CALIFORNIA ICEBERG LETTUCE RESEARCH PROGRAM

April 1, 2011 - March 31, 2012

EPIDEMIOLOGY AND CONTROL OF LETTUCE DROP CAUSED BY *SCLEROTINIA*

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SUMMARY

In addition to the continuing objective of isolate maintenance and sclerotial inoculum supply to the breeding program there were two new objectives the past year and included: a) evaluation of a detached leaf assay to identify resistance against *Sclerotinia minor* and b) to determine the mechanisms of variability in *Sclerotinia sclerotiorum* populations from California. Over the past few years, in addition to maintaining *Sclerotinia minor* isolates, we have been supplying large quantities of sclerotia for the field evaluation of lettuce germplasm and breeding lines from Ryan Hayes' program against lettuce drop resistance. These efforts have identified resistance to *S. minor* in Eruption, and a number of breeding lines utilizing this and other putative sources of resistance have been generated. The crosses generated from Eruption and other lines have yielded progeny that possess desirable levels of resistance. While the field screening has been rigorous and the breeding lines have shown improvements in resistance in desired lettuce backgrounds, only one generation can be advanced each year because field screening is conducted once each year. We therefore decided to screen elite breeding lines and parents in the greenhouse and also attempted to develop a detached leaf assay in the laboratory that has been successful in other hosts of *S. sclerotiorum*. Evaluations of leaves from 18 parents and germplasm lines, 5 $F_{3:4}$ and 10 $F_{4:5}$ progenies from crosses involving Eruption, 15 RILs from Salinas x PI251246 crosses showed that all leaves regardless of the pedigree were colonized by *S. minor*. Thus, the detached leaf assay is unsuitable to discriminate low levels of resistance in lettuce. We therefore will have to rely on field and greenhouse screening as has been done for many years. *Sclerotinia sclerotiorum* is a homothallic fungus and therefore each isolate contains both mating types (*MAT1 and MAT2*) and is able to form. Over many years of screening nearly 250 isolates of *S. sclerotiorum*, we have determined that a majority of isolates are self-fertile and form apothecia. However, approximately 30% of the California isolates failed to form apothecia. Our analysis of the mating type genes of these isolates revealed that a part of the *MAT* locus is inverted relative to isolates that are self-fertile. Thus, these isolates require a partner with the opposite mating type to form apothecia and thus might be obligate out-crossers. This may be an ingenious avenue adopted by the pathogen to maintain the genetic diversity in the population. This result potentially has major implications for the epidemiology of the disease and breeding for disease resistance.

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PROJECT TITLE: **EPIDEMIOLOGY AND CONTROL OF LETTUCE DROP CAUSED BY *SCLEROTINIA* SPECIES**

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OBJECTIVES:

1. To evaluate a detached leaf assay to identify resistance against *Sclerotinia minor*.
2. To determine the mechanisms of variability in *Sclerotinia sclerotiorum* populations from California.

PROCEDURES AND RESULTS:

Objective 1: To evaluate a detached leaf assay to identify resistance against *Sclerotinia minor.*

Greenhouse experiments involving 18 parents and germplasm lines, 5 $F_{3:4}$ and 10 $F_{4:5}$ progenies from crosses involving Eruption, 15 RILs from Salinas x PI251246 crosses were being conducted to evaluate horticultural characteristics. Four healthy leaves each were collected from 4-week-old plants in Styrofoam cups in the greenhouse were collected, wiped in alcohol and placed in a Petri plate containing moist sterilized Whatman filter paper. A single mycelial plug from a 3-4-day-old *S. minor* culture on PDA with the mycelium touching the leaf was placed on each leaf and incubated at room temperature. The lesion development on each leaf was monitored daily and scored at 48 and 72 hrs after inoculation. Lesion sizes were measured in two directions and averaged. The leaves from each line that received a plain agar plug served as non-inoculated controls. There were three replications for each lettuce line.

