling to strengthen the elongating stipe until mature apothecia develop. During the early stages of stipe formation, polyketide synthase gene BcPKS15 and the neighboring genes belonging to this cluster are specifically expressed but the bioactive polyketide that they produce is still unknown. In nature, a B. cinerea apothecium will develop in the top layer of soil in early spring. The polyketide produced by the BcPKS15 gene cluster might function as a bioactive compound that protects a fruiting body from colonization by antagonistic bacteria and fungi or from predation by insects. Once stipe elongation is complete, the MAT1-1-5 and MAT1-2-4 genes produce a heterodimeric transcription factor which activates the genes that are important for apothecial disk development. At this stage the karyogamy process is likely to begin. During apothecial disk development, another (zinc finger) transcription factor will be activated, which might be under direct control of the MAT1-1-5/MAT1-2-4 transcription factor complex. Many of the genes that are expressed specifically in apothecial disks have no homologs outside the genus Botrytis or the family of Sclerotiniaceae, hence their function remains elusive. The main biological function of the apothecial disk is to produce asci loaded with ascospores. The data in Chapter 2 present, to our knowledge, the first analysis of the transcriptome of ascospores ever. We observed a remarkable change in gene expression between the apothecial disks and the ascospores, with ~5000 genes being differentially expressed. Among the genes that were specifically expressed in ascospores, were several
Chapter 6

genes encoding potential virulence factors such as pectinolytic enzymes, proteolytic enzymes, monosaccharide transporters and ABC transporters that might participate in the efflux of antimicrobial plant defense compounds. The ascospore transcriptome data clearly suggest that they are primed to infect host plants.

**Dual mating behavior**

In the early 1990s several studies reported that unusual mating behavior may occur in *B. cinerea* (Beever and Parkes 1993; Van Der Vlugt-Bergmans et al. 1993; Faretra et al. 1988). This unusual behavior is referred to as ‘dual mater’, and it describes a strain able to mate both with a partner of opposite mating type and a partner of similar mating type but unable to self-fertilize. Faretra et al. (1988) were the first to describe dual mater behavior in *B. cinerea*. In their study, 213 field strains were crossed with tester strains (SAS56 of the *MAT1-1* type and SAS405 of the *MAT1-2* type). Of the 213 field strains 187 behaved as heterothallics and 34 strains as dual maters (Faretra et al. 1988). van der Vlugt-Bergmans et al. (1993) crossed 8 strains isolated from tomato, rose or gerbera with the same tester strains, of which 5 strains behaved as heterothallics and 3 strains as dual maters. Finally, Beever and Parkes (1993) crossed the same tester strains with 50 field strains and found 41 strains to behave as heterothallics and 9 strains as dual maters. In further studies, Faretra and Pollastro (1996) showed that sexual crosses between two heterothallic strains could yield single ascospore progeny that behaved as dual maters, at a frequency of 6%. No further studies were performed to characterize the progeny from successful crosses from the dual mater strains that were described by Faretra et al., (1988), van der Vlugt-Bergmans et al., (1993) and Beever and Parkes (1993). From a cross between SAS56 (*MAT1-1*, Benomyl-sensitive, Vinclozolin-sensitive) and SAS405 (*MAT1-2*, Benomyl-resistant, Vinclozolin-resistant), performed by van der Vlugt-Bergmans et al. (1993), 81 progeny were sampled from one apothecium. I identified the mating type alleles of the 81 progeny by PCR and observed that they segregated normally (*MAT1-1* = 38, *MAT1-2* = 43). The progeny was tested for presence of fungicide resistance markers (Table 1), which are genetically unlinked (Faretra and Pollastro 1991). The segregation of fungicide resistance markers did not significantly deviate from a normal distribution.
Table 1: Segregation of fungicide resistance in the progeny of cross SAS56 (MAT1-1) x SAS405 (MAT1-2).

