Asexual Genetic Exchange in the Barley Pathogen *Rhynchosporium secalis*

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Accepted for publication 22 November 2006.

ABSTRACT

Forgan, A. H., Knogge, W., and Anderson, P. A. 2007. Asexual genetic exchange in the barley pathogen *Rhynchosporium secalis*. *Phytopathology* 97:650-654.

The causal agent of barley scald, *Rhynchosporium secalis*, is a haploid anamorphic ascomycete with no known sexual stage. Nevertheless, a high degree of genetic variation has been observed in fungal populations on commercial barley cultivars and parasexuality has been suggested to contribute to this variation. In order to test whether asexual genetic exchange can occur, isolates of *R. secalis* were transformed to hygromycin B resis-

tance or phleomycin resistance. Mixtures of transformants were co-inoculated either on agar or in planta and screened for the occurrence of dual-antibiotic-resistant colonies. No dual-antibiotic-resistant colonies resulted from mixing transformants of different fungal isolates. In contrast, with transformants originating from the same fungal isolate, asexual exchange of markers was demonstrated on agar plates and in planta. This is the first definitive evidence of asexual genetic exchange in *R. secalis*.

Additional keywords: anastomosis, parasexual exchange, transformation, vegetative incompatibility.

Rhynchosporium secalis (Oudem.) J. J. Davis is an imperfect fungus that is able to colonize a variety of wild grasses, but has become notorious by causing scald on rye and, in particular, on barley (*Hordeum vulgare* L.), where yield losses of 1 to 10% are common but can reach up to 40% (40). No teleomorph has been identified for *R. secalis*, but phylogenetic analyses based on the sequence of the ribosomal internal transcribed spacer region revealed that the fungus is an anamorphic Ascomycete with very close relationships to the teleomorph discomycete genera *Tapesia* and *Pyrenopeziza* (13).

Despite the apparent lack of a sexual stage, *R. secalis* displays a high level of genetic variability when compared with other asexually reproducing plant-pathogenic fungi (22). In fungal populations from diverse geographical origins, a high number of pathotypes could be differentiated on sets of differential plants containing defined resistance genes (2,3,8,15,16,18,27,32,36,42, 46). This genetic variability was supported by isozyme (14,21, 24), restriction fragment length polymorphism (22), and amplified fragment length polymorphism (45) analyses. How this genetic variation is generated in *R. secalis* is not understood; however, such understanding has direct consequences for the development of strategies to control the disease, in particular those involving breeding for host resistance.

Many fungi are able to undergo the parasexual cycle of somatic recombination (29), which initially was regarded as an alternative to sexual recombination. Parasexuality is the sequence of events beginning with the fusion of vegetative hyphae (anastomosis) from two different haploid mycelia, followed by cytoplasmic mixing, nuclear migration, nuclear fusion (karyogamy) that produces a heterozygous diploid nucleus, and, finally, reestablishment of the haploid state through repeated loss of whole chromosomes or

segments. Prior to haploidization, genetic exchange occurs through mitotic recombination or the exchange of chromosomes. Events occurring during a parasexual cycle also may include deletions, loss of chromosomal elements, or loss of entire chromosomes during the mitoses that follow karyogamy. Such loss may lead to an aneuploid or haploid state with a novel complement of chromosomes.

Mutation caused by mitotic events is common in fungi (48). Evidence for the occurrence of the parasexual cycle in fungal plant pathogens under laboratory conditions is well documented in *Bipolaris sorokiniana* (44), *Erysiphe graminis* (23), and *Magnaporthe grisea* (47). It also has been suggested that parasexuality may contribute significantly to variation in *R. secalis* (1,3, 4,6,7,25). However, until recently, the lack of suitable markers has prevented definitive studies of genetic exchange. Previous attempts to demonstrate parasexuality in *R. secalis* have involved recovering novel virulence specificities (17) or isozyme patterns (25) from co-inoculations of barley with multiple *R. secalis* isolates. These studies were not conclusive, however, because it was not known whether the novel virulence specificities or isozymes that were observed were a result of changes to nuclear genes, or whether cytoplasmic DNA was responsible. In addition, it was not possible to tell whether alternative forms of the isozymes were present and had been expressed during the experiment.

