Invest Agrar: Sist Recur For (2004) 13 (1), 105-117

The Canadian *Ophiostoma* genome project

L. Bernier[1]*, C. Breuil[2], W. E. Hintz[3], P. A. Horgen[4], V. Jacobi[1], J. Dufour[1], M. Aoun[1], G. Bouvet[1], S. H. Kim[2], S. Diguistini[2], P. Tanguay[2], J. Eades[3], S. Burgess[3], P. de la Bastide[3], M. Pinchback[3] and Y. Tadesse[4]

[1] *Centre de Recherche en Biologie Forestière. Université Laval. Sainte-Foy. G1K 7P4 Québec. Canada*
[2] *Department of Wood Science. University of British Columbia. V6T IZ4 Vancouver (British Columbia). Canada*
[3] *Department of Biology. University of Victoria. V8W 2Y2 Victoria (British Columbia). Canada*
[4] *Department of Botany. University of Toronto at Mississauga. L1L 1C6 Mississauga (Ontario). Canada*

Abstract

The Canadian Ophiostoma Genome Project, which was initiated in 2001, is a collaborative effort between research teams in four different universities. Its general objective is to conduct a large-scale identification and analysis of genes controlling important aspects of the life cycle of Ophiostomatoid fungi. To this end, several expressed sequence tag (EST) libraries were obtained for the Dutch elm disease pathogen *Ophiostoma novo-ulmi* and the sapstainer *O. piceae*, following partial, single-pass automated sequencing of complementary DNA clones. The largest EST library, prepared from yeast like cells of *O. novo-ulmi* grown at 24 °C, contains over 3,400 readable sequences and serves as a general reference library for Ophiostomatoid fungi. Smaller, specific EST libraries were constructed from mycelia of *O. novo-ulmi* grown at suboptimal temperatures, from perithecia formed in laboratory crosses, as well as from *O. piceae* grown on different carbon sources. Ongoing bioinformatic searches in public databases have so far identified over 750 *Ophiostoma* unique ESTs which show significant homology with other fungal genes of known function, although a high proportion of *Ophiostoma* ESTs are either orphans (no match to any known gene) or show homology to genes of unknown function. In addition to EST analysis, differential expression of selected genes and structural genomics are also being studied.

Key words: *Ophiostoma,* Dutch elm disease, sapstain, functional genomics, expressed sequenced tag, genetic mapping, bioinformatics.

Resumen

El proyecto genómico del hongo *Ophiostoma*

El programa canadiense sobre el genoma de *Ophiostoma*, iniciado en 2001, es una colaboración entre equipos de investigación de cuatro universidades diferentes. Su objetivo general es el desarrollo de la identificación y análisis a gran escala de los genes que controlan algunos aspectos importantes del ciclo vital de los hongos de *Ophiostoma*. Con este fin, se ha obtenido diversas bibliotecas de marcadores de secuencias expresadas (bibliotecas EST) para la el patógeno de la grafiosis *Ophiostoma novo-ulmi* y para el hongo de tinción vascular *O. piceae*, seguido de una secuenciación automática parcial de un único paso de clones complementarios de ADN. La mayor biblioteca EST, preparada a partir de conidios de *O. novo-ulmi* cultivadas a 24 ºC, contiene más de 3.400 secuencias legíbles, y sirve como biblioteca de referencia para los hongos de *Ophiostoma*. Se han desarrollado bibliotecas específicas menores a partir de micelios de *O. novo-ulmi* cultivados a temperaturas sub-óptimas, a partir de los peritecios formados en cruces realizados en laboratorio, así como a partir de *O. piceae* cultivado en distintas fuentes de carbón. Las búsquedas bioinformáticas en bases de datos públicas han permitido identificar hasta ahora más de 750 EST exclusivos de *Ophiostoma*, lo que muestra una significativa homología con otros genes fúngicos de función conocida, aunque una alta proporción de los EST de *Ophiostoma* son o bien huérfanos (no relacionados con ningún gen conocido), o bien muestran homología con genes cuya función es desconocida. Además del análisis EST, la expresión diferencial de genes seleccionados y la estructura genómica están siendo también estudiadas.

Palabras clave: *Ophiostoma*, grafiosis, tinción vascular, functional genomics, expressed sequenced tag, genetic mapping, bioinformática.

* Corresponding author: louis.bernier@rsvs.ulaval.ca
Received: 21-09-03; Accepted: 09-12-03.

Introduction

In April 1996, came the announcement that the entire genome of baker's yeast, *Saccharomyces cerevisiae*, had been sequenced (Goffeau *et al*., 1997). Over 600 scientists from Europe, Japan and North America were involved in this international project, which can be rightly viewed as a milestone, as it marked the first time an eukaryotic genome was sequenced. The choice of *S. cerevisiae* was no mere coincidence, given the biological features of this organism (e.g. small genome, rapid life cycle) and the extensive amount of data produced since the 1930's by the large community of investigators (several thousands) studying yeast metabolism and genetics. With this accomplishment, the discipline of fungal genomics could no longer be considered an abstraction and has been expanding ever since. The nuclear genomes of additional fungal species, including filamentous fungi such as *Neurospora crassa* (Galagan *et al*., 2003), have been sequenced and it is likely that the list will expand rapidly over the next few years through both publicly- and privately financed initiatives.

Genomics, however, encompasses far more than genome sequencing since the latter, far from being an end in itself, is in fact the starting point for further investigations on the architecture and evolution of the nuclear genome, on the regulation of various metabolic activities, as well as the dynamics of populations and species. While it is true that studies on the aforementioned topics predate the onset of genomics, the development of high-throughput methods for the processing and sequencing of genomic DNA, coupled to the ever increasing power of bioinformatics, has resulted in a quantum leap in the way we can envision and carry out genetic investigations on eukaryotic organisms.

In this paper, we first present the case for *Ophiostoma* spp. as good candidate species for genomic studies. Then, we will highlight the overall strategy underlying our ongoing Ophiostoma genome project and present some of the results we have obtained to date.

Ophiostomatoid fungi are good candidates for genomic studies

In a paper presented at the 1992 Dutch Elm Disease Workshop (Bernier, 1993), we highlighted several of the attributes which, in our view, made *O. ulmi* and *O. novo-ulmi* attractive «model systems» for the study of tree pathogenic fungi. These included biological features (haploid genome, heterothallism, yeast-mycelium dimorphism), as well as methodological considerations such as the rapid and abundant production of biomass on a variety of solid and liquid media, the possibility of conducting sexual matings under laboratory conditions, the availability of protocols for chemical, physical or insertional mutagenesis, and the development of elm calli for studies of host-pathogen interactions. We believe that what we wrote then remains true today for the Dutch elm disease pathogens, and can be extrapolated to several other members of the genus *Ophiostoma*. Ten years ago, however, it seemed obvious to us that researchers in the field of Dutch elm disease had not taken full advantage of the genetic tractability of *O. ulmi* and *O. novo-ulmi*. Have things changed during the last decade?

