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Effect of surface wettability on germination and gene expression in *Cronartium quercuum* f. sp. *fusiforme* basidiospores

L.G. Baker[a,*], P. Spaine[b], S.F. Covert[c]

[a]*Department of Genetics, University of Georgia, Athens, GA, USA*
[b]*USDA Southern Forest Research Station, University of Georgia, Athens, GA, USA*
[c]*Daniel B. Warnell School of Forestry and Natural Resources, University of Georgia, Athens, GA, USA*

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Abstract

Cronartium quercuum f. sp. *fusiforme* is an obligate pathogen of pine and oak. Its basidiospores are specifically adapted to recognize and establish infections on the pine host. Depending on environmental cues, the basidiospores can germinate directly, which typically leads to infection of pine, or indirectly, which usually results in formation of secondary basidiospores. We investigated how changes in surface wettability, or hydrophilicity, affect basidiospore germination. When we decreased surface wettability, the direct germination and total germination (direct + indirect) frequencies increased. Additionally, there was a critical threshold between 42% and 54% wettability, at which the basidiospores switched from direct to indirect germination. We conclude that the germination type of *C. q. fusiforme* basidiospores is influenced by the hydrophilicity of the surface upon which they land. To gain insight into gene expression during basidiospore germination, we made two suppression subtraction hybridization (SSH) libraries enriched for genes expressed during each type of germination. A total of 172 unique gene sequences were recovered from the two expression libraries. Annotation of these libraries indicates that they include several clones that may encode rust-specific or basidiomycete-specific functions.
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Keywords: *Cronartium quercuum* f. sp. *fusiforme*; Basidiospore; Germination; Wettability; EST

1. Introduction

The heteroecious rust fungus *Cronartium quercuum* (Berk.) Miyabe *ex* Shirai f. sp. *fusiforme* is the causative agent of the pine disease fusiform rust. This disease is characterized by woody, spindle-shaped galls that reduce the value and weaken the wood of the pathogen's primary hosts, loblolly pine (*Pinus taeda* L.) and slash pine (*Pinus elliottii* Engelman) [1,2]. In the last 50 years, fusiform rust has become a serious threat to commercial forestry in the southeastern United States [3]. It is credited with causing $25 million to $135 million in losses annually, with losses varying depending on how the trees are utilized [1,4]. Improved silviculture, breeding [2,3] and the identification of resistance genes in pine [5–7] have all aided in the reduction of losses due to fusiform rust, but it is not yet clear if these methods will allow for durable control of fusiform rust throughout the pathogen's range. Furthermore, intensive pine plantation management practices, such as weed control and fertilization, are associated with elevated rates of fusiform rust [3,8–12]. In this context there is a need for basic research that could support the development of novel *C. q. fusiforme* control methods.

C. q. fusiforme basidiospores are the infectious agents that establish fusiform rust disease on pine. They are made on the leaves of *C. q. fusiforme*'s alternate host, oak, and are transported to pine by the wind. As in other pathogenic fungi, successful spore germination in *C. q. fusiforme* requires a combination of specific environmental signals, such as correct humidity and temperature [4,13–15]. Furthermore, depending on the surface upon which *C. q. fusiforme* basidiospores land, they undergo one of two types of germination [13–15]. If they land on a pine needle or on polystyrene, they send out long, thin germ tubes

*Corresponding author. Tel.: +1 314 977 9216; fax: +1 314 977 9205.
E-mail addresses: bakerlg2@slu.edu (L.G. Baker), pspaine@uga.edu (P. Spaine), covert@uga.edu (S.F. Covert).

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more than 95% of the time and are said to germinate "directly" (Fig. 1a; [13–15]). Submerging *C. q. fusiforme* basidiospores in water for less than 1 min increases the frequency of direct germination, apparently because it removes water-soluble inhibitors of direct germination [13,14]. In nature, this type of germination is associated with penetration of the succulent stem or branch tissues and ultimate infection of pine [16]. However, if *C. q. fusiforme* basidiospores land on glass, they often produce short, thick germ tubes with secondary basidiospores at their tips (Fig. 1b; [13–15]). This type of germination is known as "indirect" germination. Secondary basidiospores can be ejected from the tips of their germ tubes and germinate if they land on a suitable host or substrate (Fig. 1b; [13–15]). Depending upon the landing surface and water availability, the basidiospores of other rust fungi also germinate directly or indirectly [17–22]. Therefore, the production of secondary basidiospores during indirect germination appears to be an adaptation that provides rust fungi with a second opportunity to land in an environment hospitable to direct germination [22].