With the advent of a transformation system for *R. secalis* that uses dominant selectable markers (31), it has been possible to directly investigate asexual genetic exchange in this system. This article provides the first definitive evidence of asexual genetic exchange in *R. secalis*. The exchange and stable integration of antibiotic markers between isolates of this fungus is consistent with the model of parasexual genetic exchange (10). Fungal isolates were transformed to hygromycin B and phleomycin resistance, respectively, and both within-isolate and between-isolate crosses were carried out on defined culture media and in planta. In both situations, marker exchange was observed in a within-isolate cross, whereas crosses between isolates did not yield marker exchange.

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MATERIALS AND METHODS

Fungal isolates. Three isolates were randomly selected from a collection of 65 wild-type isolates of *R. secalis* supplied by H. Wallwork, South Australian Research and Development Institute, Adelaide. Isolates were cultured from single spores on lima bean agar (kanamycin at 50 µg/ml) in the dark at 17°C. Long-term storage of cultures was on agar plugs in cryovials (Nunc, Rochester, NY) in 20% glycerol: 17% skim milk powder at -80°C.

Preparation of protoplasts. Ten 90-mm petri dishes containing 20 ml of liquid Fries medium supplemented with 7.5% sucrose and kanamycin at 50 µg/ml were inoculated with approximately 10⁶ conidia and incubated for 3 weeks at 17°C with gentle shaking. Protoplasts were prepared for transformation according to existing protocols (31), with minor modifications. After the final centrifugation step, the pellet was carefully resuspended in 20 ml of Driselase/β-glucanase at 10 mg/ml (InterspeX, San Mateo, CA) enzyme solution. The enzyme solution was prepared immediately prior to the commencement of protoplasting and filter sterilized through a 0.22-µm filter. The mycelia/enzyme solution was incubated at 25°C for 2 h with gentle shaking. The resulting protoplasts then were separated from the mycelial debris by filtering into a 50-ml Falcon tube through a sterile glass Büchner funnel (porosity grade 1) packed with sterile glass wool. The debris was rinsed with 30 ml of protoplasting buffer (0.7 M KCl, 50 mM CaCl₂ · H₂O, 10 mM Tris HCl, pH 7.5) and the remaining protoplasts were squeezed from the glass wool using a sterile 10-ml pipette. The protoplast suspension was spun at 2,000 rpm in a bench-top centrifuge (Heraeus Megafuge 1.0R; Heraeus, Langensfeld, Germany) for 5 min and the supernatant discarded. This centrifugation was repeated and the remaining protoplast pellet gently resuspended in 500 µl of protoplasting buffer. A hemacytometer was used to determine the protoplast concentration. A 1 × 10⁸ protoplast/ml suspension (≈400 µl) was required for each transformation experiment.

Transformation. Plasmids for transformation were obtained from the Fungal Genetics Stock Center (School of Biological Sciences, Univ. of Missouri, Kansas City). Plasmid pBC-hygro contains the hygromycin B phosphotransferase gene (*hph*) from *Escherichia coli* under the control of the *Neurospora crassa cpc-1* promoter and the *Aspergillus nidulans trpC* terminator, and plasmid pBC-phleo contains the phleomycin resistance gene (*ble*) from *Streptoalloteichus hindustanus* under the control of the *A. nidulans gpd* promoter and the *Saccharomyces cerevisiae CYC1* terminator (41). Protoplasts were transformed as previously described (31), although in this case, a restriction enzyme-mediated integration step was included (38). Twenty units of restriction enzyme (*HindIII* or *BamHI*) was added to the protoplast suspension along with the plasmid DNA, and kanamycin at 50 µg/ml was used in the selective media following the regeneration step.

Asexual cross conditions. Asexual crosses were performed on V8-juice agar or in planta. To prepare V8-juice agar, Campbell's V8 juice was diluted 1:4 with sterile water and filtered through Miracloth (Calbiochem, Darmstadt, Germany). The pH was adjusted to 6.0 with 1 M NaOH prior to addition of 2% (wt/vol) agar and sterilization by autoclaving. To ensure that genetically homogeneous cultures were being used, single-spore isolates were grown from each transformant prior to its use in the crossing experiments. Spores of hygromycin-resistant (*hyg*^R) and phleomycin-resistant (*phleo*^R) transformants were mixed 1:1 and plated onto V8-juice agar (kanamycin at 50 µg/ml) at a spore density of 1 × 10⁵ spores/ml. To prepare the co-inoculation mixtures, spores of the *hyg*^R transformants of isolates 5 and H2.5 were mixed pairwise with spores of the *phleo*^R transformants of isolate 5. In addition, the *hyg*^R transformants of isolate 6 were mixed with the *phleo*^R transformant 5P1 of isolate 5. Mixtures of one *hyg*^R and one *phleo*^R transformant were grown on V8-juice agar

