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Disruption of the Subtilase Gene, *albin1*, in *Ophiostoma piliferum*

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Wood sapstaining fungi produce multiple proteases that break down wood protein. Three groups of subtilases have been identified in sapstaining fungi; however, it is not known if these groups have distinct physiological roles (B. Hoffman and C. Breuil, Curr. Genet. 41:168-175, 2002). In this work we examined the role of the subtilase Albin1 from *Ophiostoma piliferum*. Reamplification of cDNA ends PCR was used to obtain the *albin1* gene sequence. The encoded subtilase is probably extracellular and involved in nutrient acquisition. This gene was disrupted with an *Agrobacterium tumefaciens*-mediated transformation system. Two of the disruptants obtained had significantly lower levels of proteolytic activity, slower growth in bovine serum albumin, and significantly reduced growth on wood. Thus, *albin1* plays an important role in *O. piliferum*'s ability to acquire nitrogen from wood proteins.

Sapstaining fungi infect wood and produce a dark blue to black discoloration that lowers the value of the wood. These fungi also are of concern in some importing countries, as they may be pathogenic. The forest products industry spends millions of dollars annually on fungicides to control sapstaining fungi, but these chemicals do not have the duration of effectiveness desired and present significant environmental concerns. Biological control agents, such as Cartapip-97, an albino strain of *Ophiostoma piliferum*, have been examined as an alternative method for preventing sapstain (4, 47). However, these agents may grow slowly and have not been shown to be reliable under field conditions. To develop new strategies for controlling sapstain and to improve the available biological control agents, we need to better understand how sapstaining fungi and biological control agents grow on wood and modify wood color.

Sapstaining fungi cannot degrade structural components in wood. Instead, they utilize nonstructural components, including triglycerides, fatty acids, and proteins (14, 32, 36, 41, 48). Nitrogen levels in trees are low, and easily assimilated nitrogen sources, such as ammonia, are insufficient to support fungal growth (21, 28). Trees store most of their nitrogen in an organic form as proteins and amino acids (25, 39). To extract nitrogen from these protein sources and sustain their growth on wood, sapstaining fungi produce several extracellular proteases.

Subtilases, a type of serine protease, are the dominant extracellular proteases produced by the sapstaining fungus *Ophiostoma floccosum* (1). Subtilase genes are common in sapstaining fungal species, and three major groups of fungal subtilases have been identified (16). One group contains intracellular subtilases thought to play a variety of housekeeping roles (22, 27, 33). The other two groups contain extracellular fungal subtilases, several of which are involved in pathogenesis (5, 22, 27, 40, 43).

In this study we focused on the commercially important sapstaining fungus *O. piliferum*, which was used to develop the albino biological control agent Cartapip-97. This species is saprophytic and causes only cosmetic defects in wood. The subtilase gene *albin1* from this fungus has been partially sequenced (16), is regulated by exogenous nutrients and the ambient pH (17), and may be involved in the degradation of exogenous protein sources. Disruption of genes, including the *SAP* genes (*SAP1* to *SAP6*) in *Candida albicans* and the subtilase genes of *Aspergillus fumigatus* and *Aspergillus awamori* (11, 19, 30, 37, 44), has been used to determine the physiological roles of several fungal proteases.

Our objective in this study was to determine the role of an extracellular protease produced by the sapstaining fungus *O. piliferum* in the acquisition of organic nitrogen from wood. We hypothesized that disruption of the gene encoding this enzyme should reduce the growth of the fungus on organic nitrogen and wood. The function of this enzyme is important in designing stain control strategies and in improving the efficiency of biocontrol agents.