<table>
<thead>
<tr>
<th></th>
<th>Vinclozolin-sensitive</th>
<th>Vinclozolin-resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benomyl-sensitive</td>
<td>27 progeny (11 MAT1-1, 16 MAT1-2)</td>
<td>13 progeny (6 MAT1-1, 7 MAT1-2)</td>
</tr>
<tr>
<td>Benomyl-resistant</td>
<td>11 progeny (7 MAT1-1, 4 MAT1-2)</td>
<td>30 progeny (13 MAT1-1, 17 MAT1-2)</td>
</tr>
</tbody>
</table>

SAS56: Benomyl-sensitive and Vinclozolin-sensitive
SAS405: Benomyl-resistant and Vinclozolin-resistant

To identify dual mater strains in this progeny, each isolate of the 81 progeny was backcrossed to their parents SAS56 (MAT1-1) and SAS405 (MAT1-2), and self-fertilized. One single ascospore progeny isolate, named RS26 (carrying a MAT1-2 allele), produced apothecia when sclerotia derived from RS26 were incubated in water under apothecium development-promoting conditions and was thus considered homothallic. A sibling from the same cross between SAS56 and SAS405, the isolate named RS11 (carrying a MAT1-2 allele), was able to mate with both its parents, SAS56 and SAS405, but was not homothallic and was thus considered to show dual mater behavior. The sequences of MAT1-2 alleles of RS26 and RS11 were identical to that of SAS405 (not shown).

I used a genetic approach to study the aberrant sexual behavior in isolates RS26 (homothallism with a MAT1-2 allele) and RS11 (dual mating with a MAT1-2 allele), assuming that these traits are controlled by a single Mendelian locus, and considering the following possible scenarios:

1. If homothallic strains produce an apothecium, ascospores are formed but there is clonal propagation because meiosis occurs between two identical genotypes. Progeny obtained from an apothecium produced by a homothallic strain should be identical to its parent.

2. If dual mating is genetically controlled by the MAT locus itself or by a different, closely linked locus, dual mating behavior should occur in half the progeny from a cross of a dual mater with any other strain. Furthermore, the dual mating behavior should co-segregate with the MAT allele derived from the dual mater parent.

3. Crossing RS11 with a different strain carrying a MAT1-2 allele should result in progeny carrying exclusively MAT1-2 alleles, of which half would behave as dual maters and the other half as normal heterothallic strains, only able to mate with MAT1-1 strains).
4. If dual mating is genetically determined by a locus unlinked to the *MAT* locus, it should be possible to transmit the dual mating behavior from the genetic background of a *MAT1-2* strain into a *MAT1-1* strain, for example by back-crossing RS11 (*MAT1-2*) to its parent SAS56 (*MAT1-1*), and testing the progeny with a *MAT1-1* allele for dual mating behavior.

Figure 2 illustrates the experimental design that was used to study the dual mating behavior in *B. cinerea* and it summarizes the results obtained. Details of the experiments and the results are provided in the following paragraphs.

**Figure 2**: Schematic approach followed to study the genetics of dual mating behavior in *B. cinerea*. Details are explained in the text.
Self-fertilization of homothallic strain RS26 (carrying the MAT1-2 allele) resulted in the production of apothecia, from which 15 single ascospore progeny were sampled and analysed for the segregation of fungicide resistance markers and MAT alleles (Table 2). All 15 progeny were resistant to both benomyl and vinclozolin, like their parent RS26, suggesting that they could have been derived from homothallic sexual reproduction. Remarkably, however, it appeared that eight of the 15 progeny contained a MAT1-1 allele, while only seven carried a MAT1-2 allele alike their parent RS26. Six out of the 15 progeny were also tested for the segregation of alleles of microsatellite markers developed for B. cinerea by Fournier et al. (2002). All progeny tested carried the parental RS26 alleles of markers BC1 (Figure 3), BC4 and BC10 (not shown), as was expected. For marker BC2, however, only one of the six progeny analyzed carried the parental allele, while the remaining 5 progeny carried an alternative allele (Figure 3).

