USE OF INTERNAL TRANSCRIBED SPACER PRIMERS AND FUNGICIDE TREATMENTS TO
STUDY THE ANther-SMUT DISEASE, MICROBOTRYUM VIOLACEUM (=USTILAGO
VIOLACEA), OF WHITE CAMPION SILene ALBA (=SILene LATIFOLIA)

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We report the construction and use of polymerase chain reaction (PCR) primers for detection of Micro-
botryum violaceum in Silene alba. Such primers show that the pathogen is present in the aboveground bud
meristems, flower pedicels, and flower-stem internodes but not in the flowering-stem leaves and roots of
flowering plants. Use of primers also showed that the pathogen was absent from plants that had been inoculated
with the fungus but remained healthy. The fungicides Folicur and Bayleton cured plants of the disease, and
the absence of the fungus from such cured plants was confirmed using the PCR primers.

Keywords: anther smut, Bayleton, Folicur, ITS primers, Silene alba, Ustilago violacea.

Introduction

Plant pathogens are considered to be important in deter-
mining the abundance and distribution of their hosts (Burdon
1987; Antonovics 1994); but compared with the study of them
in crop plants, the study of pathogens in natural plant pop-
ulations has been relatively neglected. However, the anther-
smut fungus Microbotryum violaceum Deml & Oberwinkler
(=Ustilago violacea (Pers.) Fuckel) and its dioecious host Silene
alba (Miller) Krause (=Silene latifolia Poiret) have been studied
extensively as a model system for understanding host-pathogen
interactions in natural populations (Alexander and Antonovics
1988; Elmqvist et al. 1993; McCauley et al. 1995; Alexander
et al. 1996; Antonovics et al. 1996; Bierie and Honders 1996;
Shykoff and Kalitz 1997). This host is also known to have
substantial genetic variation in terms of its resistance to M.
violaceum (Alexander 1989; Thrall et al. 1995; Bierie and An-
onovics 1996), but the genetics of this resistance are not well
understood.

The study of host-pathogen interactions in this species is
hindered by the fact that infection by the pathogen is not
macroscopically apparent until the plant flowers (Alexander
1989) and by the fact that diseased individuals are sterilized.
Diseased plants can only be recognized when they produce
dark-smudged anthers. When inoculations are carried out in
the seedling stage or on vegetative rosettes, disease detection
requires a long waiting period (from several weeks to several
months) until the plant flowers. Sometimes individuals only
flower in the second season, or they require additional long-
day treatment in the greenhouse in order that flowering may
be induced. Even when inoculations are carried out at the
flowering stage, it may be several weeks or months before the
disease is expressed because the fungus has to penetrate and
grow into newly developing floral primordia in order to pro-
duce disease symptoms. Thus, Alexander et al. (1993), using
teliospore inoculations of flowers and flower buds, found that
the latent period varied from 13 to 166 d, with an overall
average (n = 108) of 82 d.

Using light microscopy of thin tissue sections, Audran and
Batcho (1980) showed that the mycelium was intercellular and
abundant in the terminal and lateral meristems but was “nearly
nonexistent” in differentiated leaves and was absent from
phloem and xylem. When both teliospore and sporidial in-
oculum was placed on the leaf surface and incubated under
cool, moist conditions, hyphae of M. violaceum could be readily
visualized on the plant surface (Hood and Shew 1997). However,
they have been notoriously difficult to detect within the
plant. We have tried the staining protocols used by Sinha
et al. (1982) to detect smut in sugarcane, but these protocols
did not produce any evidence of M. violaceum in diseased S.
alba (M. Hood, personal communication).

Nilsson et al. (1994) used near-infrared reflectance spec-
trometry of rosette leaves and principal component analysis of
the spectral data to distinguish plants of S. dioica that were
either infected or not infected with M. violaceum. Although
the technique distinguished healthy and infected plants with
substantial accuracy (>96%), it has the disadvantage that the
instrumentation is expensive, and its broader applicability to
other species (such as S. alba) is unknown.

Here we report a reliable polymerase chain reaction (PCR)
technique for detecting the pathogen within the host. We use
PCR primers for the internal transcribed spacer (ITS) regions
of ribosomal DNA of M. violaceum to amplify DNA sequences
of the pathogen and, thus, to recognize its presence within host
plants.

