

USE OF INTERNAL TRANSCRIBED SPACER PRIMERS AND FUNGICIDE TREATMENTS TO STUDY THE ANTHR-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 *Microbotryum 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 determining 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 populations 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; Biere and Honders 1996; Shykoff and Kaltz 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; Biere and Antonovics 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-smutted 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 produce 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 inoculum 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 spectrometry 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 genetics of host resistance is that diseased *S. alba* plants are usually sterile. Infection by the pathogen leads to the production of diseased flowers in both sexes. The diseased male flowers produce anthers that are filled with teliospores but no pol-

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len. In female flowers, the pathogen induces a sex switch, the flowers produce spore-filled anthers that have developed from residual staminodes, and the ovary aborts at an early stage (Alexander 1989; Thrall and Antonovics 1995; Scutt et al. 1997). When inoculations are performed at the seedling stage, plants are usually completely diseased and sterile when they first flower. Inoculations of flowering stems will produce a partially diseased individual, and such individuals are often seen in the field and in experimental populations, but frequently the disease spreads to the whole plant over a period of a few weeks. Individuals characterized as susceptible to the disease on the basis of such symptoms are therefore not usually available for use in subsequent crosses. Therefore, we report on the use of fungicides to cure infected plants and discuss the combined use of PCR detection and fungicides as a method for facilitating studies of host-resistance structure.

Material and Methods

Seed Germination and Inoculation

Surface-sterilized seeds of *Silene alba* were grown on agar plates with Murashige and Skoog medium (Murashige and Skoog 1962). Sporidial lines of each mating type (a1 and a2) of *Microbotryum violaceum* (strains 28 and 29; Alexander 1989) were grown on potato dextrose agar (PDA; Difco, Detroit) at 24°C for 48 h. Conjugation between the two mating types (a1 and a2), which is a prerequisite for infection (Fischer and Holton 1957), was carried out as follows. Sporidial concentrations of each mating type were standardized by mixing one loopful of a1 sporidial culture into 1 mL deionized water in an Eppendorf tube; this was followed by vortexing and counting of spores using a hemacytometer. Appropriate dilutions were carried out to obtain 500 sporidia per μL of each mating type. These were then mixed, and 10-d-old seedlings were inoculated by placing 1 μL of inoculum on the apical meristem between the two cotyledons. Inoculation was carried out at 18°C for 48–72 h. The inoculated seedlings were transplanted into pots on Metro-Mix 200 soil (Grace-Sierra, Malpitas, Calif.) and placed in a greenhouse until flowering.

Fungicide Treatment

Twenty diseased flowering plants (10 per fungicide) were sprayed with the triazole fungicides Folicur (synonyms: Tebuconazole [α -[2-(4-chlorophenyl)ethyl]- α -(1-1-dimethyl-ethyl)-1H-1,2,4-triazole-1-ethanol]) and Bayleton (synonyms: triadimefon [1-(4-chlorophenoxy)-3,3-dimethyl-1-(1H-1,2,4-triazol-1-yl)-2-butanone], a gift from Bayer Company) at the recommended concentrations of 6 mg/mL and 3 mg/mL, respectively (D. Komm, personal communication). Once a week for 4 wk, fungicide was sprayed onto each plant using a hand-operated plastic spray bottle until plants were well covered and there was run-off (ca. 120 mL per plant). In addition, 10 mL of fungicide was poured onto the soil in each pot. The treated plants, including infected flowers, were then trimmed and transplanted into new pots, which were transferred to a disease-free chamber. The plants were monitored twice each week until new flowers emerged.

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'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS 4-B (5'-CAG-GAGACTTGTACACGGTCCAG-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 MgCl_2 , 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).

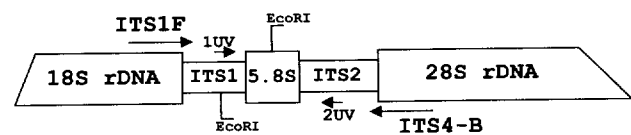


Fig. 1 Schematic diagram of the rDNA of *Ustilago violacea*. Open boxes = ribosomal subunits, arrows = position of PCR primers (ITS 1-F on the 18S, ITS 4-B on the 28S, ITS 1-UV on the ITS 1, and ITS 2-UV on the ITS 2 regions). Note the *EcoRI* sites within the 5.8S subunit and ITS 1 region.