Table 2: Segregation of MAT alleles and fungicide resistance in progeny of self-fertilized strain RS26 (MAT1-2).

<table>
<thead>
<tr>
<th>Mating types</th>
<th>Benomyl (10ug/ml)</th>
<th>Vinclozolin (5ug/ml)</th>
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<tr>
<td>RS26 (Parent)</td>
<td>MAT1-2</td>
<td>+</td>
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<tr>
<td>Progeny from selfing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32-1</td>
<td>MAT1-2</td>
<td>+</td>
</tr>
<tr>
<td>32-2</td>
<td>MAT1-2</td>
<td>+</td>
</tr>
<tr>
<td>32-3</td>
<td>MAT1-1</td>
<td>+</td>
</tr>
<tr>
<td>32-4</td>
<td>MAT1-2</td>
<td>+</td>
</tr>
<tr>
<td>32-5</td>
<td>MAT1-2</td>
<td>+</td>
</tr>
<tr>
<td>32-6</td>
<td>MAT1-1</td>
<td>+</td>
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<tr>
<td>32-7</td>
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<tr>
<td>32-8</td>
<td>MAT1-1</td>
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</tr>
<tr>
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<td>32-10</td>
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<tr>
<td>32-15</td>
<td>MAT1-1</td>
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Figure 3: Segregation of microsatellite markers BC1 and BC2 (Fournier et al., 2002) in six progeny from homothallic strain RS26. Markers used are indicated at the top. Strains analyzed are the parental isolates B05.10, SAS405, SAS56, the homothallic strain RS26 and the progeny from self-fertilized RS26 (32-1 until 32-7), as indicated at the bottom of the lanes. All progeny tested have the same allele for microsatellite marker BC1 as their parent RS26. For marker BC2, progeny isolate 32-2 has the same allele as its parent RS26 and isolates B05.10 and SAS56 (indicated by red arrows), while the other five progeny have the same allele as isolate SAS405 (indicated by green arrows).

Based on the analysis of microsatellite markers (Figure 3), progeny produced from self-fertilization of RS26 probably resulted from contamination. Therefore, RS26 was excluded for further study.

The dual mater strain RS11 (carrying a MAT1-2 allele) is able to mate with both its parents SAS56 and SAS405 but is not homothallic. RS11 was sensitive to benomyl and resistant to vinclozolin, whereas its parents were either sensitive (SAS56) or resistant (SAS405) to both fungicides, thus validating that RS11 is a sexual recombinant of these two strains. However the alleles for six microsatellite markers (Fournier et al. 2002) in RS11 were all identical to the alleles of its parent SAS405, while RS11 contained none of the alleles derived from SAS56 (not shown).
Two separate apothecia were sampled from a cross between RS11 (MAT1-2) and SAS405 (MAT1-2) and a total of 50 (from apothecium 1a) and 39 (from apothecium 2a) progeny were sampled from these apothecia, respectively. All 50 progeny from apothecium 1a (Figure 2) possessed a MAT1-2 allele. To analyse whether the progeny were sexual recombinants of these parents, the segregation of fungicide resistance markers in the progeny was analyzed (Table 3). The progeny were all sensitive to benomyl, even though parental strain SAS405 was resistant to benomyl. Also, half of the progeny was sensitive to vinclozolin although both parents were resistant to this fungicide. The results of fungicide resistance segregations were in disagreement with the phenotypes of the parents, and this observation could not be explained. When sclerotia from 30 of the 50 progeny from apothecium 1a were incubated in water under apothecium-inducing conditions, four progeny showed the emergence of apothecial stipes which, however, failed to develop into disks. When the same 30 progeny were tested in backcrosses to SAS56 and SAS405, all behaved as regular heterothallic strains.

In apothecium 2a from the same cross, it was observed that 9 out of 39 progeny carried a MAT1-1 allele whereas both parents in this cross carried a MAT1-2 allele (Figure 2). The presence of the MAT1-1 allele in this progeny cannot be explained.