Fortunately, the answer is yes, as researchers have been making increasing use of genetic and molecular approaches in their investigations on the *Ophiostoma* nuclear genome. In particular, transformation-mediated mutagenesis and the numerous technical variations on the polymerase chain reaction (PCR) method have become powerful tools for the study of *O. ulmi* and *O. novo-ulmi*, as well as other Ophiostomatoid fungi. For example, studies carried out on the individual genome have allowed the identification of putative pathogenicity genes (Et-Touil *et al*., 1999), genes involved in yeast-mycelium dimorphism (Pereira *et al*., 2000), or genes coding for chitin synthesis (Hintz, 1999) in *O. novo-ulmi*. Some of the genes involved in melanin production (and hence pigmentation) have also been cloned and analyzed in the phylogenetically related saprobe *O. floccosum* (Eagen *et al*., 2001; Wang and Breuil, 2002), whereas genes controlling nitrogen uptake have been characterized in a few sapstaining *Ophiostoma* species (Hoffman and Breuil, 2002, 2004). Cloning and manipulation of the gene encoding cerato-ulmin (Bowden *et al*., 1994), a class 2 hydrophobin which was deemed a pathogenicity factor in *O. novo-ulmi* (Richards, 1993), suggested that cerato-ulmin was important for overall parasitic fitness (Temple *et al*., 1997) rather than for pathogenicity *per se* (Bowden *et al*., 1996), although more recent work carried out on cerato-ulmin producing transformants of the saprobe *O. quercus* (Del Sorbo *et al*., 2000) may call for a critical reexamination of the effect of cerato-ulmin in hardwoods. Our understanding of the genetic diversity and population structure of various spe-

cies of *Ophiostoma* has also benefited significantly from the application of molecular methods (Hintz *et al.*, 1993; Pipe *et al.*, 1995; Hoegger *et al.*, 1996; Gagné *et al.*, 2000). Thanks to an ever-growing number of nucleotide sequences deposited in public data banks, phylogenetic relationships among Ophiostomatoid fungi are also being deciphered (Pipe *et al.*, 1997; Harrington *et al.*, 2001; De Beer *et al.*, 2003; Hausner and Reid, 2003).

The common thread that runs through the examples cited above is that they involved analyses carried out on either one locus coding for a specific product or trait, or a relatively small set of loci, usually of the anonymous type that is provided by restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNAs (RAPDs). If one assumes that the *Ophiostoma* nuclear genome contains around 8000-10000 genes (Kupfer *et al.*, 1997; Galagan *et al.*, 2003) distributed over 30 to 40 million nucleotides (Dewar *et al.*, 1997), it is clear that the above studies focused on and made use of only a tiny portion of the *Ophiostoma* genome.

Strategy underlying the Canadian *Ophiostoma* genome project

It is with the above thought in mind that the Canadian Ophiostoma genome project was planned during the year 2000, with the general objective of conducting a large-scale identification and analysis of genes controlling important aspects of the life cycle of Ophiostomatoid fungi. From the outset, it was decided that this would not be a full genome sequencing project, as necessary funding was not available. Rather, the sequencing effort was directed on arrays of genes whose expression was necessary for growth and substrate utilization, host-pathogen interaction, and propagation. The methodological implication of this decision meant that the material submitted for sequencing had to be obtained from expression libraries, i.e. complementary DNA (cDNA) libraries derived from populations of messenger RNA purified from *Ophiostoma* growing either in pure culture or on a plant host. Another decision was to obtain partial, rather than full, nucleotide sequences. Thus, emphasis was placed on obtaining expressed sequence tags (ESTs) representing the coding regions (or portions thereof) of the genes mentioned above. These ESTs would then, in turn, allow the identification of genes with known function through bioinformatic searches in public data banks. Genes of interest could then be genetically and physically mapped (Dewar *et al.*, 1997; Dusabenyagasani *et al.*, 2000) and subjected to expression profiling studies. Another decision with important implications for the project was the choice of the organisms to be studied, namely the pathogen *O. novo-ulmi* and the phylogenetically related saprobe *O. piceae*. This facilitated the cooperation among the four participating laboratories (Bernier, Hintz and Horgen, specializing on *O. novo-ulmi*; Breuil, specializing on *O. piceae*) and also allowed us to verify whether results obtained for one species could easily be transposed to another one. The Canadian Ophiostoma genome project took off in February 2001, following successful review by the Natural Sciences and Engineering Council of Canada (NSERC) which awarded funding for three years under its Genomics programme. The following sections describe, in general terms, some of the progress accomplished so far.

Specific objectives

The Canadian Ophiostoma project was designed to meet the following specific, short-term objectives: 1) the construction of cDNA libraries from *O. novo-ulmi* and *O. piceae*; 2) the obtention of ESTs by single-pass, partial sequencing of clones from these cDNA libraries; 3) the study of differential gene expression by Real-Time PCR; and 4) the production of a saturated consensus genetic map for Ophiostomatoid fungi, using both coding and non-coding segregating loci. Longer-term objectives of the project were the eventual construction of an Ophiostoma EST microarray for large-scale profiling studies, as well as the design of specific studies on genes that are differentially expressed, using both gene disruption- and gene over-expression approaches. Expressed sequence tags (objective #2) were sought for the following aspects of *Ophiostoma* metabolism: a) yeastlike growth; b) mycelial growth; c) response to temperature shifts; d) carbon utilization; e) pigmentation; f) fruit body formation; and g) pathogenicity.

Selection of *Ophiostoma* strains and construction of genomic libraries

Three representative strains were selected for the construction of genomic and/or expression libraries.

These included aggressive strain H327 (Brasier *et al.*, 1998; Et-Touil *et al.*, 1999) representing the pathogen *O. novo-ulmi*, whereas strain AF413104 was chosen for the saprobe *O. piceae*. Strain Q412T was selected as a representative of *O. ulmi*, the less aggressive pathogen responsible for the first pandemic of Dutch elm disease. Strain Q412T can be crossed to some *O. novo-ulmi* strains and generate viable F_1 progeny which are needed for genetic mapping (Dusabenyagasani *et al.*, 2000). Additional strains of *O. ulmi* and *O. novo-ulmi* were also used in specific experiments (see below).

Genomic libraries containing high molecular weight inserts (12-18 kb) in λ phage were constructed for all three reference strains and stored at –80 °C. One of the uses of these libraries is to permit the recovery of full-length genomic clones for ESTs of particular interest, thus enabling us to obtain complete sequences for these genes and regulating regions adjacent to them.

Construction and analysis of reference *Ophiostoma novo-ulmi* expression libraries

Complementary DNA libraries were constructed from polyadenylated (polyA) RNA isolated from both mycelial and yeast forms of *O. novo-ulmi* H327 grown at 24 °C. Since these libraries, constructed in W.E. Hintz' laboratory, were to serve as reference libraries, we tried to recover as many cDNA clones as possible from each library. Mycelial colonies were grown for 3 days on autoclaved cellophane placed over solid complete medium for *Ophiostoma* (OCM) (Bernier and Hubbes, 1990). Yeast cultures were obtained by incubating the fungus for 3 days in liquid OCM with shaking. Approximately 200-250 mg of either mycelium or yeast cells were used for each RNA isolation.