(kanamycin at 50 µg/ml). At 2 weeks post inoculation, spores were harvested from V8-juice agar by adding sterile H₂O (2 ml) to the culture plate and scraping the spores with the edge of a scalpel blade. To remove mycelial fragments, the spore suspension was washed through a sterile 10-ml syringe lightly packed with glass wool and resuspended in 1 ml of sterile H₂O. Spore suspensions were transferred to lima bean agar containing either a mixture of hygromycin at 100 µg/ml and phleomycin at 20 µg/ml or neither of these antibiotics.

In planta crosses were performed on 10-day-old seedlings of universally scald-susceptible barley cv. Clipper (5), each planted in a 500-ml cylindrical tissue culture container. The seedlings were misted with a 0.1% Tween 20 solution, and then 40 µl of spore suspensions containing 10⁵ spores/ml of pairwise mixtures of *hyg*^R and *phleo*^R transformants was applied to the surface of leaves using a pipette. The tissue culture containers were sealed and incubated in a growth chamber (Sanyo MLR-350HT; Sanyo Electronic Co. Ltd., Osaka, Japan). After an initial dark period of 48 h at 17°C to encourage spore germination, the plants were kept under a 12-h light, 12-h dark regime at 17°C. Leaf sections were removed at 2 weeks post inoculation and placed onto dual-selection lima bean agar. If fungal growth was observed to occur from the leaf section onto the agar, the colony was subcultured on dual selective lima bean agar. In both in vitro and in planta experiments, inoculations containing only an *hyg*^R transformant or a *phleo*^R transformant served as controls.

DNA manipulations and analysis. Genomic DNA was prepared from 2- to 3-week-old *R. secalis* liquid cultures (4 × 20 ml of Fries medium supplemented with 7.5% sucrose and kanamycin at 50 µg/ml). The cultures were decanted and excess medium removed by blotting. The fungal tissue then was ground to a fine powder under liquid N₂, transferred to a 2-ml Eppendorf tube, and mixed with 1 ml of extraction buffer (1% lauryl sarcosine, 100 mM NaCl, 10 mM EDTA, 100 mM Tris/HCl, pH 8.5) as thawing began. Total nucleic acids were extracted once with phenol/chloroform (25:24) and once with phenol/chloroform/isoamyl alcohol (25:24:1), and then precipitated by the addition of 0.7 volumes of isopropanol and 0.1 volumes of 3 M Na-acetate, pH 4.8. The nucleic acids were washed with 70% ethanol, air dried, and resuspended in 100 µl of R40 (RNAse at 40 µg/ml in 10 mM Tris/HCl and 1 mM EDTA, pH 8.0).

Primers for the polymerase chain reaction (PCR) screening of primary transformants and putative recombinants were designed to amplify sequences internal to the *hph* and *ble* genes in pBC-hygro and pBC-phleo, respectively. The *hph* primers HPHfwd (5'-CGC AAG GAA TCG GTC AAT AC-3') and HPHrev (5'-ACA TTG TTG GAG CCG AAA TC-3') amplify a 202-bp fragment. The *ble* primers BLEfwd (5'-CAA GTT GAC CAG TGC CGT TC-3') and BLErev (5'-CGC TGA TGA ACA GGG TCA C-3') amplify a 148-bp fragment. For Southern blot hybridization, ³²P-radiolabeled probes were generated by random priming using a DECAprime kit (Ambion, Austin, TX) according to the manufacturer's instructions. PCR product containing sequences internal to the selective markers (*hyg*^R and *phleo*^R) was used as template for probes. The *hph* probe was generated from a 749-bp fragment that was amplified using primers hphLONGfwd (5'-CAG CGA GAG CCT GAC CTA TT-3') and hphLONGrev (5'-GGC GTC GGT TTC CAC TAT C-3'). The *ble* probe was generated from a 349-bp fragment that was amplified using primers bleLONGfwd (5'-CCC CTA CAA CGA CCA TCA AA-3') and BLErev. PCR to generate probe templates was performed in a Corbett PC960G thermocycler (Corbett Life Science, Sydney, Australia). Products were checked on 2.0% agarose and cleaned using an UltraClean PCR cleanup kit (MoBio, West Carlsbad, CA) prior to use as template for radiolabeling.