**Table 3**: Segregation of fungicide resistance in the progeny of cross RS11 (MAT1-2) x SAS405 (MAT1-2) sampled from apothecium 1a.

<table>
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<th>Vinclozolin-sensitive</th>
<th>Vinclozolin-resistant</th>
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<td>Benomyl-sensitive</td>
<td>26 progeny (all MAT1-2)</td>
<td>24 progeny (all MAT1-2)</td>
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<td>Benomyl-resistant</td>
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RS11: Benomyl-sensitive and Vinclozolin-resistant  
SAS405: Benomyl-resistant and Vinclozolin-resistant

Dual mater strain RS11 was also backcrossed to SAS56, in order to examine whether the dual mating behavior could be transmitted to a genotype with a MAT1-1 allele (Figure 2). From apothecium 1b, I sampled 50 progeny and observed that 19 contained a MAT1-1 allele and 31 a MAT1-2 allele. To test for dual mating behavior of this progeny, all isolates with the MAT1-1 allele were backcrossed to SAS56 and 20 isolates with the MAT1-2 allele were
backcrossed to SAS405 (Figure 2). For both sets of backcrosses, two produced apothecia, suggesting that ~10% of the progeny in apothecium 1b behaved as dual maters.

Altogether, the results of the genetic analysis of dual mating behavior were puzzling. In some cases, results clearly indicated contamination, for example if a cross between two strains with identical \( MAT \) alleles yielded progeny with the opposite \( MAT \) allele. After the first indication of contamination, I took technical measures to reduce the chance of contamination in cultures and crosses. Even after taking such measures, some of the results remained difficult to interpret. It was impossible to reconstruct at which stage of the process the contamination might have occurred, over the five months period that is required for a cross to be completed. I decided not to include these data in an experimental chapter in the thesis but rather include them in the general discussion section.

**Endophytic fungi with necrotrophic capability**

Fungi are often classified into necrotrophs, hemi-biotrophs, biotrophs or endophytes based on their interaction with their host plant. Necrotrophs are pathogens obtaining nutrients by triggering programmed cell death in their host (Mengiste 2012). Biotrophs are pathogens that cause minimal damage to their host plant and disease symptoms usually occur as a result of nutrient depletion (Spoel et al. 2007; Glazebrook 2005; Hancock and Huisman 1981). Endophytic fungi colonize plant tissues without causing visible symptoms of disease or stress (Delaye et al. 2013; Rodriguez et al. 2009; Purahong and Hyde 2011), and endophytes are often considered to protect plants from drought, or attack by pathogens and pests, by their ability to produce antimicrobial secondary metabolites.