Why do *C. q. fusiforme* basidiospores germinate directly on polystyrene and indirectly on glass? One obvious difference between these two substrates is their hydrophilicity, which can be measured as "percent wettability". Hydrophobic surfaces, such as polystyrene, have a low wettability score, whereas hydrophilic surfaces, such as glass, have a high wettability score [23]. Therefore, the first objective of this study was to investigate how surface wettability influences *C. q. fusiforme* basidiospore germination. Towards this end, polystyrene and glass substrates were manipulated to increase or decrease their surface wettabilities. In addition, to better understand how *C. q. fusiforme* responds to differing surfaces, two suppression subtraction hybridization (SSH) libraries enriched for cDNAs expressed in either directly or indirectly germinating basidiospores were made. These sequences are predicted to include determinants of the different germination responses. This work establishes a foundation for understanding the environmental and molecular regulation of basidiospore germination and sporulation.

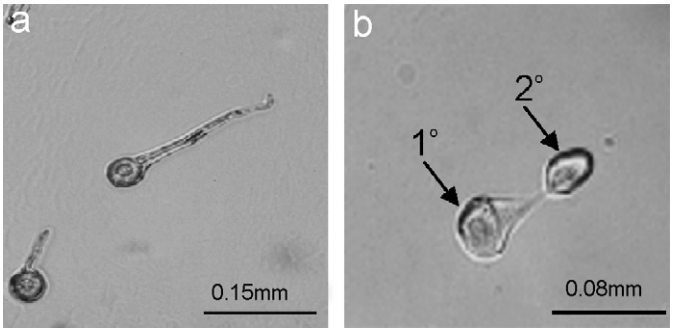

Fig. 1. *C. q. fusiforme* direct (a) and indirect (b) basidiospore germination. Primary and secondary spores are indicated by arrows in panel B.

2. Materials and methods

2.1. Measurement of surface wettability

The percent wettability of polystyrene (Fisher Bio Sciences), Nunclon® Δ polystyrene (NUNC), and glass surfaces was determined by averaging the diameter of spreading drops from a series of methanol dilutions as described elsewhere [23]. Glass slides were coated with dimethyldichlorosilane (DMS) and diphenyldichlorosilane (DPS) as described by Terhune and Hoch [23] with the following exception; instead of being baked at 400 °C, the glass slides were cleaned with detergent and then rinsed at least 3× with deionized water. For ease of use, polystyrene and Nunclon® Δ polystyrene petri-plates were cut into approximately 3″ × 1″ sections.

2.2. Basidiospore production

Leaves of *Quercus rubra L.* were inoculated with *C. q. fusiforme* aeciospores (mixed gall collection, Asheville, North Carolina and Clarke County, Georgia) at the USDA Forest Service Resistance Screening Center (Asheville, North Carolina) according to the method of Knighten et al. [24]. Oak leaves bearing telia were collected approximately 3–4 weeks after inoculation and hydrated in a polystyrene petri dish for 36 h at 20 °C in the dark.

For the wettability tests, basidiospores from the hydrated leaves were cast directly onto three to four surfaces of different wettability for 5 h at 20 °C in the dark. The oak leaves were removed from the hydration chambers and the chambers resealed. The basidiospores were allowed to germinate for an additional 5 h at 20 °C, in the dark. Two hundred and fifty spores per surface were scored in 13 replicate experiments for germination and germination type. Statistical analysis of the germination data was prepared using the student's paired *t*-test, $p = 0.05$.