Results

The entire leaf surface regardless of the line from which the leaf originated was fully colonized by *S. minor* within 96 hours post-inoculation. The data collected was unable to discriminate the lines that were evaluated even though some of these lines had proven moderately resistant in field evaluations. Thus, at least in lettuce the detached leaf assay is unsuitable to discriminate low levels of resistance.

Objective 2: To determine the mechanisms of variability in *Sclerotinia sclerotiorum* populations from California.

Sclerotinia sclerotiorum is a homothallic fungus that reproduces sexually to produce apothecia. The fungus therefore carries both mating type idiomorphs (*MAT1-1* and *MAT1-2*) as in most homothallic ascomycetes. Our previous study funded by the Board revealed that *S. sclerotiorum* is highly variable in the lettuce fields of California and the reasons or the sources of this variability remain unknown. The majority of isolates tested produce apothecia but a small number of the isolates are unable to produce apothecia despite satisfying the conditions for the production of apothecia. The reasons for this self-infertility are unknown. The goal of this study was to determine why some strains of *S. sclerotiorum* from lettuce in the San Joaquin Valley fail to form apothecia. We focused our attention on the *S. sclerotiorum* mating type (*MAT*) region, the major regulator of sexual reproduction.

Methods

This study was based on 279 *S. sclerotiorum* isolates of which 214 were from lettuce in California. The remaining 65 isolates were from at least eight other substrates (canola, cauliflower, dry bean, field soil, pepper, soybean, sunflower, tobacco) from twelve states (California, Georgia, Illinois, Kansas, Minnesota, Missouri, North Dakota, Nebraska, Ohio, South Dakota, Washington and Wisconsin), or were part of an ordered tetrad of *S. sclerotiorum* strain 1B331 obtained in the lab. Isolates were grown on potato dextrose agar medium (PDA) and potato dextrose broth (PDB) at room temperature. Stocks were maintained as sclerotia at 4˚C. Mycelia were grown at room temperature in 25 ml PDB on a lab bench, inoculated with three to four-day-old PDA cultures, and harvested after four days. For PCR, DNA was extracted by FastDNA extraction kit. For Southern blotting, a standard phenol-chloroform protocol was used. Total RNA was extracted using TRIZOL following the manufacturer's protocol. The integrity of the RNA was verified by agarose gel electrophoresis. Nucleic acids were quantified using a NanoDrop system and the DNA concentration was adjusted to 2-10 ng/µl in PCR grade water for PCR amplification and to 5-8 µg for Southern hybridization.

Cloning of *MAT* loci and phylogenetic analyses

Complete *MAT* locus coverage of *S. sclerotiorum* strains was obtained by PCR using primers designed based on the *S. sclerotiorum* strain 1980 *MAT* locus sequences. Three overlapping PCR reactions targeted the *MAT* locus and parts of the flanking *APN2* and *SLA2*, using primer pairs MAT_1887F / MAT_6555R, MAT_6042F / MAT_ 11930R and MAT_11655F / MAT_14587R. PCRs were performed in 50 µl reactions consisting of 25 µl PCR Master Mix, 1.5 µl of each primer (10 pmol/µl), 5 µl of genomic DNA (2 ng/µl) and 17 µl of PCR grade water. PCR amplifications were carried out with the following conditions: Initial denaturation at 95˚C for 10 min, followed by 35 cycles of 95˚C for 20 sec, 60˚C for 30 sec, and 60˚C for 5 min, with a 10 min final extension at 72˚C. Aliquots of PCR products (6µl) were separated on a 1% agarose gel by electrophoresis, gels were stained with ethidium bromide and visualized under a UV trans-illuminator. PCR products were sequenced with the PCR primers and additional internal primers were designed to generate complete sequencing coverage in both directions. Sequences were assembled by Geneious v4.8.5.

The most parsimonious trees for all the *MAT* genes were inferred using PAUP v.4.0b 10 with default settings including treating gaps as missing data. Alignments were generated in

Geneious v4.8.5 invoking CLUSTAL X version 2.1. All four alignments contained five taxa, including *B. cinerea* as outgroup, as well as *S. sclerotiorum* strains 1980, 44Ba1, 44Ba12 and 44Ba18.