Prehybridization and hybridization were carried out using ULTRAhyb (Ambion) hybridization solution at 42°C in hybridization bottles with constant rotation. Prehybridization was

carried out for 1 h and hybridization for 16 h. Radiolabeled probe was used without removal of unincorporated nucleotides. Following hybridization, the membranes were washed twice for 5 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at 42°C, then twice for 15 min in 0.1× SSC and 0.1% SDS at 42°C. Following washing, the membranes were exposed to X ray film (Kodak X-Omat Xk-1; Eastman Kodak Co., Rochester, NY) at –80°C in the presence of enhancer screens. Membranes that were to be probed again were stripped for 15 min in a boiling 0.5% SDS solution that was removed from the hotplate when the membrane was added to the solution. Restriction digests of fungal genomic DNA were run on 0.8% agarose gels, 0.5× Tris-acetate-EDTA (TAE) (1× TAE = 40 mM Tris-acetate, 1 mM EDTA, pH 8.0) for 8 h at 40 V. DNA was transferred to positively charged nylon membranes (34). DNA was fixed to the membrane using a GS-Genelinker (Biorad, Hercules, CA) as recommended by the manufacturer.

RESULTS

Fungal transformants. Experiments were designed to test the hypothesis that asexual genetic exchange occurs in *R. secalis* by examining whether dominant genetic markers (antibiotic resistance) are exchanged between transformants of a single fungal isolate as well as between transformants of different isolates. For this purpose, three fungal isolates (numbers 5, H2.5, and 6) were chosen from a fungal collection due to their vigorous growth in culture. They were transformed to hygromycin and phleomycin resistance, respectively, using a modification of the transformation protocol for *R. secalis* developed by Rohe et al. (31). Isolate 5 was transformed to either hygromycin or phleomycin resistance, and isolates H2.5 and 6 to hygromycin resistance. Three putative isolate-5 transformants from hygromycin selection (5H1, 5H2, and 5H3) and two from phleomycin selection (5P1 and 5P2) were

Fig. 1. Ethidium bromide-stained agarose gel of polymerase chain reaction products derived from primary transformants 5H1, 5H2, 5H3 (lanes 1–3), 5P1, 5P2 (lanes 4 and 5), and dual-resistant strains 5H1*5P1, 5H1*5P2, and 5H3*5P1 (lanes 6–8); and wild-type isolate 5 (lane 9). Primer set HPHfwd/HPHrev (top) was used to generate a 202-bp band internal to the *hph* gene. Primer set BLEfwd/BLErev (bottom) was used to generate a 148-bp band internal to the *ble* gene.

chosen for further analysis. In addition, an isolate-H2.5 *hyg*^R transformant (H2.5H1) and four isolate-6 *hyg*^R transformants (6H1, 6H2, 6H3, and 6H4) were selected for use in the crossing experiments.

Presence of the genes conferring antibiotic resistance to the fungal transformants was confirmed by PCR using genomic DNA as a template. Each of the *hyg*^R transformants yielded a 202-bp product corresponding to a sequence internal to the *hph* gene, and each of the *phleo*^R transformants yielded a 148-bp product corresponding to an internal *ble* sequence (Fig. 1). No such bands were amplified from fungal wild-type DNA. All tested transformants retained the ability to grow under selection after five subcultures on nonselective media, indicating that the transformants were mitotically stable. In addition, PCR banding patterns as well as hybridization patterns remained consistent for transformants cultured in the absence of selection (data not shown).

The pairwise co-inoculation of *hyg*^R and *phleo*^R transformants was carried out under conditions presumed to be favorable for asexual exchange and for detection of asexual recombination events. As the first step in the parasexual cycle, fungal hyphae must undergo anastomosis (29). Optimizing the in vitro inoculation density enabled the maximum opportunity for hyphal fusion events to occur. V8-juice agar was chosen as the growth medium for in vitro crossing because it produces extensive vegetative growth of *R. secalis* and retains 20% of the spore production observed on lima bean agar (37). Therefore, this combination of factors enabled a high potential for anastomosis while also providing sufficient spores to isolate putative recombinants.