For the gene expression studies, oak leaves with telia were hydrated for 30–36 h while suspended over polystyrene or glass petri chambers at 20 °C in the dark. Because basidiospores on different leaves were formed at variable rates, the spores in each chamber were classified into one of three germination categories: "most spores non-germinating", denoted by a lack of germ tubes; "most spores germinating", denoted by the presence of germ tubes at least 1–2 μm in length; or "almost all spores germinated", denoted by the production of hyphal masses on polystyrene and the development of secondary basidiospores on glass. To ensure that the full suite of developmental stages from each surface was represented in the subsequent RNA samples, representatives of all 3 germination categories were pooled together in equal amounts in each final sample. A rubber policeman and 200 μl of diethyl pyrocarbonate treated deionized water were used to loosen the basidiospores from the surfaces. The water containing the basidiospores was aspirated from the surfaces and flash

frozen in liquid nitrogen. Samples were stored in –80 °C until RNA extraction.

2.3. RNA extraction

Pools of *C. q. fusiforme* basidiospores were ground using micro-plastic pestles (Kontes Glass Co.; Vineland, NJ) and 450–600 μm acid-washed glass beads (Sigma; St Louis, MO). RNA was extracted as previously described [25]. RNA samples were DNAse treated, cleaned with phenol:-chloroform (3:1), and quantified using the Ribogreen RNA quantification kit (Molecular Probes, Eugene, OR). RNA quality was determined using formaldehyde denaturing gels.

2.4. SSH library construction

Two SSH cDNA libraries (direct and indirect) were made from 1 μg of total RNA using the Smart PCR cDNA Synthesis Kit and the PCR-Select cDNA Subtraction Kit (CloneTech Laboratories Inc; Palo Alto, CA) following the manufacturer's directions. The cDNAs were ligated into the TOPO TA pCRII vector (Invitrogen; Carlsbad, CA) and 960 clones were picked from each library. Plasmid DNA was extracted from all clones using Rapid Extraction Alkaline Lysis (R.E.A.L.) Prep 96 (Qiagen; Valencia, CA).

2.5. DNA sequence analysis

The SSH libraries were sequenced using a 96-well format ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1 or 2 (Applied Biosystems; Foster City, CA). Unincorporated dye terminators were removed from the samples using QIAquick 96 PCR Purification Kit (Qiagen; Valencia, CA). The libraries were sequenced at the University of Georgia, Genome Analysis Facility, Athens, GA. The sequences from each SSH library were assembled into contigs using SeqMan (DNAStar; Madison, WI). Consensus sequences from the contigs were used for BLAST searches (blastx and tblastx) against the NCBI nr and EST databases, using the BLOSUM62 matrix (June 2006). BLAST hits with *E*-values $<10^{-5}$ were judged to be indicative of significant homology. cDNA sequences from the longest read of a single clone within each contig were deposited into the NCBI EST database (Accession nos. 910990–911173). Additionally, all sequences generated during this study were deposited into GenBank (Accession nos. 42312331–42312269).

3. Results and discussion

3.1. Effect of surface wettability on C. q. fusiforme germination

The wettability of the polystyrene and glass surfaces used in this study was $21\pm2.3\%$ and $54\pm1.6\%$ (mean $\pm$ SE), respectively, and each caused basidiospores to germinate as previously reported (Figs. 1 and 2; [14]). To determine if surface wettability influences the germination type of *C. q. fusiforme* basidiospores, glass slides were coated with either DMS or DPS, which decreased the surface wettability to $14\pm2.0\%$ and $20\pm1.8\%$, respectively. Additionally,