PolyA mRNA was isolated using the MicroPolyA Pure mRNA Purification kit (Ambion). Isolation from ~200 mg fresh mycelium yielded 9.0 µg polyA RNA, as indicated by spectrophotometric analysis. After quantification, 8.0 µg of mycelium polyA RNA was used for first strand cDNA synthesis. Isolation from ~200 mg fresh yeast cells yielded between 1.0 and 2.5 µg polyA RNA. After quantification, 3.0 µg of yeast polyA RNA was used for first strand cDNA synthesis. Complementary DNA was then synthesized from polyA RNA using the pBluescript II XR (SK+) cDNA Library Construction kit (Stratagene). The cDNA was size fractionated by agarose gel electrophoresis into a low molecular weight (LMW) fraction containing inserts ranging in size from roughly 0.5 to 2 kb, and a high molecular weight (HMW) fraction containing inserts ranging from 2 to 5 kb. Fragments were directionally cloned into pBluescript II vector pre-digested with restriction enzymes *Eco*RI/*Xho*I. Ligated constructs were transformed into *Escherichia coli* DH12S cells by electroporation. The resulting *O. novo-ulmi* mycelium and yeast libraries were stored in a 15% glycerol stock at -80°C.

Each library (mycelium LMW and HMW; yeast LMW and HMW) was titered on selective media. Mycelium LMW and HMW libraries were estimated to contain roughly 12000 and 200 clones, respectively, whereas corresponding estimates for yeast LMW and HMW libraries were 22000 and 200 clones, respectively. Twelve clones from each library were chosen at random and screened for *Ophiostoma* cDNA inserts. Preliminary, small-scale, analysis of mycelium libraries suggested that the HMW fraction contained very few authentic inserts, whereas all 12 clones from the LMW library seemed to contain authentic insert of varied size within the appropriate 0.5 kb-2 kb range. The subset of 12 yeast LMW clones that were screened for insert all contained authentic insert up to 2.2 kb in length. The heuristic BLASTX algorithm from the Basic Local Alignment Search Tool package (Altschul *et al.*, 1997) was used to interrogate the NCBI GenBank public database. Eight of the clones appeared to be novel sequences as they did not align with any sequences in the database. None of the 12 clones appeared to be duplicated, thus indicating that the library was of high complexity. High-throughput plasmid isolations of clones from the yeast LMW library were carried out. As of October 2003, nearly 5000 clones from this library had been sequenced at the U. of Victoria sequencing facility, with a success rate of 74.1% (3405 readable sequences out of 4596 clones analyzed).

Bioinformatic searches using BLASTX showed that the 3405 *O. novo-ulmi* yeast LMW readable ESTs included a total of 1576 ESTs which shared significant homology ($E \leq 10^{-5}$) to sequences deposited in public databases. Thus, 46.3% of the readable ESTs were represented by a previously sequenced homologue from another organism. This set of 1576 ESTs is not truly representative of the number of new homologous ESTs generated, however, due to duplicate sequencing within the dataset (redundancy). Duplicate sequences were thus identified and removed, leaving a total of 878 unique ESTs (55.7% of the total homologous sequences) which showed significant homology to sequences

identified in other species. Of these 878 sequences, 711 ESTs matched sequences with known functions. The remaining 167 ESTs shared homology with sequences of unknown functions, including hypothetical proteins and open reading frames (ORFs), predicted proteins, unknown proteins, and putative proteins.

Of the 3,405 readable ESTs, 1,829 did not show significant homology to any sequences in public databases. These sequences are thus novel sequences from *Ophiostoma* spp., or orphan sequences. A proportion of these ESTs which do not match known sequences appear to be junk sequences and are likely due to the ligation of random fragments of DNA into the holding vectors during the creation of the EST library. However, they may also represent non-coding RNAs, or RNAs with unknown function. We estimate the number of junk sequences to be ~20-25% of the 1,829 non-homologous ESTs, meaning that there are ~1,350-1,450 orphan sequences in the dataset. If one assumes that the level of sequence duplication within this subset of data is similar to the level observed within the homologous subset of data (~44.3%), then approximately 575-625 of these 1829 ESTs are likely to be non-duplicated (unique) orphan sequences. The high number of orphan ESTs could be due to the small amount of fungal sequence data available in public databases. However, it is likely that a number of these ESTs are specific to Ophiostomatoid fungi. The importance of these sequences will be revealed through further analysis by comparing with phase specific libraries, by analysis of subtractive libraries, and by microarray analysis.

The complexity of the dataset is illustrated by the diversity of ESTs which share homology with previously identified sequences from other species. The genes from the yeast *S. cerevisiae* have been classified into 14 functional groups (Neumann and Dobinson, 2003), and numerous representatives from each of these functional groups are evident within our dataset. For instance, EST sequences which would belong in the METABOLISM group include glyceraldehyde-3-phosphate dehydrogenase and glyoxylase I. The ENERGY group is represented by cytochrome C and pyruvate kinase, the TRANSCRIPTION group by histone H4, the PROTEIN SYNTHESIS group by translation initiation factor, the CELL CYCLE/DNA PROCESSING group by DNA mismatch repair protein MutS, and the PROTEIN FATE group by oligosaccharyltransferase and α-mannosidase. Numerous representatives of the remaining eight functional groups are also evident within the dataset. In addition, we have already identified a number of ESTs which are of particular interest since they might play a significant role in the parasitic fitness of Ophiostomatoid fungi. These include the previously sequenced gene cerato-ulmin, the septin B gene which is involved in cellular division and hyphal growth, the oligosaccharyltransferase gene which is necessary for protein glycosylation (an important essential pathway), superoxide dismutase, chitin synthase which is involved in cell wall formation, and genes likely involved in plant cell wall degradation, such as glucan 1,3-β-glucosidase, endo-1,4-β-glucanase, and glucoamylase.

The *O. novo-ulmi* yeast LMW EST sequencing project has thus far generated close to 1,500 non-duplicated (e.g., unique) sequences. Given that the genomes of filamentous fungi generally contain between 8,000 and 10,000 genes, we have thus sequenced between 15-20% of the total number of genes within the *O. novo-ulmi* genome. Since EST analysis will identify only those sequences that are expressed, we expect the total number of genes that will be unveiled in yeast cells of *O. novo-ulmi* to be somewhat less than the total number of genes in the genome. Approximately 44% of the current dataset is duplicate sequence data. This level of redundancy was initially very low (less than 10% redundancy within the first 500 ESTs generated) but will increase as the sequencing project continues, due to the accumulation of ESTs in the dataset. We are currently generating non-duplicated, highly diverse sequence data that are 35-45% unique, meaning that new non-redundant ESTs are still being generated fairly efficiently and thus fairly cost-effectively. We expect to be able to effectively produce sequence data until the level of unique data falls below 10%. Thorough analysis of the reference yeast LMW library and of additional, specific libraries (see below) should thus yield many more unique ESTs that will provide a more complete representation of the *O. novo-ulmi* genome.

Construction and analysis of specific *Ophiostoma novo-ulmi* expression libraries

Additional expression libraries were prepared from *O. novo-ulmi* H327, namely from mycelial colonies grown at optimal (22 °C) and sub-optimal (15 and 31 °C) temperatures, and from fruiting bodies produced either asexually (synnemata) or sexually (perithe-

cia). However, unlike the reference mycelium and yeast libraries described above, these specific libraries prepared in L. Bernier's laboratory were not meant to include an exhaustive number of cDNA clones. Rather, they were deliberately enriched for cDNAs that were expressed differentially, and subtractive techniques were thus used to eliminate clones which represented genes involved in basal metabolism. This approach had the obvious advantage of minimizing sequencing costs, as it theoretically eliminated many redundant ESTs present in the yeast and mycelium LMW reference libraries.