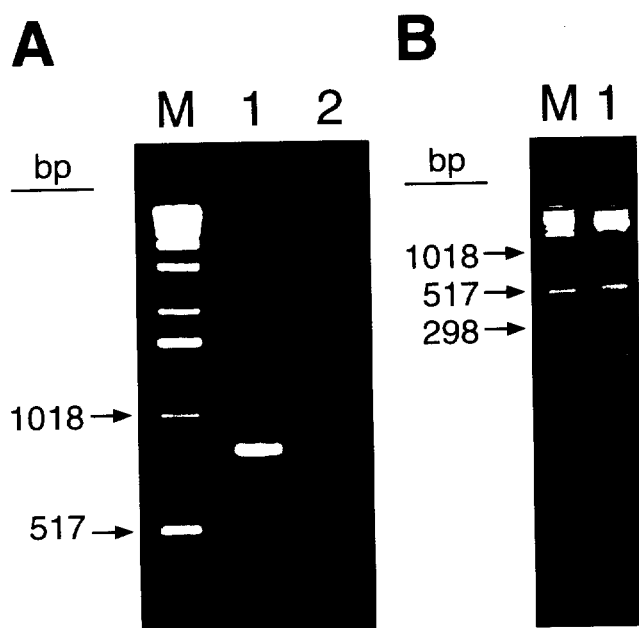


Fig. 2 A, PCR amplification from the ITS regions using ITS 1-F and ITS 4-B primers. M = molecular marker; lane 1 = bud meristems from the infected plant; lane 2 = single colony of *Ustilago violacea*. B, M = molecular marker; lane 1 = *EcoRI* restriction digestion product.

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 *SpeI* 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 *EcoRI*. Restriction digests consisted of 10 units of enzyme (2 μ L), 10 μ L of miniprep DNA from pCR 2.1, 1X (3 μ L) restriction buffer (Boehringer Mannheim), and 15 μ L 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 SD5, 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 sequence was as follows: 5'-CAA-CTCTGTGCACTCTAATGGG-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, htgs) 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'-TTCCCAGGCCA-GCCATTACACCC-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 basidiomycetes, 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).

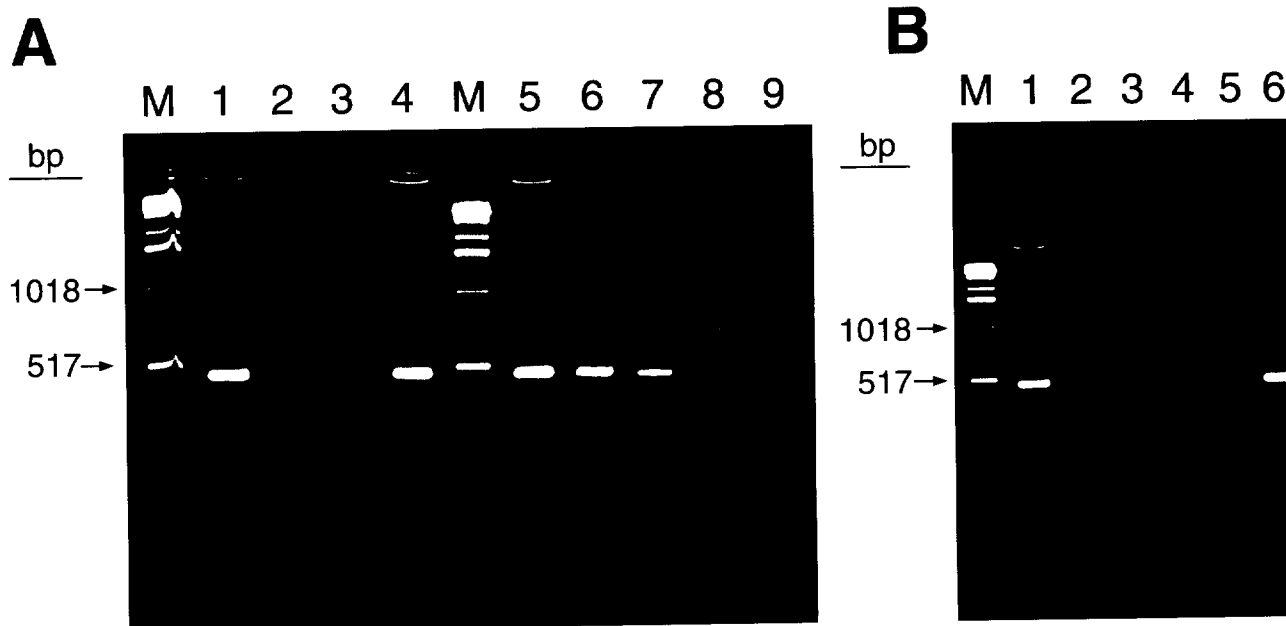


Fig. 3 PCR amplification of ITS sequences using primers ITS 1-UV and ITS 2-UV from different tissues of infected *Silene alba*. A, M = molecular marker; lanes 1 and 4 = infected bud meristems and smutted bud; lanes 2 and 3 = bud meristems from uninoculated plants and bud meristems from inoculated healthy (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; Biere and Antonovics 1996; Biere 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. Komm, 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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