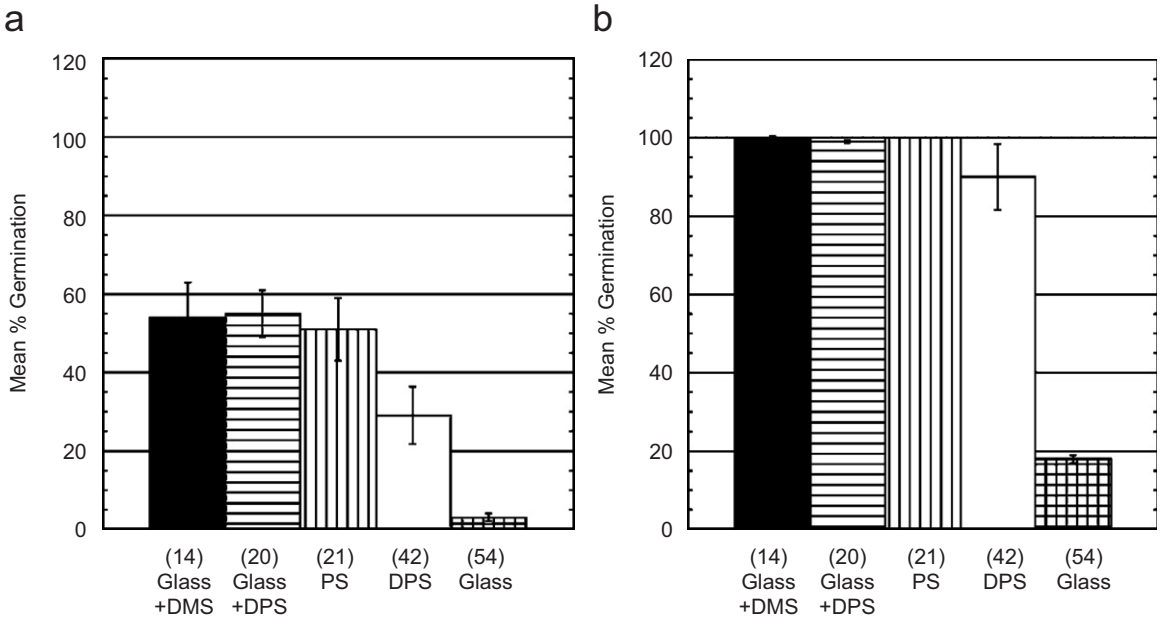

Fig. 2. Frequency of *C. q. fusiforme* basidiospore germination on surfaces with different wettabilities. (a) Percentage of basidiospores that germinated either directly or indirectly. (b) Percentage of germinated basidiospores that germinated directly. DMS = dimethyldichlorosilane, DPS = diphenyldi-chlorosilane, PS = untreated polystyrene, Δ PS = Nunclon^{TM} Δpolystyrene. Error bars indicate standard error and parentheses indicate surface wettability of treatments.

Nunclon® Δ polystyrene, a relatively hydrophilic polystyrene that is sold for certain tissue culture applications, provided an intermediate wettability of 42 ± 2.3%.

When hydrated *C. q. fusiforme* basidiospores were cast on these different surfaces and allowed to germinate for up to 10 h, the highest frequencies of germination (50–55%) occurred on the three hydrophobic surfaces, i.e., on both types of silane-treated glass (DMS and DPS) and polystyrene (Fig. 2a). In contrast, only 3% of the spores germinated on untreated glass, which was the most wettable surface in the study (Fig. 2a). This frequency of germination was much lower than that observed previously (77.6%) for spores cast directly on glass and incubated for shorter periods of time [14], but differences in the genotypes or ages of the telial columns might account for this between-experiment variation. In our study, the majority of *C. q. fusiforme* basidiospores germinated regardless of surface if the combined casting and germination time was extended beyond 10 h (data not shown), indicating that most spores were viable. The frequency of germination on Nunclon™ Δ polystyrene was intermediate between that of the most hydrophobic and most wettable surfaces (Fig. 2a). The differences between germination frequency on Nunclon™ Δ polystyrene and untreated polystyrene ($p < 0.05$) or untreated glass ($p < 0.004$) were statistically significant. Furthermore, as wettability increased from 42% (Δ polystyrene) to 54% (untreated glass), the proportion of germinated spores that germinated directly decreased significantly from 100% to 18% ($p < 0.001$, Fig. 2b). These data indicate that surface wettability is an important determinant of basidiospore germination frequency and germination type in *C. q. fusiforme*, and more specifically that conditions of low wettability are associated with the developmental changes that promote host infection.

Although many researchers have studied post-germination surface recognition and signaling in rust fungi (i.e. *Uromyces* species), there have been few studies on the signals that trigger spore germination in rusts. In our study, both the frequency of germination and the frequency of direct germination decreased dramatically between 42% and 54% wettability, suggesting that *C. q. fusiforme* basidiospores respond to a critical wettability threshold at some point between these two values. Similarly, *Colletotrichum graminicola* and *Phyllosticta ampelicida* spore germination frequencies drop significantly when surface wettability rises across a relatively narrow threshold [26,27].