MATERIALS AND METHODS

Fungal and bacterial strains and growth conditions. Fungal isolate *O. piliferum* 156-112 was obtained from the University of British Columbia Department of Wood Science (Vancouver, British Columbia, Canada) (23). Fungi were grown at 18 to 23°C for 2 to 5 days on malt extract agar (MEA) (Oxoid Ltd., Basingstoke, Hampshire, England) plates, which were overlaid with cellophane membrane backing (Bio-Rad, Life Science, Mississauga, Ontario, Canada) when mycelia were grown for DNA extraction. Transformants were grown on 2% skim milk agar (SMA) (Difco, Oakville, Ontario, Canada) plates to assess their extracellular protease activity. To determine the growth of the transformants on bovine serum albumin (BSA) and BSA-NH_4Cl-glucose media, precultures were grown at 20 to 23°C for 5 days in malt extract broth (Oxoid) (17). Cultures were filtered through three layers of sterile cheesecloth, and the yeast and spore cells were collected by centrifugation at 3,000 × *g*, resuspended at concentration of 1×10^8 cells/ml in water, and left overnight at 20°C. One milliliter of the cell suspension was used to inoculate 20 ml of minimal medium at the appropriate pH containing BSA or BSA, NH_4Cl, and glucose, which was incubated at 20°C for the appropriate time. Culture biomass was determined by weighing mycelia that were filtered through a 70-μm-pore-size nylon cell strainer (Falcon) and oven dried at 70°C overnight.

Growth on wood was determined as described by Fleet et al. (14). Sterilized sapwood blocks were inoculated with fungal cores taken from actively growing mycelia and incubated in moist chambers for 2 weeks. Three wood blocks were infected for each transformant.

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Escherichia coli strain TOP10 (Invitrogen Life Technologies, Carlsbad, Calif.) was used in all cloning experiments and was grown on Luria-Bertani agar plates containing 1 μg of ampicillin per ml or no antibiotic, as appropriate. *Agrobacterium tumefaciens* GV3103 was grown, stored, and transformed as described by Pikaard (http://www.biology.wustl.edu/pikaard/).

PCR, RACE-PCR, multiplex PCR, and multiplex reverse transcription (RT)-PCR. PCRs were carried out with a Hybaid TouchDown thermocycler (InterScience, Oakville, Ontario, Canada). For PCRs we used 200 ng of template DNA and 30 cycles of 96°C for 45 s, 56°C for 45 s, and 72°C for 45 s. For reamplification of cDNA ends PCRs (RACE-PCRs), a SMART-RACE cDNA amplification kit (CLONTECH, Bioscience, Windsor, Ontario, Canada) was used. One microgram of total RNA was used to prepare 3′ and 5′ RACE-ready cDNAs, which were amplified according to the manufacturer's instructions. Reamplification of DNA fragments was performed by using the gene-specific primers pP1-1 (5′-ACTTTGGCAGCGTCGTCGACAT-3′) and pP1-2 (5′-CAACGTAGTTGATGCCGGCAATG-3′) and the supplied nested universal primer with an annealing temperature of 68°C. To perform RACE-PCRs, we designed sets of gene-specific primers from partial sequence data for *albin1* (16). PCR of 3′ cDNAs with the gene-specific primers resulted in amplification of an appropriate amplicon. No amplification product was obtained from the 5′ reaction with *albin1*, due to the lack of a full-length transcript in the samples. We therefore designed a degenerate primer, PRO 1 (5′-ACICA[A/G]ACIGG[T/C]CC[T/C]TGG-3′), based on the homologous sequence (T/E)T(Q/E)(S/K)GAPW at the N terminus of other mature fungal subtilase sequences. We then used this primer in conjunction with the *albin1* gene-specific primer wP1-4, and a suitable amplicon was produced. All of the bands generated were cloned, sequenced, and deposited in the GenBank database.

Multiplex PCRs were performed with the ribosomal DNA-specific primers NL1, NL2, wP1-1 (19), and dP1-2 (5′-CCTCATGGTCGGTGTGGCAAAGTA-3′). For reactions we used a 40:1 ratio of the wP1-1–dP1-2 primer pair to the NL1-NL2 primer pair, as this ratio was empirically found to provide successful amplification of both genes. For reactions we used 40 amplification cycles as described previously, and the reactions were repeated three times.

Contaminating DNA was removed from the total RNA for multiplex RT-PCRs by adding 1 μl of 1 M $MgCl_2$ and 10 U of DNase I FPLCpure (Amersham, Baie d'Urfé, Québec, Canada) and incubating the preparation at 37°C for 30 min. RT was carried out with Superscript II (Invitrogen) and a poly-T_{20} primer. cDNAs were purified with a QIAquick Cleanup kit (Qiagen). cDNAs were stored at −20°C for short-term storage or at −80°C for longer times. PCRs with primers wP1-1 and wP1-4 and PCRs with primers NL-1 and Nl-2 were performed as described above.