Another technical difficulty associated with studies of the
signatures of host resistance is that diseased S. alba plants are
usually sterile. Infection by the pathogen leads to the pro-
duction of diseased flowers in both sexes. The diseased male flow-
ers produce anthers that are filled with teliospores but no pol-

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DNA Extraction

DNA from different host tissues of diseased and healthy plants was extracted following the protocol of Dellaporta et al. (1983). Fifty milligrams of host tissues was used per extraction. The following tissues were investigated: bud meristems, flower pedicels, flowering-stem leaves, flower-stem internodes, and roots of flowering plants. Bud meristems (destined to produce flowering branches) were dissected from isolated small buds (<5 mm) in the leaf axils of flowering stems by removing the surrounding developing leaves.

For the pathogen, we carried out PCR amplification using our standard stock lines (no. 28 and no. 29). However, for the PCR reactions, DNA was not extracted directly. Instead, cells from a single sporidial colony of both mating types from the PDA plate were directly transferred to the PCR reaction mix before amplification. For transferring fungal cells to the PCR reaction mix, the tip of an inoculating needle was touched to the surface of a single colony and was then swirled in the reaction mixture. If too many cells are transferred, the PCR reaction fails because the primer : template ratio is too low. In all past studies and in this study, there was no evidence that ITS sequences differed between the mating types.

PCR Primers

The basidiomycete-specific ITS primers ITS 1-F (5'-CTTGGTCATTAGAAAGTAA-3') and ITS 4-B (5'-CAGGAGACTTTGACACGTTCCG-3') were used for initial amplification (Gardes and Bruns 1993). The primers were synthesized by Genosys, and they amplified a product of 838 base pairs (bp). This product includes the 3’ end of 18S rDNA, the ITS 1 region between the 18S rDNA and 5.8S rDNA, the 5.8S rDNA, the ITS region 2, and a partial sequence from the 28S rDNA (fig. 1).

PCR Amplification and Cloning

Amplifications were performed in 50-μL reaction volumes in a Perkin-Elmer DNA thermal cycler 480. The reaction mixture contained 1X PCR buffer (Boehringer Mannheim, Indianapolis), 200 μM of each deoxyribonucleotide triphosphate, 2 μM of each of the primers, 4 mM MgCl₂, and 2.5 units of Taq DNA polymerase (5 U/μL; Boehringer Mannheim). Fifty microliters of mineral oil was placed on top of each reaction mixture before PCR amplification. The reaction was performed for 35 cycles with an initial denaturation period of 3 min (at 94°C) and a final extension period of 10 min (at 72°C).

Fig. 1 Schematic diagram of the rDNA of Ustilago violacea. Open boxes = ribosomal subunits, arrows = position of PCR primers (ITS 1-F on the 18S, ITS 4-B on the 28S, ITS 1-UV on the ITS 1, and ITS 2-UV on the ITS 2 regions). Note the EcoRI sites within the 5.8S subunit and ITS 1 region.
The first 13 cycles consisted of 60 s at 94°C followed by 30 s at 62°C for annealing and 60 s at 72°C for extension. In the subsequent 13 cycles, the extension time was increased from 60 to 90 s, and in the last nine cycles, the extension period was for 2 min.

The DNA from the PCR product was cloned in a pCR 2.1 vector and transformed into competent *E. coli*, following the manufacturer’s protocol (Invitrogen, San Diego, Calif.). Ten white colonies were randomly selected from the Luria-Bertani (LB) agar plates and cultured overnight in an LB medium with ampicillin. The Miniprep Kit (Qiagen) was used to extract plasmid DNA. The insert was cut from the plasmid using the unique restriction sites Spel and XbaI of the polylinker. In order to confirm that the cloned insert contained the correct ITS sequence, the cut insert was digested with the restriction enzyme *EcoR*I. Restriction digests consisted of 10 units of enzyme (2 μL), 10 μL of miniprep DNA from pCR 2.1, 1X (3 μL) restriction buffer (Boehringer Mannheim), and 15 μL of DI water. The digestion mixture was incubated at 37°C for 3 h. The digested DNA products were electrophoresed on a 2% agarose gel at 80 mA for 1 h. In order to confirm insert size, the original amplified PCR product was run side by side on the same gel. Gels were stained in ethidium bromide to visualize the DNA. The insert in the purified plasmid DNA was sequenced at the Iowa State University’s DNA Sequencing Facilities. The complete sequence of the PCR product (838 bp) that resulted from the ITS 1-F and ITS 4-B primers has been submitted to the GenBank at the National Center for Biotechnological Information (accession no. AF082990).

In order to confirm that the PCR protocols had been successfully applied, even when amplification of fungal DNA was not evident (e.g., in healthy, uninoculated plants or in specific tissues of diseased plants), PCR was carried out using the plant-specific primers NS3 and NS4 (Gardes and Bruns 1993). In all cases, the protocols successfully amplified plant DNA and the lack of amplification of fungal DNA was interpreted as evidence that the pathogen was absent.