Although the basidiospores of *C. q. fusiforme* may sense and respond to surface wettability directly, they may also be influenced by the length of time that water stands on different surfaces. For example, the infection of hybrid poplar leaves by *Melampsora larici-populina* is positively correlated with the duration of leaf surface moisture, which in turn varies according to the wettability of different poplar clones, i.e., water or dew droplets on the most wettable clones are relatively broad and quick-drying, while those on the least wettable clones are mounded and more persistent [28]. It is possible, therefore, that hydrophobic surfaces promote direct germination of *C. q. fusiforme* basidiospores because they allow standing water to accumulate to a height that submerges the spores and rinses away the soluble factors that otherwise inhibit direct germination.

Manipulating the surface properties of plant tissues may provide us with novel methods for rust disease control. For example, selecting for leaves or needles with high surface wettability in plant breeding programs might provide protection against a wide range of rust pathotypes by inhibiting germination and/or infection [28]. Alternatively, it might be possible to control disease by treating nursery seedlings with chemicals that temporarily increase surface wettability and thus inhibit direct germination.

3.2. Genes expressed during direct and indirect germination

To gain a better understanding of how substrate wettability affects gene expression in germinating *C. q. fusiforme* basidiospores, two SSH libraries enriched for sequences expressed in either directly or indirectly germinating basidiospores were made. Nine hundred sixty clones from each library were sequenced and assembled into overlapping contigs. Of the 172 different cDNA contigs identified in the libraries, 68 were unique to the direct library, 87 were unique to the indirect library, and 17 were common to both libraries. Based on BLAST search results, 62 of the deduced amino acid sequences from these contigs were homologous to entries in the NCBI non-redundant or EST databases (Table 1). The majority (61%) of the homologous sequences came from other basidiomycetes, like *Ustilago maydis* and *Cryptococcus neoformans*, but a significant number (24%) also came from a variety of ascomycetes (Table 1). The *C. q. fusiforme* contigs were placed in annotation categories based on their homology to characterized proteins, predicted proteins with conserved domains, or genes expressed in particular fungal tissues (Table 2). The largest single annotation category was composed of hypothetical proteins of unknown function and many other categories were comprised of proteins predicted to be involved in basic cellular metabolism (Table 2). However, among the categories defined by expression patterns, there were several contigs that are candidates for rust-specific or basidiomycete-specific functions, both of which represent poorly understood aspects of fungal biology.

Within the "Expressed by rust fungi *in planta*" category (Table 2), contigs C676 and I732 were homologous to two sequences previously identified as being expressed by *C. q. fusiforme* in pine stems [29], and C694 was homologous to a sequence expressed by the wheat rust pathogen, *Puccinia graminis*, in wheat leaves [30](Table 1). These three contigs may encode rust-specific proteins. The I732 sequence is not homologous to any other protein or EST sequences at NCBI, C676 is only similar to several other alleles of the