The 29 species described in the genus *Botrytis* are all considered to be necrotrophic pathogens, except for *B. deweyae*, which grows as an endophyte in *Hemerocallis* species and only under particular conditions can cause disease (Chapter 5). Fungi with such behavior are also referred to as “latent pathogens” (Hyde and Soytong 2008; Álvarez-Loayza et al. 2011; Eaton et al. 2011; Delaye et al. 2013). Other examples of endophytes known to be latent pathogens are *Phomopsis citri*, *Fusicoccum aesculi*, *Ladiodiplodia theobromae* (Wright 1998) and *Phomopsis viticola* (Mostert et al. 2000). There is no information on the mechanisms that control the switch in behavior from endophyte to pathogen. Sieber (2007) proposed
that endophytes have co-evolved with their hosts and can thus not be highly virulent, and symptoms are observed only very rarely and to a limited extent. The occasional pathogenic behavior of endophytes may be influenced by abiotic or biotic factors (Sieber 2007). Our observation that *B. deweyae* can grow as an endophyte inside *Hemerocallis* but may also cause very severe necrotic lesions on young leaves, leading to total destruction of the plant, argues against the hypothesis by Sieber (2007) that endophytes cannot be highly virulent. Further arguments against this hypothesis are provided by several reports that even *B. cinerea* (Barnes and Shaw, 2003; Sowley et al., 2010) is able to grow as an endophyte in host plants without causing disease symptoms and yet such isolates can be highly aggressive in infection experiments (Sowley et al., 2010; van Kan et al., unpublished observations). The capacity of *Botrytis* species to act as devastating necrotrophic pathogens, but also display an endophytic lifestyle suggests that the classification of fungi into pathogens or endophytes might need reconsideration. For a better understanding of the lifestyle of *B. deweyae*, analysis of its genome and its gene expression profile during endophytic and necrotrophic growth on *Hemerocallis* is needed. Studies on the endophytic fungus *Pestalotiopsis fici* using genomic and transcriptomic approaches revealed that the genome of *P. fici* contains up to 74 gene clusters related to secondary metabolite production, including 27 polyketide synthase genes (Wang et al. 2015). This number of secondary metabolite gene clusters is much higher as compared to *B. cinerea* and *Sclerotinia sclerotiorum* with a necrotrophic lifestyle (each ~40 secondary metabolite gene clusters) and to *Neurospora crassa* with a saprotrophic lifestyle (31 gene clusters). Many studies have revealed that endophytic fungi can be interesting resources for production of novel bioactive compounds (Li et al. 1996; Zou et al. 2000; Silva et al. 2005; Phongpaichit et al. 2007; Lösgen et al. 2007; Gu et al. 2007; Bérdy 2005). It will be interesting to study whether also *B. deweyae* contains more polyketide synthase genes than the 20 PKS genes present in *B. cinerea* (Kroken et al. 2003).

**Perspectives**

Fruiting bodies of fungi are attractive and useful for human beings, not only for their beauty or their nutritional value and culinary quality, they are also a source of natural products with beneficial properties. Metabolites from fungal fruiting bodies, or their chemical derivatives, are exploited as medicine or food additives. Many of these compounds are called “secondary metabolites”. Genome sequences of fungi have revealed the enormous potential
of fungi to produce secondary metabolites, of which only some have been identified and chemically characterized. *Botrytis cinerea* is no exception to this rule: of the 43 putative secondary metabolite gene clusters, the products are identified for less than 10 of them. The polyketide produced in *B. cinerea* primordial stipes by the secondary metabolite gene cluster, including the *BcPKS15* gene is just one of the examples of unknown and unexplored metabolites with potentially interesting properties. Other examples of secondary metabolite gene clusters that are exclusively expressed in fungal fruiting bodies were reported in *Sordaria macrospora* (Nowrousian 2009; Schindler and Nowrousian 2014), and *Coprinopsis cinerea* (Plaza et al. 2014).

A different perspective of the insights into *B. cinerea* fruiting body development might be in disease control. *B. cinerea* apothecia have rarely been detected in the field, yet studies on the population genetics of *B. cinerea* (Giraud et al. 1997) have revealed that sexual reproduction occurs much more frequently than previously assumed. However, sexual reproduction not only maintains or increases genetic diversity, the sclerotia also act as overwintering structures and in early spring, ascospores act as propagules for dispersal and infection on newly emerging plant tissues. By understanding the mechanisms that control sexual development in *B. cinerea*, it might be feasible to identify a step in apothecium development as a target for control this ubiquitous, devastating pathogen.
References


Barnes SE, Shaw ME (2003) Infection of commercial hybrid *Primula* seed by *Botrytis cinerea* and latent disease spread through the plants. Phytopathology 93: 573–578.


General discussion


Chapter 6


Zhang S, Xia XY, Kim B, Keyhani NO (2011) Two hydrophobins are involved in fungal spore coat rodlet layer assembly and each play distinct roles in surface interactions, development and pathogenesis in the entomopathogenic fungus, Beauveria bassiana. Molecular Microbiology 80(3):811-26.

Summary
The genus *Botrytis* comprises 29 species, predominantly necrotrophic pathogens that infect a single host plant species with two exceptions: *Botrytis cinerea*, which can infect more than 200 host plants, and *Botrytis pseudocinerea* which causes disease on several unrelated host plants.