**Designing Primers with Enhanced Specificity for *M. violaceum***

The ITS 1-F and ITS 4-B primers were intended to be specific for the higher fungi and basidiomycetes (Gardes and Bruns 1993) but also produced some faint bands in healthy plants (data not shown). Gardes and Bruns (1993) also noted that in several instances these primer pairs did amplify bands from a variety of plants.

In order to enhance the sensitivity and the reliability of PCR-based methods to detect *M. violaceum*, we therefore designed a sense and antisense primer pair with enhanced specificity for the pathogen, as follows. We obtained DNA sequences from (GenBank) of the ITS regions of isolates of *M. violaceum* from different host species. The hosts, host-strain, and accession numbers were as follows: Silene dioica: strain SD4, U41458; strain SD1, U41457; strain SD2, U41459; strain SD3, U41460; Silene virginica: strain SV1, U41462; Silene otites: strain SO1, U41464; and Dianthus carthusianorum: strain DC1, U41463. All the sequences were aligned with GCG software (version 9.1, Genetics Computer Group, Madison, Wisc.) using the command “pileup.” Regions of dissimilarity between the sequence from *S. alba* and the sequences from these other isolates were selected to design a 23-bp sense primer, which we termed ITS 1-UV. The primer sequences was as follows: 5'-CAA-ACTCCTGTGCACCTCTAATGGG-3'. In order to confirm further that the ITS 1-UV primer would selectively amplify DNA from *M. violaceum* isolated from *S. alba*, an extensive search was performed with this primer sequence in the pertinent databases (nr, est, gss, hgs) of GenBank using BLAST search engine. The sequence did not show any homology with any available fungal sequences in the databases (except with *M. violaceum* from *S. alba*). The primer sequence showed some similarities within 13–17 bp of several sequences from some unrelated organisms, like polyoma virus, yeast, nematode, chicken, mouse, and human. However, Roux et al. (1998), in a study of phylogeny of Ustilaginaceae and Microbotryaceae, recently submitted several new ITS sequences of *Microbotryum* to GenBank. Interestingly, ITS 1-UV shows similarity with ITS sequences of *Microbotryum silenes-inflata* isolated from *S. vulgaris* and with ITS sequences of *M. violaceum* isolated from *S. ciliata*.

We used a similar method to design the antisense primer, ITS 2-UV. Its sequence was as follows: 5'-TTCCCAAGGCGC-GCCATTACACC-3'. The BLAST search with this primer sequence showed similarities only with *M. violaceum* from *S. alba* and from several other *Silene* species (S. dioica, S. virginica, S. otites, and S. vulgaris). However, considering the sequences of the ITS 1-UV and ITS 2-UV primers together, only *M. violaceum* from *S. alba* and *S. vulgaris* would be expected to produce any PCR product because other species did not share sequences at both primers.

This primer pair is expected to produce a 485-bp PCR product from *M. violaceum* isolated from *S. alba*. In order to verify
that there would be no dimer/secondary structure formation between the sense and antisense primers, we used the Oligotech software of Oligos, Etc.

**Results**

**Detection within Host**

The primers ITS 1-F and ITS 4-B amplified a product of 838 bp from Silene alba bud meristems infected with Microbotryum violaceum (fig. 2A, lane 1). A similar band was produced when M. violaceum was used directly in the PCR amplification (fig. 2A, lane 2). The product from the infected tissue was cloned using the TA Cloning Kit and was subsequently sequenced. Several restriction sites for EcoRI are conserved in the rDNA of fungi; specifically, almost all ascomycetes, including M. violaceum from S. alba, share an EcoRI site within the 5.8S rRNA gene. Therefore, the cloned PCR product was treated with EcoRI. It is evident from the diagnostic cut (fig. 2B, lane 1) that the amplified product does have EcoRI sites. Sequencing of the product showed that there were two EcoRI sites: one was within the 5.8S sequence and another was within the ITS 1 sequence.

The designed primer pair ITS UV-1 and ITS UV-2 was successful in producing the expected 485-bp PCR product when used on DNA isolated from the bud meristems of diseased S. alba (fig. 3, panel A, lane 1). The identical PCR product was obtained when DNA was amplified from fungal spores collected from the same plant (fig. 3A, lane 4). In order to confirm that this primer pair had amplified the correct region, the PCR product was sequenced. The sequence showed that the primer had successfully amplified the expected region. Bud meristems (fig. 3A, lane 2) from uninoculated plants as well as from inoculated S. alba that had remained healthy for several months (fig. 3A, lane 3) did not produce any PCR products with these primers.