PCR screening for *MAT* inversion

All *S. sclerotiorum* isolates were screened for presence of the *MAT* inversion using inversion-specific primer pair Type-IIF / Type-IIR targeting a 1306 bp fragment that included parts of *MAT1-1-1/MAT1-2-1* (Type-IIF: 5'-CGTTTAAGGGAAATCCAGA-3', Type-IIR: 5'-ACGTGCATCCAAGAAGACGC-3') (Figure S1). PCR screening for absence of the inversion and thus the presence of a complete *MAT1-1-1* was done in all isolates using primer pair MAT1-1-F / MAT1-1-R generating a 671 bp PCR product. PCR conditions were as for *MAT* locus cloning, except the extension temperature and time were 72˚C and 60 sec, respectively. PCR reactions were performed on a Bio-Rad DNA Engine thermocycler (Bio-Rad Laboratories, Hercules, CA). Aliquots of PCR products (6µl) were separated on a 1% agarose gel by electrophoresis, stained with ethidium bromide and visualized under UV trans-illuminator (Ultra-Violet Products Ltd, Cambridge, UK). Selected PCR products were sequenced to confirm target-specific amplification.

***MAT* gene expression analysis**

The expression of all *MAT* genes was assessed using primer pairs specific to each gene, in *S. sclerotiorum* strains 1B331-1, 1B331-2, 1B331-5 and 1B331-6 that contain the *MAT* inversion, and strains 1B331-3, 1B331-4, 1B331-7 and 1B331-8 that lack the *MAT* inversion. The primer pairs for each gene were as follows. *MAT1-1-5*: MAT5F / MAT5R; *MAT1-1-1*: MAT1F / MAT1R; *MAT1-2-4*: MAT4F / MAT4R; *MAT1-2-1*: MAT2F / MAT2R; *MAT1-2-1/MAT1-1-1*: FusionF / FusionR (Table S5). First-strand cDNA synthesis was done with DNAse treated total RNA using Clontech SMARTer cDNA synthesis kit following the manufacturer's instructions (Clontech Laboratories, Inc., CA). Double-stranded cDNA for all *MAT* genes was synthesized. Partial coverage was obtained for for *MAT1-1-1*, and complete coverage for *MAT1-2-1* using the gene-specific primer pairs listed in Table S5.

When more than one transcript was obtained, PCR products were cloned into pCR2.1-TOPO following the manufacturer's protocol (Invitrogen Life Technologies, Carlsbad, CA) and sequenced using both vector (M13F / M13R) and gene specific primers. Exon-intron boundaries were determined by comparison of transcript and gene sequences using CLUSTALW V2.1.

Southern blotting of *MAT* region

To assess the numbers of *MAT* loci in *S. sclerotiorum* genomes, Southern blotting was performed for *S. sclerotiorum* isolates derived from the ordered tetrad, strains 1B331-1, 1B331-2, 1B331-5, and 1B331-6 had the *MAT* inversion, strains 1B331-3, 1B331-4, 1B331-7, and 1B331-8 lacked the *MAT* inversion. Genomic DNA (8 to10 µg) was digested with *Bsa*HI that had restriction sites upstream, downstream and on the *MAT* inversion. Digests were run on 0.8% agarose gels and transferred to nylon membranes (Roche, Basel, Switzerland). A Southern probe specific to *MAT1-2-1* was generated by PCR using primers MAT1-2-F / MAT1-2-R. The probe was labeled with digoxigenin (Roche, Basel, Switzerland) according to the manufacturer's instructions, and hybridization and detection was performed according to the manufacturer instructions (Roche, Basel, Switzerland). X-ray film (Kodak, Rochester, NY) was used and developed following a 30 min exposure. The probe was expected to hybridize to 1.16 and 1.5 kb fragments in isolates with standard *MAT* loci and *MAT* inversions, respectively.