Several protocols exist for creating expression libraries that are enriched for genes of interest. We elected to use a PCR-based technique known as Suppressive Subtractive Hybridization (SSH), described by Diatchenko *et al*. (1999). In SSH (Figure 1), blunt-ended cDNAs synthesized from differentially expressed mRNAs (also known as tester RNA) are divided into two portions which are each ligated to a specific adapter. Adapters have specific primer sites that allow the amplification of the differentially expressed genes. The two sets of tester cDNAs are then each hybridized with excess driver cDNA (representing reference mRNAs) devoid of adapters. The reactions from this first hybridization are then mixed and rehybridized in the presence of excess driver cDNA, which results in the formation of several hybrid cDNAs. Following two rounds of PCR amplification with suitable primers, hybrids corresponding to differentially expressed sequences are amplified exponentially, whereas other cDNAs are either not amplified at all or only to a low level.

For the identification of genes that were differentially expressed at different temperatures, one-week old mycelial cultures of H327 were grown over autoclaved cellophane on malt extract agar (MEA) at 22 and 31 °C. Following RNA extraction (Rneasy Mini Kit, Qiagen) and cDNA synthesis (Super SMART cDNA Synthesis Kit, Clontech), ESTs that were differentially expressed at a higher temperature were thus enriched by SSH (PCR Select cDNA Subtraction Kit, Clontech), with cDNA from cultures incubated at 31 °C as tester and cDNA from cultures grown at 22 °C as driver. A total of 126 cDNA clones were recovered. For synnemata production, strain H327 was grown as described for mycelium production but without cellophane. Individual synnemata were harvested with the aid of a sterile needle, ground in liquid nitrogen, and stored at –80 °C prior to total RNA extraction. However, attempts to isolate total RNA from up to 1200 synnemata using three different protocols were unsuccessful.

For perithecia production, strain H327 was used as the female partner and grown as described for synnemata production. Once the H327 (MAT A) mycelium had covered the entire plate, it was fertilized with a yeast cell suspension of the sexually compatible (MAT B) *O. novo-ulmi* strain CKT11 grown in liquid complete medium at 24 °C with shaking. Starting from 7 days after fertilization, individual perithecia were harvested with a sterile needle, rolled over agar to remove adhering mycelium, ground in liquid nitrogen and stored at –80 °C. Following total RNA extraction from ~300 perithecia, an aliquot was electrophoresed on a formaldehyde agarose gel. Results suggested that the sample contained a very low amount of total RNA. However, a portion of the *Efα-1* gene encoding elongation factor 1 was successfully amplified by reverse transcriptase PCR (RT-PCR), indicating that the sample indeed contained RNA. A population of ESTs associated with sexual fruiting was cloned using cDNA from perithecia as tester and cDNA from mycelium as driver (Figure 1). A total of 138 cDNA clones were recovered.

A quick, small-scale test was run on the perithecium-specific library to verify subtraction efficiency by PCR analysis using primers amplifying part of the gene encoding the ubiquitous glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. A PCR product was obtained after 18 and 33 cycles in unsubtracted and subtracted libraries, respectively, suggesting that SSH had indeed reduced the abundance of constitutively expressed ESTs from the latter. Subsamples of ~120 ESTs from each of the temperature- and perithecium specific libraries were thus sequenced. Each library yielded over 90% readable sequences.

Although not completed, bioinformatic analysis of the *O. novo-ulmi* ESTs from the temperature- and perithecium specific libraries has been proceeding at an encouraging pace, as these libraries contain a much smaller number of sequences than the yeast LMW reference library. We used both BLASTX and FASTA (Pearson and Lipman, 1988) algorithms to search public databanks (including repositories of genomic clones and collections of ESTs), as well as our own *O. novo-ulmi* EST dataset. Unique sequences accounted for 92% and 85% of the clones in the SSH libraries obtained from cultures grown at 31 °C and from perithecia, respectively. The two subtracted libraries also differed markedly from each other, as they shared very few sequences: six pe-