**Tissue-Specific Detection**

In order to assess the localization of the pathogen within the host, PCR using the designed primers was carried out on DNA extracted from different host tissues of S. alba. The pathogen was detected from the inoculated plant's bud meristems (fig. 3A, lane 5), flower pedicels (fig. 3A, lane 6), and flower-stem internodes (fig. 3A, lane 7). However, tissues from the flowering-stem leaves (fig. 3A, lane 8) and root (fig. 3A, lane 9) of infected plants did not show any presence of the pathogen.

**Fungicide Treatment**

All plants treated with the fungicides produced healthy flowers. All control plants remained diseased. There was no obvious effect of the fungicide on time of flowering, although this was not quantified. The designed ITS primers were used to confirm that the fungicide-treated plants did not show any trace of the pathogen within the bud meristems of the host (fig. 3B, lanes 2–5). However, infected plants not treated with the fungicide did show the presence of the pathogen in bud meristems (fig. 3B, lane 1).

![Fig. 3](image-url)  
**Fig. 3**  
PCR amplification of ITS sequences using primers ITS 1-UV and ITS 2-UV from different tissues of infected Silene alba. A, M = molecular marker; lanes 1 and 4 = infected bud meristems and smutted bud; lanes 2 and 3 = bud meristems from uninoculated plants and bud meristems from inoculated healthy (resistant) plants; lanes 5–9 are from different tissues of infected plants, as follows: bud meristems (lane 5), flower pedicels (lane 6), flower-stem internodes (lane 7), flowering-stem leaves (lane 8), and roots (lane 9). B, M = molecular marker; lanes 1 and 6 = infected bud meristems and smutted bud (used as a positive control); lanes 2–5 = meristematic tissues from fungicide-treated plants.
Discussion

The genetic and molecular basis of disease resistance in natural populations is of central importance for understanding the mechanism by which resistance traits evolve. Study of the *Silene alba* and *Microbotryum violaceum* host-pathogen system over the last decade has generated a great deal of information about host-pathogen interactions in natural populations (Alexander and Antonovics 1988; Thrall et al. 1995; Alexander et al. 1996; Antonovics et al. 1996; Bier and Antonovics 1996; Bier and Honders 1996). However, studies to understand the molecular and genetic basis of this interaction have been hampered by slow progress in understanding the mechanisms of infection and in developing techniques for the detection of the pathogen within the host. The use of PCR primers therefore provides a useful method for detecting the pathogen in vivo. Moreover, our strategy of first using a general ITS primer developed in the context of standard systematic studies and then developing primers with enhanced specificity may be useful to other systems. Our designed primers showed enhanced specificity as they reliably amplified DNA from *M. violaceum* in *S. alba*, and we never obtained amplification of plant DNA. This resulted in a much lower likelihood of false positives, in contrast with the occasional amplification of plant DNA when generalized basidiomycete primers are used.

Using this PCR technique, we were unable to detect the pathogen in plants that were inoculated but that failed to develop disease symptoms. This indicates that resistant plants restrict the growth of the fungus rather than tolerate its presence. However, it is currently unknown at which stage resistance to infection is expressed. Our techniques will allow us to detect if, for example, the fungus is initially able to grow in resistant plants but is then cleared or if the resistance mechanism acts at the point of hyphal penetration. We have often speculated that the absence of disease symptoms on *S. alba*, either in experimental studies or in natural populations, did not preclude the possibility that these plants were still infected but were asymptomatic. Our results indicate that individuals that are asymptomatic during flowering are free of the disease, but our sample size was small. It would be interesting to use the PCR detection method to study whether there is an appreciable frequency of asymptomatic infected individuals in a larger sample or in natural populations. Moreover, our approach could also be used to develop an early detection method for the presence of the fungus following seedling or vegetative inoculation, one that would not require that researchers wait for plants to flower.

Up to this point, it has not been possible to make crosses between infected (and putatively susceptible) and healthy (putatively resistant) plants because infected plants are sterile. Our experiments show that treating infected plants with the fungicides Folicur and Bayleton results in cured plants that possess fully fertile flowers. The fungicide Folicur is generally used to cure loose smut of wheat, and Bayleton is used to cure powdery mildew and rust diseases in crops (D. Komn, personal communication). The fungicides act by inhibiting ergosterol biosynthesis in the fungus (Keon and Hargreaves 1996). Our use of the PCR primers confirmed that the cured plants no longer showed any sign of infection. The methods we have developed here will therefore facilitate the process of crossing experiments between diseased and healthy plants.

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Literature Cited