Cloning and sequence analysis. PCR products were cloned with a TOPO-TA cloning kit (Invitrogen) used according to the manufacturer's protocol. Inserts were amplified, purified, and sequenced with a Perkin-Elmer 480 DNA thermal cycler by using an ABI PRISM BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Woodbridge, Ontario, Canada) at the Nucleic Acid and Protein Service Laboratory (University of British Columbia). Nucleotide sequences were analyzed with an Applied Biosystems ABI 373 DNA sequencer (PE Applied Biosystems). The resulting sequences were analyzed for similarity with other known protein sequences by using the BLAST search engine at the National Center for Biotechnology Information website. ClustalW (46), the compute pI/Mw tool, SignalP, Genescan (8), ORFfinder, and the predict protein server were used for further sequence analyses.

Ergosterol analysis. Ergosterol extraction and analysis were based on the method of Pasanen et al. (34). A Saturn 3800 series gas chromatograph-mass spectrometer (Varian, Palo Alto, Calif.) with a 30-m DB-5 column was used for the analysis. Identification of the analytes was based on their retention times and mass spectra. The cholesterol-trimethylsilyl internal standard and ergosterol-trimethylsilyl eluted at 16.8 and 17.8 min, respectively. A blank sample of hexane, pyridine, and bis-trimethylsilyl-trifluoroacetamide had no peaks in this region. Integrated peak areas were determined by using SaturnView, version 5.52 (Varian). A calibration curve was developed based on the ratio of the ergosterol and cholesterol peak areas and was used to determine the concentrations of ergosterol in the samples.

Disruption vector construction. Restriction digestion and ligation were performed by using standard protocols (3). The wP1-1–wP1-4 primer pair was used to amplify *albin1*, and the gene was cloned into the pCR2.1 TOPO TA vector (Invitrogen) to generate the vector pwP1. The hygromycin B resistance cassette was amplified from the vector pCB1004 with primers Thga-1 (5′-GTCGACGTGGAGGTAATAATTGACGACGATA-3′) and Thga-2 (5′-GTCGACAACGTTTTCCAATGATGAGCAC-3′) (9). These primers amplified 1.6 kb of the hygromycin cassette and added AccI restriction sites on either side of the amplicon. This amplicon was cloned, and then it was excised with Acc1 and ligated into AccI-digested pwP1 to generate the vector pwP1-HPH. This vector contained 589 bp (residues 13 to 602) of *albin1* flanking the 5′ end of the HPH expression cassette and 244 bp (residues 603 to 847) of *albin1* flanking the 3′ end. The disruption cassette created was excised from this vector by digestion with HindIII/Xba1 and ligated into HindIII/SpeI-digested pCAMBIA-0380 (Cambia, Canberra, Australia) to create the vector pCwP1-HPH.

***A. tumefaciens*-mediated transformation.** Plasmid pCwP1-HPH was transformed into *A. tumefaciens*, and the resulting isolates were grown at 26°C for 2 days in minimal media containing 50 μg of kanamycin per ml with shaking at 300 rpm (17, 18). Sufficient cells were added to induction medium supplemented with 200 μM acetosyringone to obtain an optical density at 600 nm of 0.15 and grown for 6 h at 26°C with shaking at 300 rpm (7). Yeast-like cells of *O. piliferum* were grown in 2% malt extract at 20°C with shaking at 200 rpm for 5 days. Cells were filtered through three layers of cheesecloth, centrifuged at 2,000 × *g* for 5 min, washed twice with sterile distilled water, and resuspended at a concentration of 1×10^8 cells/ml. Equivalent volumes (10 ml) of the *A. tumefaciens* cells and fungal cells generated were mixed. One milliliter of the mixture was spread onto cellophane sheets (Bio-Rad) laid on induction medium agar plates supplemented with 200 μM acetosyringone and incubated for 5 days at 20°C. The cellophane sheets with cells were transferred to MEA plates supplemented with 300 μg of hygromycin per ml, 200 μM cefataxime, and 100 μg of moxalactum per ml to select for transformants and to kill the remaining *A. tumefaciens* cells. Transformants were picked from the plates with a sterile needle, transferred to MEA supplemented with 300 μg of hygromycin per ml, and subcultured as single spores.