Table 1
Top BLAST hits for *C. q. fusiforme* cloned sequences

Contig name[a]	Length (amino acids)	Top BLASTx or tBLASTx Hit [Organism] (Accession #)[b]	BLAST E Value	No. of Clones[c]
C671	369	Hypothetical protein UM04145.1 [*Ustilago maydis*] (XP_760292); signal peptide; expressed in germinating teliospores	8E-13	130
C676	222	G32 Galled pine stem suppression subtraction hybridization library [*Pinus taeda*/*Cronartium quercuum* f. sp. *fusiforme*] (CN852372)	2E-92	68
C679	354	Unnamed protein product [*Kluyveromyces lactis*] (XP_453836)	1E-22	27
C680	257	Hypothetical protein FG10252.1 [*Gibberella zeae* PH-1] (XP_390428)	7E-09	14
C699	283	Putative senescence-associated protein [*Pisum sativum*] (BAB33421)	2E-45	5
C694	205	2_g01 SSH-library of infected wheat leaves [*Puccinia graminis*] (CV191710)	2E-19	25
C724	216	Putative cytoplasm protein [*Cryptococcus neoformans* var. *neoformans* JEC21] (AAW42472); predicted GTPase	6E-11	14
C702	179	Putative secreted protein [*Ixodes scapularis*] (AAY66614)	7E-06	13
C714	199	Unnamed protein product [*K. lactis*] (XP_453844)	3E-06	11
C741	207	cEgh16 from germinating conidia [*Blumeria graminis*] (AAB05211)	2E-08	18
D725	128	Hypothetical protein [*Rattus norvegicus*] (XP_580193)	2E-05	1
D805	85	Putative sphingosine hydroxylase [*C. neoformans* var. *neoformans* JEC21] (XP_568900)	2E-23	1
D816	182	Chitinase [*Puccinia triticina*] (AAP42832)	7E-10	6
D818	150	Carboxypeptidase Y [*Pichia pastoris*] (CAA61240)	2E-15	1
D819	818	Hypothetical protein CAGL0M13651 g [*Candida glabrata* CBS138] (XP_449937); peptidase S10 domain	7E-15	18
D820	202	Hypothetical protein UM03291.1 [*U. maydis* 521] (XP_759438)	8E-05	2
D824	177	Pepsin IIB [*Gadus morhua*] (P56272)	3E-06	1
D825	178	Hypothetical protein CNK01730 [*C. neoformans* var. *neoformans* JEC21] (AAW46332); fasciclin extracellular domain	8E-05	1
D832	67	Uf298 haustorium-specific cDNA library similar to senescence-associated protein, mRNA sequence [*Uromyces viciae-fabae*] (DR010476)	2E-21	1
D834	230	Ribosomal protein L21 homolog [*S. pombe*] (BAA24802)	3E-49	3
D837	111	Predicted small heat shock protein [*Paxillus filamentosus*] (AAT91263)	5E-10	1
D839	103	Hypothetical protein DEHA0F10439 g [*Debaryomyces hansenii* CBS767] (XP_460775); glutathionine peroxidase domain	1E-06	1
D841	192	Alpha-glucosidase precursor, Maltase [*S. pombe*] (Q9C0Y4)	2E-54	1
D842	212	cDNA from germinating urediniospores [*Phakopsora pachyrhizi*] (DN740039)	1E-28	2
D845	132	Hypothetical protein UM04972.1 [*U. maydis*521] (XP_761119); thioredoxin-like protein	2E-35	1
D847	131	Hypothetical ribosomal protein UM04828.1 [*U. maydis* 521] (XP_760975)	2E-18	1
D857	185	Exo-beta-1,3-glucanase [*Lentinula edodes*] (BAD97446)	1E-26	1
D860	185	Hypothetical protein UM03045.1 [*U. maydis* 521] (XP_759192); expressed in filamentous diploid library	4E-17	1
D861	110	Hypothetical protein UM00662.1 [*U. maydis* 521] (XP_756809)	8E-12	1
D864	118	Chain D, Human Glyoxalase I [*Homo sapiens*] (1QIPD)	4E-17	1
D865	118	Hypothetical protein UM06061.1 [*U. maydis* 521] (XP_762208)	1E-08	1
I673	78	Predicted tissue specific transplantation antigen P35B [*Homo sapiens*] (AAH01941); epimerase domain	1E-06	1
I683	70	Putative phospholipid:diacylglycerol acyltransferase [*C. neoformans* var. *neoformans* JEC21] (AAW46497)	9E-09	1
I693	213	Hypothetical protein FG04972.1 [*Gibberella zeae* PH-1] (XP_385148)	2E-08	7
I700	99	Putative endopeptidase [*C. neoformans* var. *neoformans* JEC21] (AAW44743)	5E-10	1
I701	90	Putative acetyl-Coenzyme A acyltransferase 2 [*C. neoformans* var. *neoformans* JEC21] (AAW40978)	4E-10	1
I704	197	Hypothetical protein UM00173.1 [*U. maydis* 521] (XP_756320); nodulin-like domain	3E-06	5
I707	198	Conserved hypothetical protein [*C. neoformans* var. *neoformans* JEC21] (AAW41163)	4E-05	3
I708	140	Putative ribosomal protein S19 [*Pleurotus ostreatus*] (CAD10794)	9E-17	2
I709	175	Malate dehydrogenase [*Paracoccidioides brasiliensis*] (AAP37966)	5E-48	1
I710	58	Putative ATP-binding cassette (ABC) transporter [*C. neoformans* var. *neoformans* JEC21] (AAW43068)	5E-21	1
I712	105	Unnamed protein product [*Tetraodon nigroviridis*] (CAG07445); isocitrate dehydrogenase domain	1E-06	1
I715	95	Hypothetical protein UM01150.1 [*U. maydis* 521] (XP_757297); expressed in germinating teliospores	1E-06	2
I718	131	MGC78921 predicted protein [*Xenopus laevis*] (AAH72051)	2E-05	1
I719	157	Hypothetical protein SPAC222.04c [*S. pombe*] (CAB60696)	2E-17	1
I721	108	Putatve opa-interacting protein OIP2 [*C. neoformans* var. *neoformans* JEC21] (AAW41250)	8E-11	1