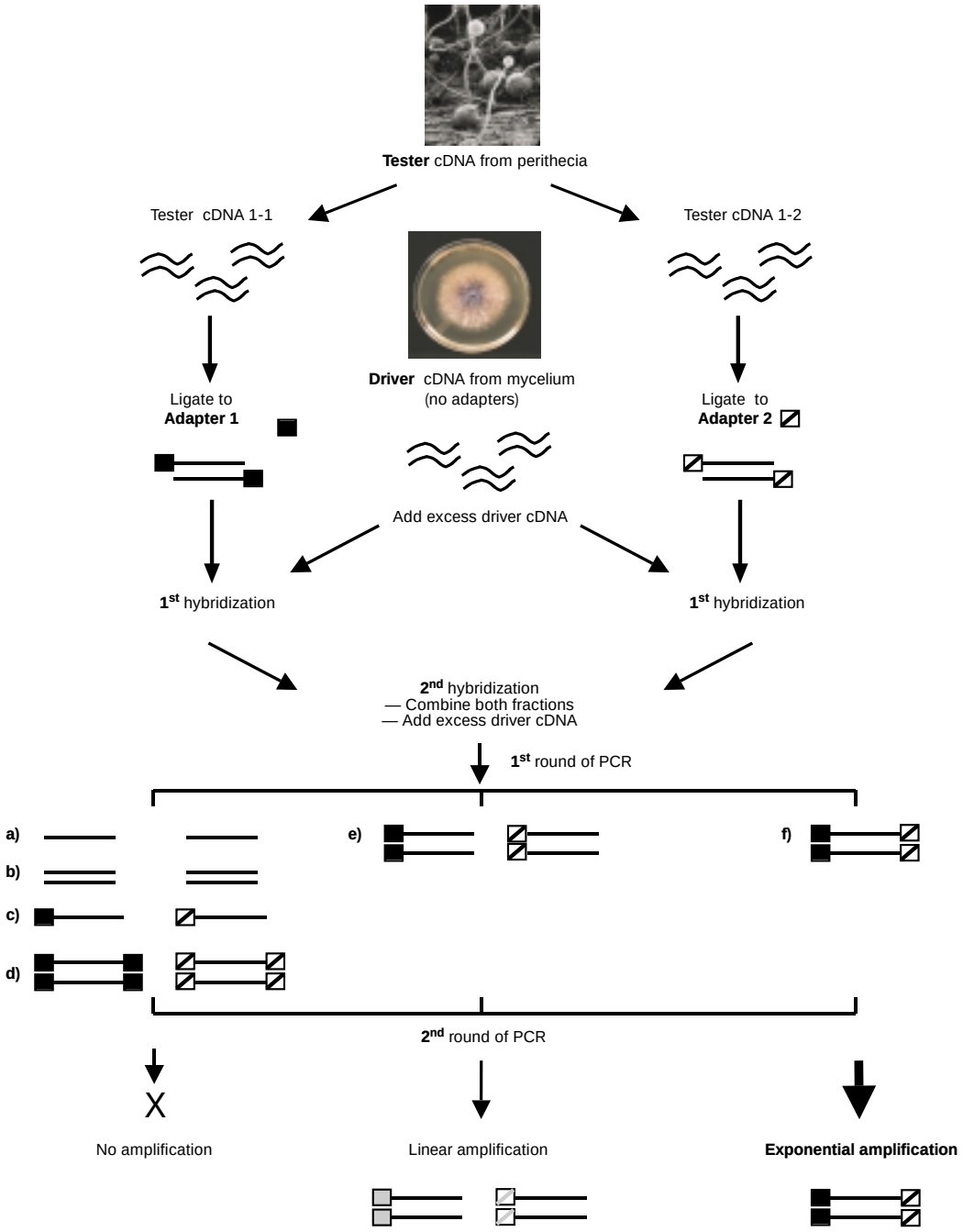

Figure 1. Application of Subtractive Suppressive Hybridisation (SSH) for constructing specific expression libraries of *Ophiostoma*. In the example illustrated here, SSH was used to recover tester cDNAs that were preferentially expressed in perithecia from a laboratory cross between two sexually compatible strains of *O. novo-ulmi*. The sample of tester cDNA was divided in two, and each fraction was first ligated to an appropriate adapter (black and hatched boxes), and then hybridized with excess driver cDNA (without adapters) from a mycelial culture. The reactions from the first hybridization were mixed and subjected to a second hybridization, again in the presence of excess driver cDNA. Two successive rounds of PCR amplification were then carried out on the resulting hybridization mixture in order to enrich it in differentially expressed genes. Among the different hybrid types present in the mixture, driver cDNAs (a, b) or single-stranded tester cDNAs containing a single adapter (c) were not amplified. Double-stranded hybrid tester cDNAs attached to identical adapters (d) on both ends were not amplified either due to the formation of stable panhandle-like structures which suppressed PCR. Tester cDNA hybrids attached to a single adapter (e) were only amplified at a linear rate. However, hybrids corresponding to differentially expressed genes (f) were amplified exponentially since they harboured different adapters on each end. (Adapted from Diatchenko *et al.*, 1999).

rithecium ESTs had homology with five temperature-related ESTs. Less than 30% of the sequences in the subtracted libraries were found in the reference yeast LMW library. Taken together, the above results suggest that SSH was indeed an efficient means for recovering unique, differentially expressed sequences. A total of 47 temperature-related ESTs were found to share significant homology (E-value $\leq 10^{-5}$) with fungal sequences deposited in public databanks. Among these, 37 *O. novo-ulmi* ESTs were highly homologous to 27 different genes with known function, whereas the 10 other ESTs were homologous to genomic sequences or ESTs with undefined function. No significant homology was found for the remaining 63 temperature-related ESTs which were readable. A high number of orphans (85 out of 125 ESTs analyzed) was also recorded for the perithecium-specific ESTs. Forty ESTs recovered from perithecia matched sequences in public data banks, including 14 sequences of known function. Interestingly, two of the latter were ESTs from mated cultures of the rice blast pathogen *Magnaporthe grisea* and from mature perithecia of *N. crassa*, respectively.

Construction of a pathogenicity EST library for *O. novo-ulmi*

Since *O. novo-ulmi* is a highly aggressive pathogen, our group was obviously interested in identifying ESTs associated with important aspect of the fungus lifestyle. In this case, however, two methodological constraints have to be dealt with in a satisfactory fashion: 1) extraction of mRNA from plant tissue; and 2) separation of fungal mRNAs from plant mRNAs. First of all, we elected to inoculate *O. novo-ulmi* to elm calli, rather than saplings, as we thought this might facilitate the subsequent extraction of RNA transcripts. Thus, both soft and hard calli were produced from the susceptible species *Ulmus americana*. Upon inoculation of soft calli with *O. novo-ulmi* H327, the fungus was observed to be growing abundantly on surface of the calli, as well as in intercellular spaces within the calli. Total RNA was successfully extracted from both inoculated and non-inoculated calli, and recovery of mRNAs is currently underway. Here again, SSH will be used in order to separate fungal transcripts from those produced by the host. A possible limitation of our approach is that not all fungal genes involved in the colonization of highly differentiated tissues in elm saplings may be expressed when *O. novo-ulmi* invades calli. However, elm calli have been shown to display chemical defense responses, such as production of phytoalexins, that are typically observed in the xylem of saplings (Yang *et al*., 1989, 1994). We thus expect to be able to recover several of the transcripts associated with pathogenicity of *O. novo-ulmi*, as well as transcripts associated with elm response to the pathogen.

Construction of *O. piceae* mycelium and yeast libraries

Suppressive subtractive hybridization was also used by C. Breuil's group to construct differential cDNA libraries of genes in mycelium and yeastlike cells of *O. piceae* AF413104. Close to 300 yeast cDNA clones subtracted against mycelium cDNA, and 70 mycelium cDNA clones subtracted against yeast cDNA were obtained. Prior to sequencing, the quality of the yeast and mycelium SSH libraries was checked by dot blot assay using 18S rDNA, 26S rDNA, a protease gene, and a melanin gene as probes. Only six rDNA clones were found among the 234 yeast cDNAs analyzed, whereas the subset of 57 mycelium cDNAs subjected to the dot blot assay included 22 rDNA clones, indicating that the mycelium library was of poor quality. Therefore, the latter was not retained for further work.

The efficacy of the SSH approach appeared to be lower for *O. piceae* than for *O. novo-ulmi*, as the 189 yeast clones of *O. piceae* which were randomly selected for sequencing contained only 32% (60/189) unique sequences. The three most abundant sequences were genes coding for cytochrome C oxidase (34 clones), glucose repressible gene protein (9 clones), and phosphatase 2a inhibitor-related protein (7 clones). The 60 unique *O. piceae* ESTs included 19 sequences with significant homology (E-value $\leq 10^{-5}$) to known genes, 24 hypothetical or predicted protein gene sequences, and four sequences with no significant match with known genes. Comparison of yeast ESTs from *O. piceae* (from which mycelium cDNA had been subtracted) and *O. novo-ulmi* (unsubtracted) showed that a glucose repressible gene found in *O. piceae* appeared to be unique to the latter.

In addition, SSH was carried out for identifying ESTs expressed when *O. piceae* was grown on three different carbon sources, namely glucose, starch and olive oil. Methodological adjustments to improve the size and diversity of cDNA clones resulted in the obtention of a high percentage (average 90%) of readable sequences, with

inserts greater than 400 bp. Bioinformatic analyses showed that over 80% of readable ESTs had significant matches ($E \leq 10^{-5}$) with genes of known function from other organisms, with a very low percentage (5%) of housekeeping genes. Furthermore, starch-derived ESTs were found to include sequences coding for cellobiohydrolase, cellobiose dehydrogenase, endoglucanase, exochitinase, maltose permease, and cellulose-growth-specific protein. The finding of genes encoding endoglucanase is especially interesting since, to our knowledge, there is no report yet of cellulase genes in *Ophiostoma* species. We hypothesize that this result might be related to the presence of impurities in the starch used in the growth medium, or to the incubation of *O. piceae* on cellophane membranes. Although the olive oil-derived EST library contained genes involved in glycerol metabolism, it did not seem to contain lipase-related genes. Using the ESTs as probes, we screened an *O. piceae* genomic library to retrieve genes coding for cellobiohydrolase, cellobiose dehydrogenase, endoglucanase, and exochitinase. Their full sequences will be determined and characterized in the near future.