Purification of genomic DNA and total RNA. Fungal DNA was purified as described by Kim and Breuil (24), with addition of a two-stage phenol chloroform extraction step prior to DNA precipitation by isopropanol. For purification of total RNA, frozen mycelia were disrupted as described by Kim et al. (23), and the total RNA was extracted by using a Trizol-based method (Invitrogen).

Southern analyses. One microgram of the wP1-1–wP1-4 amplicon was digested with NotI to remove the 5′ end of the amplicon, purified, and then labeled with 50 μCi of [α-^{32}P]dCTP (Amersham) by using Ready-To-Go labeling beads (Amersham). Unincorporated nucleotides were removed with G-50 microcolumns (Amersham). Ten-microgram portions of genomic DNAs were digested with 10 U of NotI for 16 h and separated on 1.0% agarose gels at 60 V for 8 h. DNAs were then transferred to Zeta-Probe GT blotting membranes (Bio-Rad) according to the manufacturer's recommendations. Membranes were hybridized with the *albin1* probe with the Ultrahyb solution (Ambion) at 50°C. Two room temperature washes were performed with 2× SSC–0.1% sodium dodecyl sulfate for 5 min, and then two more washes at 50°C were performed by using 0.1× SSC–0.1% sodium dodecyl sulfate for 15 min (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Protease assays. Cultures were filtered through a 70-μm-pore-size nylon cell strainer, and culture filtrates were collected. Proteolytic activity was determined by adding 50 μl of each culture filtrate to 100 μl of a solution containing 2 mg of succinylated casein (Pierce, Brookville, Ontario, Canada) per ml in 50 mM borate buffer (pH 8.5); 100 μl of buffer was added in control reactions. The reaction mixtures were incubated for 2 h at 37°C. Fifty microliters of trinitrobenzenesulfonic acid was then added to each mixture, and the reaction mixtures were incubated at room temperature for 20 min to allow color development, which was measured at 450 nm. Reactions and controls were done in triplicate. One unit of protease activity was defined as the amount of enzyme required to produce an absorbance of 1.

Nucleotide sequence accession number. The sequences of the bands generated by RACE-PCR have been deposited in the GenBank database under accession number AF413105.

RESULTS

Sequence of *albin1*. The mature Albin1 enzyme was 285 amino acids long and contained the active-site serine motif SGTSMA(T/S)PH, the active-site histidine motif GHGTH, and the active-site aspartic acid motif D(S/T)G, which are common to protease K subfamily subtilases (42). Phylogenetic analysis of this sequence showed that it grouped closely with the sequences of proteases T, K, and R from *Tritirachium album* and PR1 from *Metarhizium anisopliae* (17). This enzyme had greater than 80% amino acid identity with members of the same group and ~50% amino acid identity with other extra-