Table 1 (*continued*)

Contig name[a]	Length (amino acids)	Top BLASTx or tBLASTx Hit [Organism] (Accession #)[b]	BLAST E Value	No. of Clones[c]
I723	127	Hypothetical protein UM06102.1 [*U. maydis* 521] (XP_762249); cellulase domain	1E-10	2
I726	129	Tryptophan synthetase [*Coprinopsis cinerea*] (AAP79219)	5E-45	1
I730	125	Hypothetical protein UM05277.1 [*U. maydis* 521] (XP_761424); mitochondrial ATP synthase domain	3E-15	1
I732	80	G65 Galled pine stem suppression subtraction hybridization library [*P. taeda*/*C. q. fusiforme*] (CN852395)	1E-38	1
I736	140	Hypothetical ribosomal protein UM06388.1 [*U. maydis* 521] (XP_762535)	2E-19	1
I740	69	Hypothetical protein UM00309.1 and UM00961.1 [*U. maydis* 521] (XP_756456); expressed in filamentous diploid library	6E-05	1
I743	108	Hypothetical protein UM00309.1 [*U. maydis* 521] (XP_756456); Rieske cytochrome bc1 iron-sulfur domain	6E-05	1
I749	192	Phosphoribosyl-5-amino-1-phosphoribosyl-4-imidazolecarboxiamide isomerase [*P. pastoris*] (AAT07972)	1E-16	1
I752	160	Predicted histone H3 [*Lentinula edodes*] (BAD11819)	1E-36	2
I766	123	Uf077 haustorium-specific cDNA library similar to vacuole protein [*Uromyces viciae-fabae*] (DR010255)	3E-12	1
I769	117	Polyubiquitin [*Prunus avium*] (AAL25813)	7E-38	2
I772	144	Predicted related to stress response protein rds1p [*Neurospora crassa*] (CAD21425)	1E-09	1
I776	160	Predicted ER to Golgi transport-related protein [*C. neoformans* var. *neoformans* JEC21] (AAW41532)	2E-18	1
I777	90	Hypothetical protein UM01629.1 [*U. maydis* 521] (XP_757776)	3E-06	1
I781	105	20S Proteasome [*Saccharomyces cerevisiae*] (BAA00725)	4E-17	1
I891	250	Riboflavin aldehyde-forming enzyme [*Agaricus bisporus*] (CAB85691)	1E-14	4

[a]The first letter of each contig name denotes if the sequences in a given contig were common to both subtracted libraries, or if they were found only in the Directly germinating library or Indirectly germinating library.

[b]Descriptions of domains (if any) or expression patterns (if known) are supplied for hypothetical proteins.

[c]Number of clones making up each contig. For contigs common to both subtracted libraries, the number from each library is indicated (D = Directly germinating, I = Indirectly germinating).