Asexual exchange results. Mixtures of isolate 5-derived transformants gave rise to isolated colonies on dual-selection plates. Single-spore isolates from the resulting colonies were cultured for further analysis. *Hyg*^R transformant 5H2 failed to produce any dual-resistant colonies when co-inoculated with either of the *phleo*^R transformants. This transformant also differed markedly from transformants 5H1 and 5H3 by displaying increased hyphal growth and a colored phenotype. The reason for this has not been investigated at this stage, although it is possible that transformation-induced mutation has disrupted a component necessary for the parasexual cycle or the control of fungal development. Also, co-inoculations containing either isolate H2.5-derived or isolate 6-derived transformants did not produce dual-antibiotic-resistant colonies (Table 1). No growth of wild-type fungal isolates was observed on dual selection or under hygromycin B selection. *Hyg*^R transformants did occasionally germinate under phleomycin selection, but growth was restricted to one or, occasionally, two short germ tubes before growth ceased. The action of phleomycin is to bind and cleave DNA, and thus, it is possible that phleomycin-induced damage may not become lethal until the first round of DNA replication, which may not be until after the production of the first short germ tube. In contrast, hygromycin B, which acts on ribosomes to prevent protein synthesis, may have a more immediate and complete lethality. Recovery of dual-resistant colonies following the co-inoculation of singly resistant transformants was strong preliminary evidence that genetic exchange had occurred.

TABLE 1. Recovery of dual-antibiotic-resistant *Rhynchosporium secalis* isolates from co-inoculations of hygromycin-resistant (*hyg*^R) transformants with phleomycin-resistant (*phleo*^R) transformants^a

<i>phleo</i> ^R	<i>hyg</i> ^R							
	5H1	5H2	5H3	H2.5H1	6H1	6H2	6H3	6H4
In vitro								
5P1	+	–	+	–	–	–	–	–
5P2	+	–	+	–	nd	nd	nd	nd
In planta								
5P1	–	–	–	–	–	–	–	–
5P2	+	–	+	–	nd	nd	nd	nd

^a Symbols: + = dual-antibiotic-resistant spores recovered; – = no dual-antibiotic-resistant spores recovered; and nd = not determined.

In addition to in vitro crosses, in planta co-inoculations were performed on seedlings of the scald-susceptible cv. Clipper. As with co-inoculation on plates, hyg^R transformants 5H1 and 5H3 produced mycelia able to grow under dual selection when co-inoculated with phleo^R transformant 5P2. In contrast, transformant 5P1 did not yield dual-resistant mycelia during co-inoculation in planta. The lower inoculation density on the barley leaves may explain why the transformants that resulted in double resistant colonies in vitro did not do so in planta.

In all cases, microscopic examination was used to confirm that growth under double selection was arising from individual spores rather than from mycelia. Well-isolated, germinating single spores are readily distinguishable from mycelial fragments at ×80 magnification. In the event that isolated heterokaryotic mycelial fragments did propagate under double selection, such fragments would be expected to give rise to uninucleate two-celled spores that would be unable to survive subculture under double selection. Only spores containing nuclei that had undergone either mitotic recombination or whole or partial chromosome exchange involving the antibiotic selection markers would be expected to survive such subculture.

Transformants and dual-resistant strains were analyzed for the presence of antibiotic resistance marker genes by Southern blot hybridization. Probes generated from PCR amplicons internal to the *hph* or *ble* genes hybridized to high molecular weight genomic DNA, indicating that transferred sequences integrated into the genome and were not present as the transforming vector. To analyze transformants generated from either the *hph*- or *ble*-containing vectors, genomic DNA was digested with *Hind*III and hybridized with the PCR-generated probes. *Hind*III cuts outside both the *hph* and *ble* probes. The *hph* probe hybridized to DNA from the hygromycin-resistant transformant 5H3, whereas the *ble* probe hybridized to phleomycin-resistant transformant 5P2. The double band detected by the *ble* probe is most likely explained by two independent integration events. Both probes hybridized to DNA from the dual-resistant strain 5H3*5P2 (Fig. 2). Neither probe hybridized to DNA prepared from wild-type *R. secalis*. The binding patterns of each probe to DNA from dual-resistant isolate 5H3*5P2 are identical to each of the parental strains 5H3 and 5P2, indicating that marker DNA from one of the strains has been transferred asexually to the other. The nature of the exchange event and whether these banding patterns indicate mitotic recombination or whole or partial chromosome exchange remains to be determined.

DISCUSSION

High pathogenic variability is one of the remarkable features of *R. secalis*, but the driving force that generates the observed number of fungal pathotypes remains to be identified. In gene-for-gene systems (10), the plant defense response is triggered when the activity of single avirulence (*Avr*) genes in the pathogen is recognized by corresponding resistance (*R*) genes in the host. To bypass *R*-gene-specified recognition, the pathogen needs to mutate or delete the complementary *Avr* gene. In the barley-*R. secalis* interaction that follows the gene-for-gene logic (30), both strategies could be demonstrated for the *Avr* gene *NIP1*. Single-base mutations have been shown to incapacitate the response specifically mediated by the *R* gene *Rrs1* (9,30). In addition, the *NIP1* gene is absent from ≈45% of all analyzed fungal isolates, allowing the colonization of *Rrs1* barley (39).