Generation of the sexual state in culture

Apothecium formation was induced in a total of 38 isolates, including 29 isolates with the *MAT* inversion, and nine isolates lacking the *MAT* inversion. Sclerotia were produced on autoclaved potato pieces in 250 ml flasks, and the apothecium assay was performed. Formation of apothecial stalks was considered indicative of fertility and thus homothallism. The assay was repeated once, but only for the isolates that failed to produce apothecial stalks in the first assay.

Isolation of ordered tetrad

Eight ascospores were removed in sequence from a single ascus (ordered tetrad) of *S. sclerotiorum* strain 1B331. *Sclerotinia sclerotiorum* strain 1B331 originated from a sclerotium collected from diseased lettuce in CA, a pure culture was established by transferring emerging hyphae to PDA. A fully expanded apothecium obtained in pure culture was placed in sterile water in a 1.5 ml centrifuge tube, and gently macerated by forceps to release the asci. A single ascus containing eight mature ascospores was dissected using a scalpel. Ascospores were sequentially transferred to new PDA plates, and stored at 4˚C.

RESULTS

***Sclerotinia sclerotiorum MAT* loci**

We generated DNA sequencing coverage of the *MAT* regions of the three *S. sclerotiorum* strains 44Ba1, 44Ba12 and 44Ba18 that measured 12146 bp, 12218 bp and 12218 bp, respectively. Comparisons between homologous regions of *S. sclerotiorum* strain 1980 from GenBank and *S. sclerotiorum* strains 44Ba1, 44Ba12 and 44Ba18 were more than 99% similar, but *S. sclerotiorum* strains 44Ba12 and 44Ba18 differed from all other isolates by the presence of a 3600 bp inversion. *Sclerotinia sclerotiorum* strain 44Ba1 comprised the same ORFs as strain 1980, notably *MAT1-1-5*, *MAT1-1-1*, *MAT1-2-4* and *MAT1-2-1* located between *APN2* and *SLA2*, whereas strains 44Ba12 and 44Ba18 differed by the absence of *MAT1-1-1* and *MAT1-2-1*, the inversion of *MAT1-2-4* and the presence of fusion ORFs *MAT1-1-1/MAT1-2-1* and *MAT1-2-1/ MAT1-1-1* at the inversion boundaries (Figure 1).

Analysis of the inversion boundaries revealed the presence of identical 250 bp motifs at the inversion boundaries arranged in opposite orientation (Figure 1). The locations and orientations of the 250 bp-motifs in *S. sclerotiorum* strain 44Ba1 are congruent with their involvement in a homologous recombination event between *MAT1-1-1* and *MAT1-2-1*, resulting in the inversion of *MAT1-2-4* and the generation of the chimeric *MAT1-1-1*/*MAT1-2-1* and *MAT1-2-1*/*MAT1-1-1* observed in *S. sclerotiorum* strains 44Ba12 and 44Ba18 (Figure 1).