Table 2
Summary of *C. q. fusiforme* clones with BLASTx or tBLASTx hits

Annotation category	No. of clones
Hypothetical protein, unknown function	17
Protein degradation	6
Carbohydrate metabolism	5
Expressed by rust fungi *in planta*	5
Ribosomal protein/processing	5
Expressed in germinating spores	4
Glycolysis and respiration	3
Amino acid biosynthesis	2
Expressed in filamentous cells	2
Membrane metabolism	2
Stress response	2
Chitin metabolism	1
Chromatin	1
Efflux pump	1
Enzyme of unknown function	1
Extracellular, unknown function	1
Lipoprotein metabolism	1
Protein stabilization	1
Secretion	1
Vitamin B2 metabolism	1

same locus cloned from additional *Cronartium* species (Accession nos. DQ009562–DQ009567), and C694 is similar to only three other ESTs, all from rust fungi (data not shown). Although limited homology is not strong evidence for rust-specificity, this limited distribution of homologous sequences is nonetheless noteworthy given that the number of completely sequenced fungal genomes accessioned at NCBI currently exceeds 50 (http://www.ncbi.nlm.nih.gov/genomes/leuks.cgi). Of the two remaining members of the "Expressed by rust fungi *in planta*" category, D832 may represent a basidiomycete-specific sequence; in addition to an EST from *Uromyces viciae-fabae* haustoria (Table 1; [31]), it was similar to ESTs from many other basidiomycetes, but not from ascomycetes (data not shown). Contig I766 was most similar to another EST from *U. viciae-fabae* haustoria. However, it was also similar to ESTs from many different types of fungi and plants (data not shown), so it remains a candidate for a widespread cellular function.

The contigs similar to sequences "Expressed in germinating spores" included C671, C741, D842, and I715. Within this category, contigs C671 and I715 were homologous only to sequences from *U. maydis* (data not shown), thus they may represent functions specific to spore germination in plant pathogenic basidiomycetes. D842 may represent a sequence specific to germinating rust spores as its most similar homolog at NCBI was a sequence expressed in germinating urediniospores from *Phakopsora pachyrhizi*, the soybean rust [32]. Three ESTs detected in

poplar leaves were also highly related to D842, but these sequences did not correspond to DNA in the poplar genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html; data not shown), raising the possibility that these ESTs were derived from a microbial contaminant of poplar leaves, such as the poplar rust pathogen *M. larici-populina*. Finally, C741 was most similar to cEgh16, a gene highly expressed in germinating conidia from the obligate pathogen, *Blumeria graminis* [33]. Two cEgh16-homologous proteins in *Magnaporthe grisea*, GAS1 and GAS2, are expressed specifically in appressoria and are required for host penetration and full virulence [34], but related proteins are also found in at least one non-pathogenic fungus, i.e., *Neurospora crassa*.

To determine if the 172 cDNA sequences collected from the direct and indirect libraries were expressed in a germination-specific manner, we attempted to quantify gene expression levels with clone array hybridizations, but the very small quantities of cDNA that could be recovered from germinating basidiospores severely hampered these efforts (data not shown). In the absence of this data, the number of sequences in each contig can be used to give a qualitative approximation of gene expression levels for certain loci. For example, contigs D819 and I693 were composed of multiple sequences from either the direct or the indirect library, respectively (Table 1). This distribution suggests that that they actually are expressed in a germination-specific manner. Similarly, contigs I685, I691, D797, D801, D807, D811, and D878, which were not homologous to any entries in the NCBI databases, were represented by at least 5 clones in their respective library and none in the other (data not shown). However, many of contigs specific to either subtracted library were composed of only 1 or 2 sequences (Table 1 and data not shown), raising the possibility that they are derived from genes that are expressed at low levels in both types of germinating spores and that their apparent differential expression is simply an artifact of their infrequent representation in each library, even after subtraction of the highly expressed sequences. It is most appropriate, therefore, to think of this clone collection as a small-scale EST project, which is potentially enriched for genes expressed during direct and indirect germination of *C. q. fusiforme* basidiospores.

The 110 novel contigs in this clone collection represent an intriguing subgroup, but the lack of a stable transformation system for any rust fungus essentially prohibits their detailed functional analysis at this time. The same is true for any of the genes predicted to encode rust-specific functions. However, many other clones in the collection are homologous to proteins of unknown function in *U. maydis* or *C. neoformans*, and both of these organisms are good experimental systems for studies of gene function.

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