Genetic variation within and among field populations of *R. secalis* may occur through a mix of mutation, migration, and recombination. In the absence of a teleomorph, asexual recombination may contribute to generating novel virulence specificities in one of two ways: either by mutating a single fungal gene involved in gene-for-gene recognition or by creating novel combinations of existing virulence specificities. Therefore, asexual recombination

has been suggested to contribute to the observed variability (1,3, 4,6,7,24–26). This work describes the first direct evidence of asexual genetic exchange operating in *R. secalis* under laboratory conditions. Dual-antibiotic-resistant fungal colonies were observed to arise from mixing of single-antibiotic-resistant transformants on agar as well as after co-inoculation of susceptible host plants. Marker DNA exchanged during this process was shown to be stable for several generations by subculturing in the absence of selection, followed by the return to dual-antibiotic selection. Demonstration of asexual genetic exchange in *R. secalis* places this fungus together with the many fungi, including phytopathogens, that previously have been shown to undergo this process (e.g., *Aspergillus niger* [28], *Fusarium oxysporum* [43], and *M. grisea* [47]).

In the present study, successful genetic exchange occurred only between transformants of the same isolate. Although definitive conclusions cannot be drawn from a small data set, it is tempting to speculate that a vegetative incompatibility system may control the success of asexual genetic exchange in *R. secalis*. In Ascomycetes, the parasexual cycle requires the formation of a heterokaryon. The coexistence of two different nuclei in a single cell is controlled by a number of specific loci (termed *het* for heterokaryon incompatibility or *vic* for vegetative incompatibility) that need either to carry identical alleles or, alternatively, to interact specifically with each other to allow survival of the heterokaryon (12,35). In this report, hyg^R and phleo^R transformants of a single isolate are genetically identical except for the presence of marker DNA and alterations as a consequence of the transformation procedure. If a vegetative compatibility system operates in *R. secalis*, completion of a parasexual cycle is expected to be favored for genetically identical or at least very similar transformants. An exception to this may occur if *het* or *vic* loci were disrupted during the transformation procedure, resulting in vegetatively compatible transformants. A parasexual genetic exchange was not found between transformants originating from different isolates. The absence of asexual exchange between non-identical fungal isolates, which of course limits the width of recombination-based variability, may be explained by vegetative incompatibility. No such system has been described previously in *R. secalis*. The characterization of vegetative compatibility groups, which has been extensive in other phytopathogenic fungi such as *F. oxysporum* (19), has provided a useful diagnostic tool for pathogenicity studies in these organisms.

Although this report demonstrates that *R. secalis* is capable of parasexual exchange, the relative contribution of this process to overall genetic variation appears to be rather limited. Transpos-

Fig. 2. Southern analysis of *Rhynchosporium secalis* primary transformants 5P2 (lane 1, phleomycin-resistant [phleo^R]), 5H3 (lane 2, hygromycin-resistant [hyg^R]), and hybrid strain 5H3*5P2 (lane 3, phleo^R and hyg^R). Total DNA was isolated from 2- to 3-week-old single-spore fungal colonies and digested with *Hind*III. Radiolabeled probes derived from sequences internal to the *hph* or *ble* genes hybridized to the A, hyg^R and B, phleo^R transformants, respectively. C, Both probes hybridized to DNA from the hybrid strain 5H3*5P2.

able elements have been suggested (45) as a cause of variation in amplified fragment length polymorphism markers between isolates recovered from scald hotspots. Such elements may provide a source of variation during the vegetative phase of the life cycle. However, evidence from the analysis of the genetic structure of *R. secalis* populations (22,33), including the observed low clonality and the equal occurrence of opposite mating types in most populations (11,20), argues in favor of the existence of a teleomorph and of regular sexuality as the major source of genetic variation in *R. secalis* populations.

ACKNOWLEDGMENTS

This project was supported by an Australian Grains Research and Development Corporation Scholarship. We thank H. Wallwork and L. Scott for supplying us with scald isolates; and K. Oldach (ACPGF, Adelaide, Australia) for providing primer hphLONGfwd.

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