Chapter 1 introduces the genus *Botrytis*, its molecular phylogeny and its asexual and sexual stages, followed by a description of the organization and function of mating type loci in *Ascomycota* and an outline of the thesis.

Chapter 2 describes a genome-wide expression analysis of apothecium development in *B. cinerea* and the function of mating type genes in this process. First, the transcript levels of *B. cinerea* orthologs of *Aspergillus nidulans* and *Sordaria macrospora* genes known to be involved in sexual development were examined. Secondly, differential expression analysis was performed to compare transcriptional changes occurring at distinct stages of apothecium development. Thirdly, cluster analysis identified genes that were co-expressed in specific stages, particularly in sclerotia, three developmental stages of apothecia and mature ascospores. Functional analyses by targeted knockout mutagenesis revealed that the MAT1-1-1 gene and the MAT1-2-1 gene are both required for the initiation of sexual development. Deletion mutants in either of these genes failed to produce any sexual structures. Deletion of the MAT1-1-5 gene or the MAT1-2-4 gene resulted in normal development of stipes which, however, were defective in the formation of an apothecial disk, asci and ascospores. In addition, comparative gene expression profiling was performed for a MAT1-1-5 deficient mutant blocked at the transition from stipes to apothecial disk formation and wild type apothecial disks.

Chapter 3 describes possible roles of hydrophobin genes in development of *B. cinerea*, especially in sclerotia and apothecia. The *Bhp1*, *Bhp2* and *Bhp3* genes are required for both sclerotium and apothecium development. Deletion of *Bhp1* and *Bhp3* affected the surface hydrophobicity of sclerotia, while deletion of *Bhp1* and *Bhp2* affected apothecium development. The DNA proportion and RNA expression of hydrophobin genes in apothecia was quantified to determine the proportion of maternal and paternal contribution to
apothecium tissue. Nearly 99% of the apothecial tissue appeared to be derived from the maternal parent.

Chapter 4 describes the mating type gene organization in *B. elliptica*, which is highly similar to that in *B. cinerea*. The *B. elliptica* MAT1-1 locus contains the *MAT1-1-1* gene and the *MAT1-1-5* gene, whereas the MAT1-2 locus contains the *MAT1-2-1* gene and the *MAT1-2-4* gene. Apothecia were developed from a cross that was set up between a *MAT1-1* and a *MAT1-2* strain, which proved that *B. elliptica* is a heterothallic species. Mature apothecia developed approximately three months after fertilization. Ascospores were infectious on lily leaves and able to induce necrotic symptoms two days after inoculation.

Chapter 5 describes *Botrytis deweyae*, a novel species that can grow as an endophyte in cultivated *Hemerocallis* hybrids, but in certain conditions can cause disease referred to as ‘spring sickness’. Between 2009 and 2012, *B. deweyae* isolates were collected from several *Hemerocallis* hybrids in the greenhouse. The morphology of *B. deweyae* is described. Molecular phylogenetic analyses based on three housekeeping genes and two virulence genes revealed that *B. deweyae* is closely related to the lily pathogen *B. elliptica*. Infection experiments confirmed that *B. deweyae* is able to cause necrosis on *Hemerocallis* leaves.

Chapter 6 discusses the results presented in this thesis. A model of processes and mechanisms involved in apothecium development is proposed. A detailed discussion of the phenomenon known as dual mating behavior in *B. cinerea* is also provided.
Acknowledgements

10 September 2011 marked my life as I began my journey to the Netherlands to pursue my dream in obtaining a PhD degree. After four years of studying, researching and working, I finally succeeded in completing my PhD. During these years, there were many people that contributed to my success in completing this journey, hence I would like to convey my gratitude to them here.

To my parents, who brought me to this world and nurtured me, Mak and Opak, this success is for you. Your unconditional love for me kept me on track and inspired me. For my late father who is no longer here to share this moment, I know your prayers are always with me.