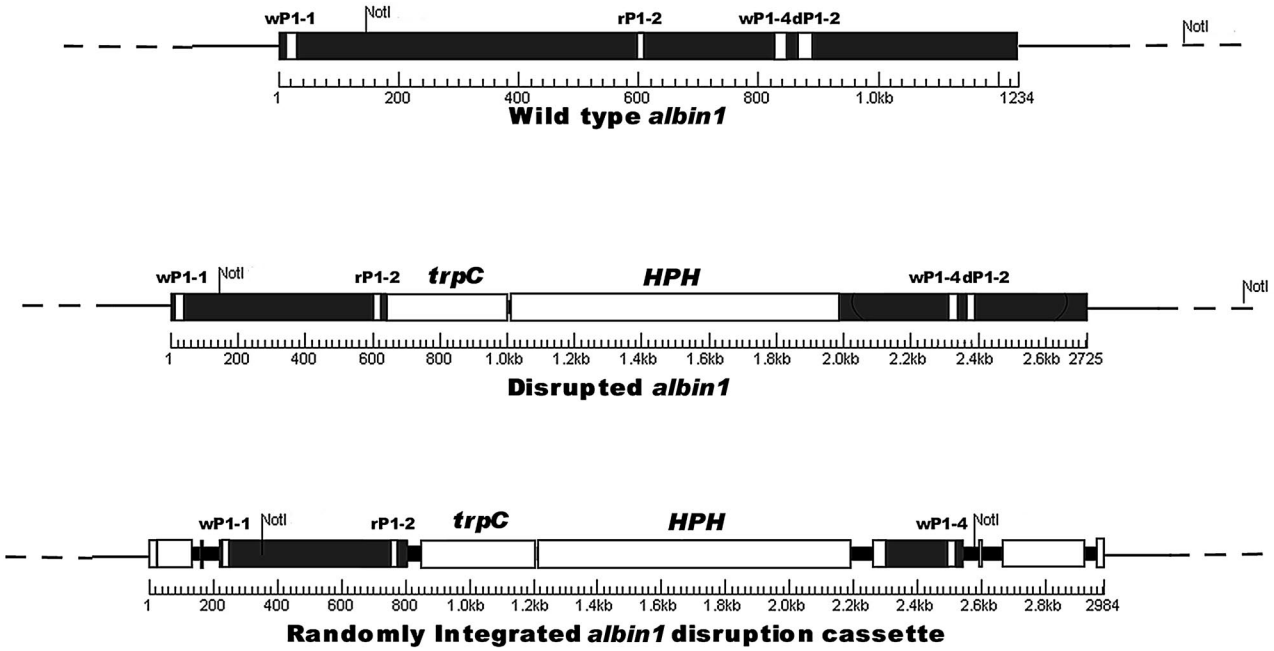

FIG. 1. Schematic representation of the wild-type *albin1* gene, a hypothetical disrupted *albin1* gene with the hygromycin expression cassette inserted into the gene, and a randomly integrated disruption cassette. Relevant primer sites are shown, as are the locations of NotI restriction sites and the location of the *albin1* probe.

cellular subtilases. The mature enzyme had a theoretical molecular mass of 33 kDa and an isoelectric point of 7.0.

Disruption of *albin1*. Approximately 50 transformants appeared on MEA plates supplemented with 300 μg of hygromycin per ml 5 to 7 days after transformation of 1×10^9 spores with *A. tumefaciens* harboring the pCwP1-HPH plasmid. The transformation was repeated twice with similar results, and 200 transformants were retained for further study. Control transformations with the pCambia-hph vector had similar transformation efficiencies. Thirty-five transformants produced little or no noticeable clearing in the SMA. Five of these transformants were morphologically similar to the wild type on SMA and MEA. These transformants retained their phenotypes on SMA and MEA and their resistance to hygromycin after three successive rounds of plating, indicating that they contained stably integrated copies of the disruption cassette.

Southern analysis was used to confirm insertion of the disruption cassette into the *albin1* locus of three transformants that produced little or no noticeable clearing in the SMA (isolates 21, 23, and 34). First NotI was used to digest the genomic DNAs from the transformants and from the wild type. This enzyme cuts *albin1* near the 5′ end, and the cut site occurs in the 5′ ends of both the wild-type gene and the disruption cassette (Fig. 1). A NotI cut site also was present between the 3′ end of *albin1* and the left T border of the disruption construct. This site would be inserted into the host genome during a random integration event; however, this site would be lost during homologous integration mediated by the 5′ and 3′ *albin1* segments. In these analyses we used a NotI-digested *albin1* probe, which removed the 5′ end of *albin1*, which reduced the number of bands in the Southern blot. The wild type produced a band at ~5.2 kb (Fig. 2). Strains with an ectopic integration had both the 5.2-kb wild-type band and a band at ~2.2 kb, which resulted when NotI cut at the 5′ and 3′ ends of the disruption cassette. Transformant 21 produced both a ~2.2-kb band and a ~6.2-kb band. The ~6.2-kb band could