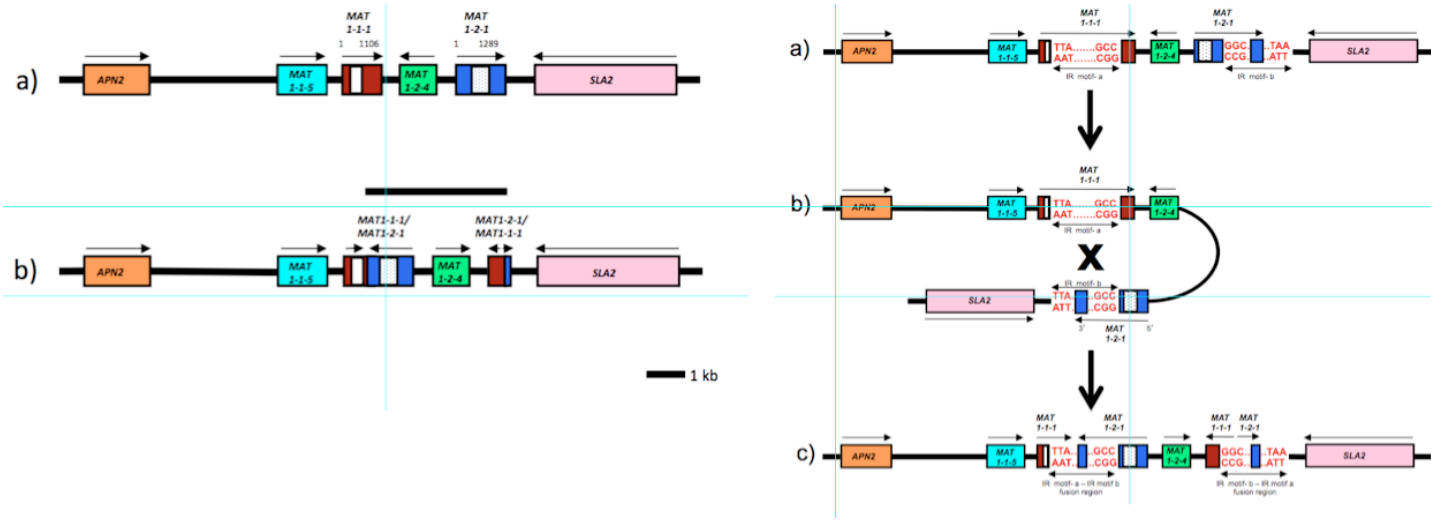

Figure 1. Map of the two types of mating type loci in *Sclerotinia sclerotiorum* (left). The standard *MAT* locus is shown in (a), the new *MAT* locus with the inversion found in this study is shown in (b), the inversion is indicated a thick black line above the gene diagram. Right: Most likely evolutionary pathway to the partly inverted *MAT* locus, the crossing over at the inverted motif in (b) gives rise to the inversion shown in (c).

MAT locus distribution in *Sclerotinia sclerotiorum* field populations

PCR screening for presence of the *MAT* inversion in *S. sclerotiorum* field populations in eight different states showed that both types of *MAT* loci were present in California, Georgia and Nebraska. None of the isolates from Washington had the *MAT* inversion, and for the remaining states only single isolates were screened. On lettuce in California, both *MAT* regions were present in almost equal proportions, 93 of the isolates tested had the inversion. However, isolates from cauliflower and pepper in California (3 and 2 isolates, respectively) contained only one of the two M*AT* loci. Isolates with MAT inversion on lettuce in California were collected as early as 1994, suggesting isolates with the inverted MAT region did not evolved recently, or were not recently introduced.

MAT loci distribution in *Sclerotinia sclerotiorum* ordered tetrad

PCR screening for presence and absence of *MAT* inversions in the eight *S. sclerotiorum* strains 1B331-1 - 1B331-8 representing an ordered tetrad displayed a *MAT* distribution pattern that is in agreement with segregation of the two types of *MAT* loci. Each sibling strain was PCR-positive for either the presence or absence of the inversion. The *S. sclerotiorum* strains 1B331-1, 1B331-2, 1B331-5 and 1B331-6 corresponding to ascospores 1, 2, 5 and 6 numbered from top to bottom of the ascus, contained the inversion, whereas *S. sclerotiorum* strains 1B331-3, 1B331-4, 1B331-7 and 1B331-8 corresponding to ascospores 3, 4, 7 and 8 lacked the inversion.

Impact of *MAT* inversion on apothecium formation

Sclerotinia sclerotiorum is generally considered to be homothallic and to produce a sexual fruiting body (apothecium) containing ascospores under favorable conditions. We investigated the influence of the *MAT* inversion on the capability to self, and found that out of the 29 isolates that had the *MAT* inversion, 18 produced apothecial stalks indicative of sexual recombination. All nine *S. sclerotiorum* isolates without the *MAT* inversion that we tested formed apothecial stalks.