Study of differential gene expression by Real-Time PCR

In parallel to constructing EST libraries for the discovery of novel genes in *Ophiostoma*, we began studying and quantifying the differential expression of known genes by Real-Time PCR (Tadesse *et al*., 2003). This relatively new technique combines reverse transcriptase-PCR with fluorescent probes in uniplex or multiplex reactions, to quantitatively measure gene expression in fungal cells recovered at different stages or in different environments. The gene encoding cerato-ulmin (*cu*), produced throughout the life cycle in all developmental stages of *O. novo-ulmi* and *O. ulmi* (Richards, 1993), was selected for monitoring gene expression using both Real-Time PCR and Northern analysis. Levels of *cu* mRNA were measured in both yeast and mycelial stages of wild-type *O. ulmi* (H5) and *O. novo-ulmi* (H327 and VA-3O) strains, whereas mutant strains H5-tf16 (an over-expressing *cu O. ulmi* mutant) and THEK5-8 (a null cu^- *O. novo-ulmi* mutant) were used as controls. Data from both Northern analysis and Real-Time PCR analysis demonstrated that there was no *cu* mRNA transcription in the null mutant. The Northern analysis clearly showed that the over-expressing mutant H5-tf16 produced dramatically more *cu* mRNA that the wild-type strains. The quantitative data from Real-Time PCR demonstrated that mycelium had twice the abundance of *cu* mRNA as compared to yeast and that the non-aggressive *O. ulmi* strain H5 produced 10 times less *cu* mRNA than the aggressive *O. novo-ulmi* strains H327 and VA-3O. The non-aggressive over-expressing mutant H5-tf16 had a *cu* mRNA abundance level that was 20 fold greater than the wild type *O. ulmi* H5 strain (Tadesse *et al.*, 2003).

The above data, obtained in P.A. Horgen's laboratory, were consistently generated on multiple Real-Time PCR runs and clearly demonstrated the value of generating reproducible quantitative abundance numbers with this technique which is now being used for studying other genes of interest represented in the *Ophiostoma* EST database. Over the longer term, however, one of the expected outputs of the *Ophiostoma* genomics project is the construction of EST microarrays which would allow the simultaneous study of thousands of genes. Microarray technology would obviously be a powerful tool for gene expression studies but specific microarrays could also be designed for other purposes such as population genomics and taxonomic identification of species from the genus *Ophiostoma*.

Structural analysis of the *Ophiostoma* nuclear genome

While a major effort was devoted to developing expertise and knowledge on *Ophiostoma* functional genomics, the project also included a structural analysis of the *Ophiostoma* nuclear genome. The latter included investigations of chromosome structure and organization, as well as genetic mapping of the nuclear genome.

We used pulsed-field gel electrophoresis (PFGE) to separate chromosomes of 71 isolates representing *O. ulmi, O. novo-ulmi, O. piceae, O. quercus, O. floccosum, O. piliferum*, and *O. setosum* (Tanguay *et al.*, 2003). A general PFGE karyotype was established for each species. Intraspecific polymorphisms that appeared as variation in the number and in the size range distribution of chromosomal bands (Dewar and Bernier, 1995) were observed in every species studied, except *O. setosum* in which the number of chromosomal bands resolved by PFGE was constant. Within a species, no consistent relation could be established between karyotype and geographic origin or host of the isolate. Variations observed in the Dutch elm disease pathogens allowed us to distinguish *O. ulmi* from *O.*

novo-ulmi and to differentiate the two subspecies (*novo-ulmi* and *americana*) within *O. novo-ulmi*. Additional work involving PFGE analyses is planned in order to better understand the mechanisms underlying large-scale chromosome rearrangements and assess the validity of the chromosome speciation model (Fischer *et al*., 2000) for Ophiostomatoid fungi.

Genetic mapping of the *Ophiostoma* nuclear genome was carried out on a population of F_1 progeny from an interspecific cross between *O. novo-ulmi* laboratory strain 416-R-14 and wild-type *O. ulmi* strain Q412T. To this day, 165 segregating markers have been mapped genetically. These include three loci identified by mutations resulting in nutritional requirements for adenine or methionine (Bernier and Hubbes, 1990) or in hypersentivity to ultra-violet irradiation (Bernier and Hubbes, 1994), 13 other coding loci represented by cDNAs, and 149 anonymous loci identified by RAPD polymorphisms (Dusabenyagasani *et al.*, 2000). The markers that we have so far mapped are distributed over 11 linkage groups. Further analysis of the mapping populations using PFGE has allowed us to identify the chromosomes represented by linkage groups (Figure 2). In its current state, the *Ophiostoma* genetic map contains mostly anonymous markers. With the availability of EST libraries and the cloning and comparison of relevant genomic sequences in *O. ulmi* and *O. novo-ulmi*, the next development will be the addition of a growing number of coding loci to the map.

Conclusion

Within the last year, the Canadian Ophiostoma genome project has been gathering momentum and, as a result, several thousands of ESTs associated with yeastlike growth, substrate utilization, temperature response, and sexual fruiting are currently being annotated and analyzed in *O. novo-ulmi* and *O. piceae*. Additional ESTs associated with the pathogenic phase of *O. novo-ulmi* should be available soon. This marks a dramatic shift from previous work that focused on either one or a few genes, or on anonymous markers. As we proceed with bioinformatic searches through public databanks, it is with growing excitement that we discover unique ESTs (over 750 so far) which show significant homology with genes that have been cloned and characterized in model fungi such as *S. cerevisiae*, *N. crassa* and *Aspergillus nidulans*, as well as in other fungal plant pathogens such as *Botrytis cinerea*, *M. grisea* and *Ustilago maydis*. Thus, it is now possible to classify large arrays of *Ophiostoma* genes according to function.

However, ongoing analyses of *Ophiostoma* ESTs are making it increasingly clear that much remains to be discovered about fungal genes. Thus, several ESTs we have analyzed so far show homology only with fungal sequences encoding hypothetical proteins in *N. crassa* or in *M. grisea*. Another numerically important subset of *Ophiostoma* ESTs is made up of orphan sequences with no significant match to sequences in public data banks. This should not come as a surprise, however, as fungal genome exploration is still at an early stage. In particular, the representation of plant pathogenic fungi in public data banks is still low and generally limited to a few species that are of importance to agriculture rather than forestry. However, our current frustration with the current state of information should decrease over time, as more sequences from more species (including a growing number of tree pathogenic fungi) become available. Moreover, it is likely that some «prize sequences» are hidden within our current *Ophiostoma* orphan ESTs: these would correspond to genes which are unique to *O. novo-ulmi* and/or *O. piceae* and some of them may be associated with traits that contribute to parasitic fitness.

The availability of large collections of ESTs is only the prelude to what should be fascinating research on the structural, functional and population genomics of *Ophiostoma* spp., including both pathogens and saprobes. For example, once an Ophiostoma gene (or EST) microarray is available, large-scale experiments can be conducted in which hundreds of different sequences are studied simultaneously. It will then be possible to identify sets of genes which are either up- or down-regulated during a specific metabolic event, such as the transition from mycelial to yeast phase. Finer analyses of genes of particular interest within these sets will be possible, using both gene disruption- and gene over-expression approaches.