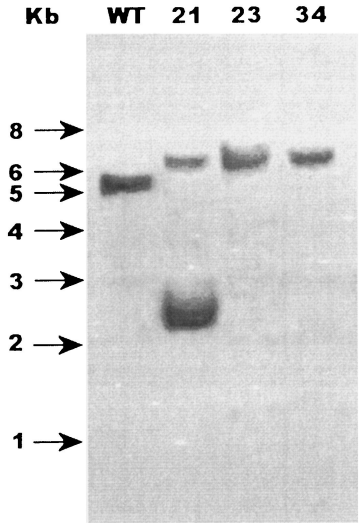

FIG. 2. Southern blot analysis of transformants 21, 23, and 34 and wild-type *O. piliferum* with an *albin1* probe. Ten micrograms of genomic DNA from each of the transformants and the wild type was digested with NotI and run on a 1% agarose gel for 8 h. The DNA was transferred and hybridized with a NotI-digested wP1-1–wP1-4 amplicon probe. WT, wild type.

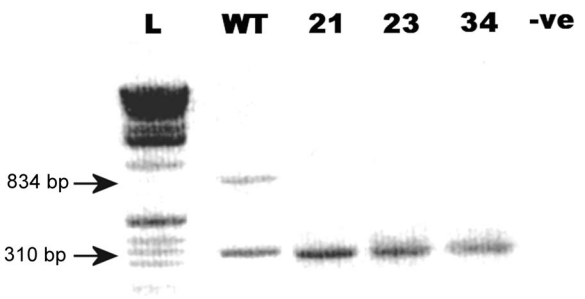

FIG. 3. Multiplex RT-PCR with *albin1*-specific and internal transcribed spacer-specific primer pairs and the cDNAs of transformants 21, 23, and 34 and wild-type *O. piliferum*. cDNA from each of the transformants and the wild type was amplified by using the NL1-NL2 and wP1-1–wP1-4 primer pairs in multiplex PCRs with a hybridization temperature of 56°C and 40 cycles of amplification. Lane L contained a 1-kb DNA ladder (Life Technologies). WT, wild type; -ve, no-RT wild-type control.

have resulted from the integration of the disruption cassette into the wild-type *albin1* locus. Transformants 34 and 23 produced only the ~6.2-kb band, indicating that the disruption cassette had specifically integrated into the *albin1* locus in these transformants.

In a multiplex RT-PCR with primers wP1-1 and wP1-4, the wild type and all the transformants produced an NL-1–NL-2 amplicon (Fig. 3), but transformants 21, 23, and 34 could not produce a wP1-1–wP1-4 amplicon, suggesting that these transformants could not produce a wild-type *albin1* transcript.

Analysis of *albin1* disruptants. The wild type and transformant 39 grew similarly in BSA media, while transformant 19 produced ~30% less biomass (Table 1). All of the other transformants grew substantially less, producing <40% of the wild-type growth. In BSA media supplemented with glucose and ammonia all of the transformants and the wild type grew similarly.

In BSA media the wild type and transformant 39 produced similar amounts of extracellular proteolytic activity per milligram of biomass produced, while transformant 19 produced ~20% more activity than the wild type (Table 1). None of the other transformants produced more than 40% of the proteolytic activity of the wild type.

TABLE 1. Growth and extracellular proteolytic activities of wild-type *O. piliferum* and selected transformants in BSA or BSA-NH_4-glucose medium

Isolate	Growth in BSA medium (mg)[a]	Growth in BSA-NH_4-glucose medium (mg)[a]	Extracellular proteolytic enzyme activity (U/mg)[a]
Wild type	340 ± 60	390 ± 55	0.00039 ± 0.00004
19	220 ± 50	360 ± 70	0.00046 ± 0.000005
21	52 ± 30	340 ± 54	0.00007 ± 0.00002
23	55 ± 36	345 ± 48	0.00016 ± 0.00001
30	122 ± 30	380 ± 58	0.00004 ± 0.00002
34	32 ± 22	350 ± 45	0.00013 ± 0.00001
39	342 ± 40	425 ± 64	0.00035 ± 0.00006

[a] A total of 10^7 yeast and/or spore cells of transformants 19, 21, 24, 30, 34, and 39 and the wild type were inoculated into BSA medium or BSA medium supplemented with glucose and ammonia and incubated for 8 days. The growth and weight of the dried fungal cultures were measured as described in Materials and Methods. The proteolytic activities of BSA cultures were measured as described in Materials and Methods.