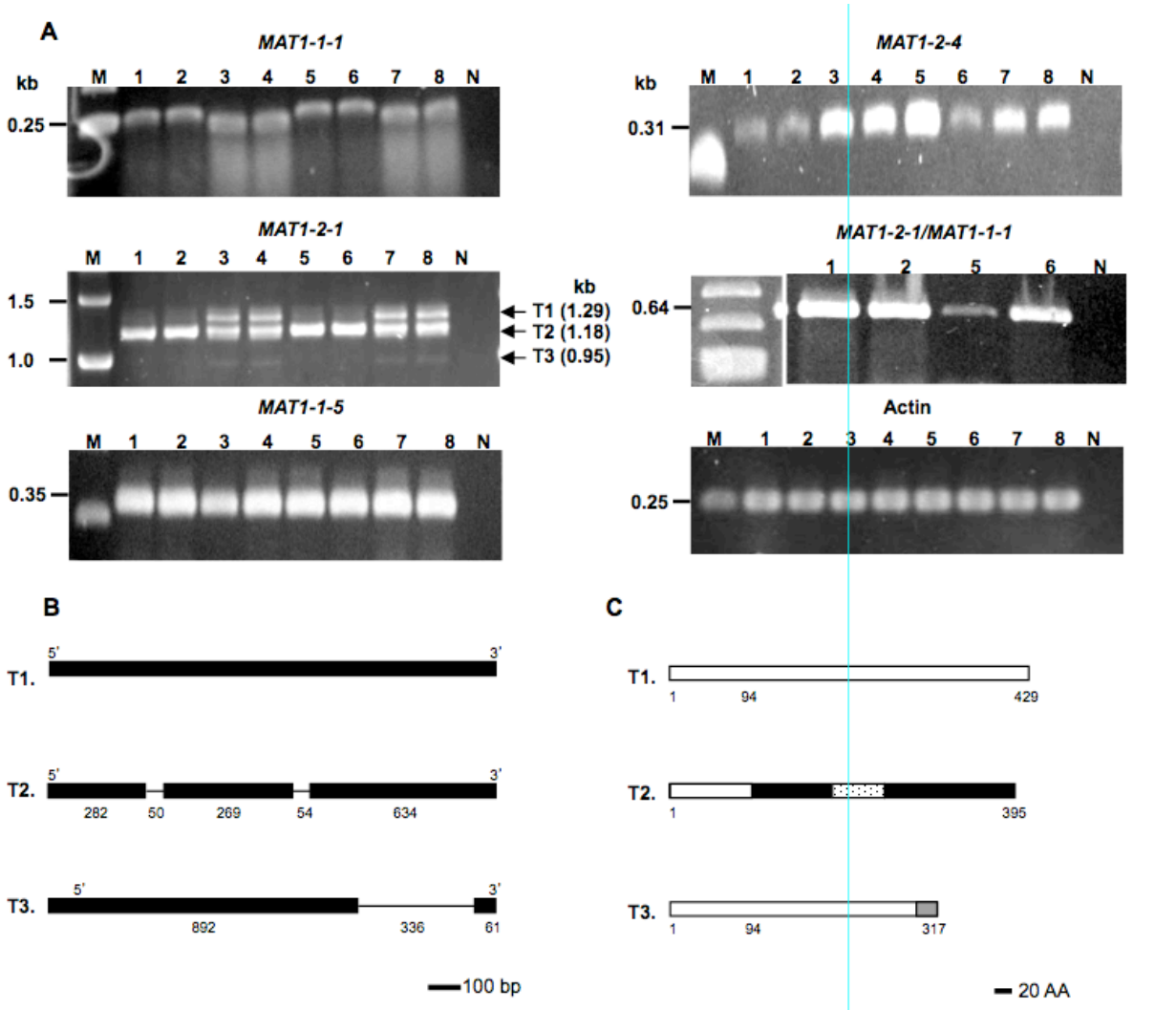

Figure 2. Mating type gene expression analysis in *S. sclerotiorum* isolates. A. Gels displaying RT-PCR results for all *MAT* genes and a control of the eight *S. sclerotiorum* strains 1B331-1 to 1B331-8 representing an ordered tetrad. B. Diagrams of *MAT1-2-1* transcripts T1, T2 and T3, exons are represented by black boxes, introns by black lines, exon and intron lengths are indicated, the diagrams are to scale. C. Conceptual amino acid translations for transcripts T1, T2 and T3. The HMG domain is indicated by the dotted box, white boxes represent amino acid identity between trancripts T1 and T3, black and gray boxes represent sequence unique to transcripts T2 and T3, respectively. The lengths of the different regions are indicated below the boxes.