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Curriculum Vitae

Razak Bin Terhem was born on the 25th of May 1985 in Sarawak Malaysia. He started his Bsc study in Forestry in the Universiti Putra Malaysia in 2006. His Bsc thesis was entitled Sexual development of Dinoderus minutus. In 2009, he continued with his MSc study in Plant Protection at the University of Queensland Australia. For his MSc thesis, he did research on Sexual development of Cochliobolus species and fungal flora on palm trees under the supervision of Dr Roger G Shivas and Dr Elizabeth Aitken. In September 2011, he started his PhD project entitled Sexual development of Botrytis species in the Laboratory of Pytopathology at Wageningen University, under the supervision of Dr. Jan van Kan and Prof. Pierre de Wit. Starting 1st of October 2015 he is a Lecturer at the Faculty of Forestry, Universiti Putra Malaysia.

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List of publications


Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Razak Bin Terhem  
Date: 14 September 2015  
Group: Laboratory of Phytopathology  
University: Wageningen University & Research Centre

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### Seminar plus

**International symposia and congresses**

- Botrytis-Sclerotinia Post-genome Workshop, Lyon, France  
  **Sep 15-17, 2011**
- XVI International Botrytis Symposium, Bari Italy  
  **Jun 23-28, 2013**
- XI International Fungal Biology Conference Karlshruhe Germany  
  **Sep 29-Oct 03, 2013**
- 12th European Conference on Fungal Genetics, Seville Spain  
  **Mar 23-27, 2014**
- EPS PhD Retreat, University of Amsterdam  
  **Jul 01-04, 2014**
- The 10th International Mycological Congress, Bangkok, Thailand  
  **Aug 03-08, 2014**

### Presentations

- NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, The Netherlands (poster)  
  **Apr 22-23, 2013**
- XVI International Botrytis Symposium, Bari Italy (talk and poster)  
  **Jun 23-28, 2013**
- XI International Fungal Biology Conference Karlshruhe Germany (poster)  
  **Sep 29-Oct 03, 2013**
- 12th European Conference on Fungal Genetics, Seville Spain (poster)  
  **Mar 23-27, 2014**
- The 10th International Mycological Congress, Bangkok, Thailand (talk)  
  **Aug 03-08, 2014**
- ALW Platform Molecular Genetics Annual Meeting, Lunteren, The Netherlands (talk)  
  **Oct 09-10, 2014**

### IAB interview

- Meeting with a member of the International Advisory Board of EPS  
  **Sep 29, 2014**

### Excursions

**Subtotal Scientific Exposure**  
19.5 credits*

### 3) In-Depth Studies

**EPS courses or other PhD courses**

- Host-Microbe Interactomics, Wageningen University, The Netherlands  
  **Nov 01-02, 2011**
- Phyto Path Trainning, European Bioinformatic Hixton Cambridge UK  
  **Sep 19-20, 2012**
- The power of RNA Seq, Wageningen University Netherlands  
  **Jun 05-07, 2013**
- Bioinformatic- User's Approach, Wageningen University Netherlands  
  **Aug 26-30, 2103**

### Journal club

**Subtotal In-Depth Studies**  
3.9 credits*

### 4) Personal development

**Skill training courses**

- Interpersonal Communication for PhD Students (ICPS), Wageningen University  
  **Dec 12-13, 2011**
- Data Management, Wageningen University  
  **Feb 24, 2014**
- Voice Matters - Voice and Presentation, Wageningen University  
  **Apr 01 & 15, 2014**
- Techniques for Writing and Presenting a Scientific Paper (TWP), Wageningen University  
  **Apr 07,08,10 and 11, 2014**
- Information Literacy including EndNote Introduction (ILP), Wageningen University  
  **Feb 17-18, 2015**

**Organisation of PhD students day, course or conference**

**Membership of Board, Committee or PhD council**

**Subtotal Personal Development**  
3.2 credits*

| TOTAL NUMBER OF CREDIT POINTS* | 34.1 |

* A credit represents a normative study load of 28 hours of study.
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