To assess the growth of the six transformants and the wild-type strain on wood, these strains were inoculated onto pine wood blocks and grown for 2 weeks. Isolates 19, 30, and 39 grew like the wild type, while isolates 21, 34, and 23 grew slowly and caused little staining of the wood blocks. Ergosterol analysis of the wood blocks inoculated with transformants 34 and 23, the two isolates with a specifically disrupted *albin1* locus, revealed that these two isolates produced ~50% less fungal biomass (6.6 ± 0.2 μg of ergosterol/g of wood) and ~40% less fungal biomass (9.1 ± 0.8 μg of ergosterol/g of wood), respectively, than the wild type (13.9 ± 0.9 μg of ergosterol/g of wood). Uninfected wood had only 0.9 ± 0.1 μg of ergosterol/g of wood.

DISCUSSION

The *albin1* gene of *O. piliferum* encodes a protein containing 285 amino acids. The inferred amino acid sequence contains active-site motifs common to protease K subfamily subtilases (42) and is in the size range previously reported for fungal subtilases (20, 27). Albin1 groups phylogenetically with extracellular subtilases T, K, R, and Pr1 from *M. anisopliae* that degrade the cuticle of the exoskeleton of host insects (35). If Albin1 is an extracellular subtilase responsible for the degradation of exogenous proteins, then the *albin1* gene may be important for the growth of *O. piliferum* on wood.

Recently, *A. tumefaciens* has been used to transform a number of fungi, including the closely related sapstaining fungi *Ophiostoma piceae* and *Ceratocystis resinifera* (45, 26). The *Agrobacterium tumefaciens*-mediated transformation efficiency of *O. piliferum* was 50 transformants per 10^7 cells, which is similar to the values reported for other fungi, including *Mycosphaerella grammicola*, *Coccidiodes immitis*, and *Fusarium venenatum* (2, 12, 49). This technique typically generates transformants with a single copy of the integrated DNA and is thought to increase targeting to homologous regions of DNA, although one of our transformants, transformant 21, had more than one copy of the disruption vector (2, 10, 12, 29, 31, 49). Approximately 17% of the *O. piliferum* transformants that we recovered had diminished extracellular activity, indicating that the expected targeting of the protease gene was occurring.

albin1 was successfully disrupted in transformants 23 and 34. No genes have previously been disrupted in any sapstaining species of *Ophiostoma*. A few genes have been disrupted in the closely related fungus *Ophiostoma novo-ulmi*; however, none of the disruptants had a significantly altered phenotype (6, 13, 15), perhaps due to the presence of redundant or equivalent genes that masked the effects of the disruption (15). Disruption of *albin1* severely impaired *O. piliferum*'s production of extracellular proteolytic activity and its growth in BSA. The restoration of the growth of the targeted disruptants in BSA media supplemented with glucose and ammonia indicated that it was solely the loss of the ability of these mutants to acquire carbon and nitrogen from exogenous protein that caused their impaired growth. The disruption of *albin1* also severely affected *O. piliferum*'s growth on wood, and the disruptants produced only one-half the biomass that the wild type produced on wood. The *albin1* disruptants could grow on wood as wood

contains amino acids and inorganic sources of nitrogen, as well as proteins. Also, the wood microenvironment may have stimulated the production of alternative proteases.

Our results show that *albin1* encodes an extracellular subtilase involved in the acquisition of nutrients from host tissues and is important in *O. piliferum*'s growth on wood. *albin1* and the enzyme that it encodes could be useful targets for the development of novel sapstain control strategies. The amount and form of nitrogen available in trees could influence the growth of sapstaining fungi. As the levels fluctuate with the season, this information may help identify high- and low-risk periods for harvesting logs and could explain some of the inconsistencies observed in field trials of the biocontrol fungus Cartapip-97 (4, 38, 39, 47).

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