MAT gene expression analysis in *Sclerotinia sclerotiorum*

RT-PCR was used to assess expression of the six *MAT* genes, including the two chimeras *MAT1-1-1/MAT1-2-1* and *MAT1-2-1/MAT1-1-1* (Figure 2). The eight isolates representing the ordered tetrad were used for RT-PCR. We found that all *MAT* genes were expressed, including the *MAT1-1-1* and *MAT1-2-1* portions of the chimeras (Figure 2). But there was alternative splicing for *MAT1-1-1* and *MAT1-2-1* that differed between isolates with and without *MAT* inversions. The *MAT1-1-1*-specific transcript variants were 205 bp or 253 bp in length, in isolates without and with *MAT* inversion, respectively (Figure 3). The length difference was due to a spliced 48 bp-intron 223 bp downstream of the *MAT1-1-1* 5'-end in isolates lacking the inversion. The intron was delimited by TA-GT and AG-CC at the 5'- end 3'-end, respectively.

For *MAT1-2-1*, a single transcript was obtained in isolates with the *MAT* inversion, but three transcript variants in isolates lacking the *MAT* inversion (Figure 2A). The first transcript variant,

variant T1, was unspliced. In variants T2, two introns were spliced as in *S. sclerotiorum* strain 1980 (Anselem et al. 2011). The first intron was 50 bp in length, started 283 bp downstream of the *MAT1-2-1* 5'-end. The second intron measured 54 bp, started 703 bp downstream of the *MAT1-2-1* 5'-end (Figure 2B). The third transcript variant, variant T3, was characterized by a spliced 336 bp-intron 893 bp downstream of the 5'-end (Figure 2B). Only variant T2 contained the DNA-binding HMG domain characteristic of *MAT1-2-1*, whereas the HMG domain was absent in variants T1 and variants T3 (Figure 2C).
The three *MAT1-1-1* transcript variants had unique amino acid translations. All three variants shared 94 amino acids at the N terminus, only variants T1 and T3 shared the subsequent 193 amino acids, and the remaining positions at the C terminus differed in all three variants (Figure 2C).

MAT* locus copy number in *Sclerotinia sclerotiorum

In order to assess whether the *S. sclerotiorum MAT* locus was single- or multi-copy, Southern hybridization was performed with a *MAT1-2-1* probe and the eight *S. sclerotiorum* strains 1B331-1 to 1B331-8 representing the ordered tetrad. *MAT1-2-1* was targeted due to the presence of three transcript variants differing in length and potentially derived from more than one *MAT* region. The results obtained were consistent with the presence of a single *MAT* locus, as all isolates only had one diagnostic Southern band reflecting the presence or absence of the *MAT* inversion.

Conclusions

We found a new type of *MAT* locus in *S. sclerotiorum*, and are the first to report that *S. sclerotiorum* has two different types of *MAT* loci, a first in fungi. We demonstrated that each *S. sclerotiorum* isolate has only one *MAT* locus, and were able to explain that isolates with the *MAT* locus inversion evolved from isolates without inversion by means of a crossing over.

The two types of *MAT* loci are present in California on lettuce in near equal proportions and isolates with different *MAT* loci are capable of outcrossing as we have shown by analyses of cultures derived from an ordered tetrad. Thus, *S. sclerotiorum* isolates from lettuce in California are able to undergo sexual recombination and evolve new traits, including traits related to pathogenicity and virulence. *Sclerotinia sclerotiorum* isolates with the inversion were collected in California on lettuce as early as 1994, and were thus not recently introduced, or did not recently evolve. These results potentially have major implications for the epidemiology of the disease and breeding for disease resistance.