Will the above analyses be available only to a «happy few»? This should not be the case, as far as members of the Canadian Ophiostoma Genome Project are concerned, as it is increasingly clear to us that the vast quantity of information suddenly unveiled by genomic approaches rapidly becomes too much to handle for a restricted group of investigators. We thus plan to make *Ophiostoma* EST data public. This should be an exciting opportunity for researchers working on Ophiostomatoid fungi and on the problems they cause on trees and wo-

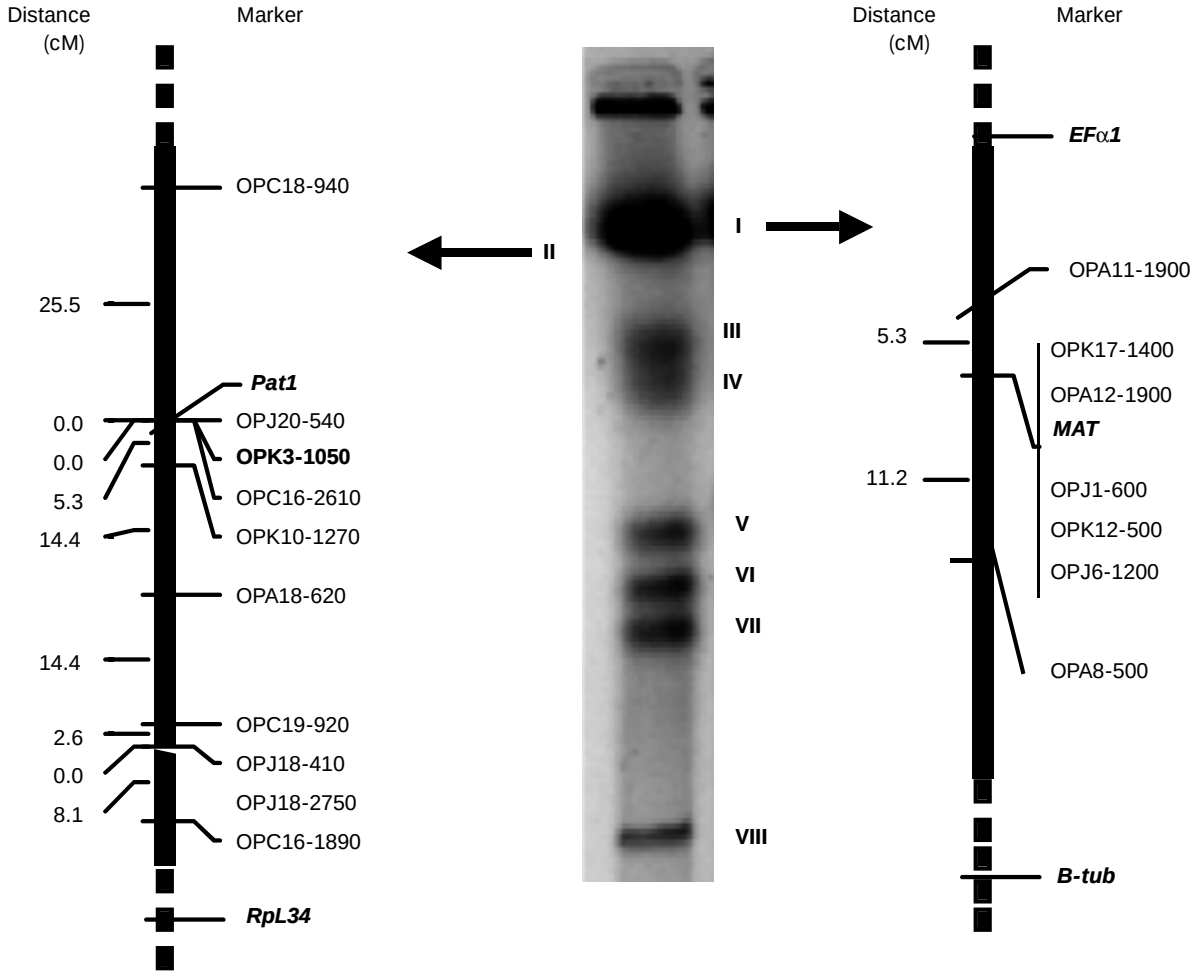

Figure 2. Partial linkage map for the Dutch elm disease fungi, Ophiostoma ulmi and *O. novo-ulmi*. The photograph at the centre of the figure shows the eight chromosomal bands (chDNAs) that can be separated by pulsed-field gel electrophoresis (PFGE) in *O. novo-ulmi*. The largest band consists of at least two chDNAs (data not shown), identified by Roman numerals I and II, whose size is larger than 5.7 million base pairs (Mb). The size of the smallest chDNA (VIII) is 1.0 Mb. A subset of 10 genetic loci located on chDNA I are shown on the right portion of the figure. The *B-tub* and *Efα1* loci, encoding b-tubulin and elongation factor α1, respectively, were cloned and hybridized to PFGE-separated chromosomes to confirm that they were located on chDNA I. The mating-type (*MAT*) locus and several RAPD loci (identified by primer and size of the amplicon) were also assigned to chDNA I, since they co-segregated with the *B-tub* and *Efα1* loci. Similar analyses showed that the *Pat1* pathogenicity locus was located on chDNA II, as it co-segregated with the *RpL34* locus encoding ribosomal protein L34, and with several RAPD loci, including those shown on the left portion of the figure. Genetic distances, in centiMorgans (cM), are indicated between closely linked loci. Dotted lines represent more distal portions of chDNAs I and II.

od products. Hopefully, these developments may also attract other researchers, thus augmenting the critical mass of scientists interested in Ophiostomatoid fungi.

Acknowledgements

The work described herein was made possible by a Genomics Grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada. The authors are indebted to Marie-Hélène Cavanagh, Jérôme Laroche, François Larochelle and Stéphane Larose at U. Laval, Ben Koop at U. Victoria, and Veronica Pereira at U. Toronto.

Literature Cited

ALTSCHUL S.F., MADDEN T.L., SCHÄFFER A.A., ZHANG J., ZHANG Z., MILLER W., LIPMAN D.J.,

1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389-3402.

BERNIER L., 1993. Conventional and molecular genetic approaches to the study of pathogenicity in *Ophiostoma ulmi sensu lato*. In: Dutch Elm Disease Research: Cellular and Molecular Approaches. Sticklen M. and Sherald J., eds. Springer-Verlag, New York, pp. 293-307.

BERNIER L., HUBBES M., 1990. Mutations in *Ophiostoma ulmi* induced by N-methyl-N'-nitro-N-nitrosoguanidine. Canadian Journal of Botany 68, 225-231.

BERNIER L., HUBBES M., 1994. Induction and genetic characterisation of ultraviolet-sensitive mutants in the elm tree pathogen *Ophiostoma ulmi* (*sensu lato*). Mycological Research 98, 943-953.

BOWDEN C.G., HINTZ W.E., JENG R.S., HUBBES M., HORGEN P.A., 1994. Isolation and characterization of the cerato-ulmin gene of the Dutch elm disease pathogen, *Ophiostoma ulmi*. Current Genetics 25, 323-329.

BOWDEN C.G., SMALLEY E., GURIES R.P., HUBBES M., TEMPLE B., HORGEN P.A., 1996. Lack of association between cerato-ulmin production and virulence in *Ophiostoma novo-ulmi*. Molecular Plant-Microbe Interactions 9, 556-564.

BRASIER C.M., KIRK S.A., PIPE N., BUCK K.W., 1998. Rare interspecific hybrids in natural populations of the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi*. Mycological Research 102, 45-57.

DE BEER Z.W., WINGFIELD B.D., WINGFIELD M.J., 2003. The *Ophiostoma piceae* complex in the Southern Hemisphere: a phylogenetic study. Mycological Research 107, 469-476.

DEL SORBO G., SCALA F., PARRELLA G., LORITO M., COMPARINI C., RUOCCO M., SCALLA A., 2000. Functional expression of the gene cu, encoding the phytotoxic hydrophobin cerato-ulmin, enables *Ophiostoma quercus*, a nonpathogen on elm, to cause symptoms of Dutch elm disease. Molecular Plant-Microbe Interactions 13, 43-53.

DEWAR K., BERNIER L., 1995. Inheritance patterns of nonhomologous chromosomes in *Ophiostoma ulmi sensu lato*. Current Genetics 27, 541-549.

DEWAR K., BOUSQUET J., DUFOUR J., BERNIER L., 1997. A meiotically reproducible chromosome length polymorphism in the ascomycete fungus *Ophiostoma ulmi* (*sensu lato*). Molecular and General Genetics 255, 38-44.

DIATCHENKO L., LUKYANOV S., LAU Y.F.C., SIEBERT P.D.,1999. Suppression subtractive hybridization: A versatile method for identifying differentially expressed genes. In: Methods in Enzymology 303: cDNA Preparation and Characterization. Weissman, S.M., ed. Academic Press, San Diego, pp. 349-380.

DUSABENYAGASANI M., DUFOUR J., BERNIER L., 2000. Approaches for genomic mapping of the Dutch elm disease pathogens. Annual Meeting of the American Phytopathological Society, New Orleans (LA). Phytopathology 90, S21 (Abstract).

EAGEN R., KIM S.H., KRONSTAD J.W., BREUIL C., 2001. An hydroxynaphtalene reductase gene from the wood-staining fungus *Ophiostoma floccosum* complements the buff phenotype in *Magnaporthe grisea*. Mycological Research 105, 461-469.

ET-TOUIL A., BRASIER C.M., BERNIER L., 1999. Localization of a pathogenicity gene in *Ophiostoma novo-ulmi* and evidence that it may be introgressed from *O. ulmi*. Molecular Plant-Microbe Interactions 12, 6-15.

FISCHER G., JAMES S.A., ROBERTS I.N., OLIVER S.G., LOUIS E.J., 2000. Chromosomal evolution in *Saccharomyces*. Nature 405, 451-454.

GAGNÉ P., YANG D.Q., HAMELIN R.C., BERNIER L., 2001. Genetic variability of Canadian populations of the sapstain fungus *Ophiostoma piceae*. Phytopathology 91, 369-376.

GALAGAN J.E. and 76 others, 2003. The genome sequence of the filamentous fungus *Neurospora crassa*. Nature 422, 859-868.

GOFFEAU A. and 632 others, 1997. The yeast genome directory. Nature 387 (Supplement), 1-105.

HARRINGTON T.C., MCNEW D., STEIMEL J., HOFSTRA D., FARRELL R., 2001. Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. Mycologia 93, 111-136.

HAUSNER G., REID J., 2003. Notes on *Ceratocystis brunnea* and some other *Ophiostoma* species based on partial ribosomal DNA sequence analysis. Canadian Journal of Botany 81, 865-876.

HINTZ W.E., 1999. Sequence analysis of the chitin synthase A gene of the Dutch elm disease pathogen *Ophiostoma novo-ulmi* indicates a close association with the human pathogen *Sporothrix schenckii*. Gene 237, 215-221.

HINTZ W.E., JENG R.S., YANG D.Q., HUBBES M., HORGEN P.A., 1993. A genetic survey of the pathogenic fungus *Ophiostoma ulmi* across a Dutch elm disease front in Western Canada. Genome 36, 418-426.

HOEGGER P.J., BINZ T., HEINIGER U., 1996. Detection of genetic variation between *Ophiostoma ulmi* and the NAN and EAN races of *O. novo-ulmi* in Switzerland using RAPD markers. European Journal of Forest Pathology 26, 57-68.

HOFFMAN B., BREUIL C., 2002. Cloning and genetic analysis of subtilases in sapstaining fungi. Current Genetics 41, 168-175.

HOFFMAN B., BREUIL C., 2004. Analysis of the distribution and regulation of three representative subtilase genes in sapstaining fungi. Fungal Genetics and Biology 41 (In Press).

KUPFER D.M., REECE C.A., CLIFTON S.W., ROE B.A., PRADE R.A., 1997. Multicellular ascomycetous fungal genomes contain more than 8000 genes. Fungal Genetics and Biology 21, 364-372.

NEUMANN M.J., DOBINSON K.F., 2003. Sequence tag analysis of gene expression during pathogenic growth and microsclerotia development in the vascular wilt pathogen *Verticillium dahliae*. Fungal Genetics and Biology 38, 54-62.

PEARSON W.R., LIPMAN D.J., 1988. Improved tools for biological sequence comparison. Proceedings of the National Academy of Sciences of the USA 85, 2444-2448.

PEREIRA V., ROYER J.C., HINTZ W.E., FIELD D., BOWDEN C., KOKUREWICZ K., HUBBES M., HORGEN P.A., 2000. A gene associated with filamentous growth in *Ophiostoma novo-ulmi* has RNA-binding motifs and is similar to a yeast gene involved in mRNA splicing. Current Genetics 37, 94-103.

PIPE N.D., BUCK K.W., BRASIER C.M., 1995. Molecular relationships between *Ophiostoma ulmi* and the NAN and EAN races of *O. novo-ulmi* determined by RAPD markers. Mycological Research 99, 653-658.

PIPE N.D., BUCK K.W., BRASIER C.M., 1997. Comparison of the cerato-ulmin (*cu*) gene sequences of the Himalayan Dutch elm disease fungus *Ophiostoma himal-ulmi* with those of *O. ulmi* and *O. novo-ulmi* suggests that the *cu* gene of *O. novo-ulmi* is unlikely to have been acquired recently from *O. himal-ulmi*. Mycological Research 101, 415-421.

RICHARDS W.C., 1993. Cerato-ulmin: a unique wilt toxin of instrumental significance in the development of Dutch elm disease. In: Dutch Elm Disease Research: Cellular and Molecular Approaches. Sticklen, M. and Sherald, J., eds. Springer-Verlag, New York, pp. 89-151.

TADESSE Y., BERNIER L., HINTZ W.E., HORGEN P.A., 2003. Real time RT-PCR quantification and Northern analysis of Cerato ulmin (*CU*) gene transcription in different strains of the phytopathogens *Ophiostoma ulmi* and *O. novo ulmi*. Molecular Genetics and Genomics 269, 789-796.

TANGUAY P., BERNIER L., BREUIL C., 2003. Electrophoretic karyotype polymorphisms within and between species of the genus *Ophiostoma*. Proceedings of the International Congress of Plant Pathology, Christchurch (NZ), Abstract 2-8/02.

TEMPLE B., HORGEN P.A., BERNIER L., HINTZ W.E., 1997. Cerato-ulmin, a hydrophobin secreted by the causal agents of Dutch elm disease, is a parasitic fitness factor. Fungal Genetics and Biology 22, 39-53.

WANG H.-L., BREUIL C., 2002. A second reductase gene involved in melanin biosynthesis in the sap-staining fungus *Ophiostoma floccosum*. Molecular Genetics and Genomics 267, 557-563.

YANG D., JENG R.S., HUBBES M., 1989. Mansonone accumulation in elm callus induced by elicitors of *Ophiostoma ulmi*, and general properties of elicitors. Canadian Journal of Botany 67, 3490-3497.

YANG D., HUBBES JR M., JENG R.S., HUBBES M., 1994. A glycoprotein isolated from culture filtrates of *Ophiostoma ulmi* as a mansonone-inducing elicitor on elm callus. Mycological Research 98, 295-300.