Isolation and Characterization of Differentially Expressed Genes in the Mycelium and Fruit Body of *Tuber borchii*

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The transition from vegetative mycelium to fruit body in truffles requires differentiation processes which lead to edible fruit bodies (ascomata) consisting of different cell and tissue types. The identification of genes differentially expressed during these developmental processes can contribute greatly to a better understanding of truffle morphogenesis. A cDNA library was constructed from vegetative mycelium RNAs of the white truffle *Tuber borchii*, and 214 cDNAs were sequenced. Up to 58% of the expressed sequence tags corresponded to known genes. The majority of the identified sequences represented housekeeping proteins, i.e., proteins involved in gene or protein expression, cell wall formation, primary and secondary metabolism, and signaling pathways. We screened 171 arrayed cDNAs by using cDNA probes constructed from mRNAs of vegetative mycelium and ascomata to identify fruit body-regulated genes. Comparisons of signals from vegetative mycelium and fruit bodies bearing 15 or 70% mature spores revealed significant differences in the expression levels for up to 33% of the investigated genes. The expression levels for six highly regulated genes were confirmed by RNA blot analyses. The expression of glutamine synthetase, 5-aminolevulinic acid synthetase, isocitrate lyase, thioredoxin, glucan 1,3- β -glucosidase, and UDP-glucose:sterol glucosyl transferase was highly up-regulated, suggesting that amino acid biosynthesis, the glyoxylate cycle pathway, and cell wall synthesis are strikingly altered during morphogenesis.

Several truffle species are harvested all over the world in significant quantities, as the organoleptic properties (i.e., taste and flavor) of their edible ascomata are highly appreciated. The fruiting of ectomycorrhizal *Tuber* depends on a complex set of variables, including metabolites and signals produced by the host plant, the nutritional status of the substrate, and unknown environmental cues (e.g., humidity and temperature). The different types of cells and tissues of fruit bodies of ascomycetes (ascomata) are the result of a differentiation process leading to the production of asci containing meiospores (30). The molecular bases of such events are largely unknown, with the exception of those in some model fungi, such as *Aspergillus nidulans* (1) and *Neurospora crassa* (26), in which an interactive cascade of developmentally regulated genes regulates sporulation.

Morphological descriptions of ascoma development in truffles are scarce and illustrate only advanced developmental stages (27). This situation is due to the hypogeous habitat of truffles, which leads to erratic sampling. In addition, symbiotic relationships are required for the development of the truffle fruit body (36), and fruit bodies cannot be produced in vitro. These features have hampered systematic studies of the molecular bases underlying fruit body development. Truffles, however, are not obligate symbionts, and some of them, including *Tuber borchii*, can be grown in pure mycelial cultures by exploitation of their limited saprotrophic capabilities. Efforts

have been made to elucidate this developmental process in *T. borchii*. A number of genes involved in cell wall formation (5, 7, 12), signal transduction (3, 11), and lipid metabolism associated with nutrient deprivation and cellular organization (35) have been characterized. Although this knowledge has led to a better understanding of some aspects of fruit body development, the molecular processes underlying fruit body initiation and maturation remain unclear. mRNA differential display has been used successfully to isolate five developmentally regulated genes in *T. borchii* (45). However, no data concerning the function of the deduced proteins encoded by these genes are yet available.

For further study of the genetics of *T. borchii* fruit body formation, we wanted to investigate the structure, function, and expression of additional genes involved in fruit body development. Therefore, cDNA clones isolated from a cDNA library of vegetative mycelium were sequenced and screened by using cDNA arrays for altered mRNA levels in the spore maturation stage of fruit body development. Even though the biological material was not homogeneous in its origin (i.e., vegetative mycelium is grown in axenic media, while fruit bodies are sampled in nature), novel information was obtained: 57 new cDNAs corresponding to up- or down-regulated genes were isolated. Transcripts with the highest increased concentrations in ascomata were involved in C and N metabolism, cell wall synthesis, and antioxidant defense mechanisms. On the other hand, genes expressed in vegetative mycelium and down-regulated in ascomata coded for unknown proteins.

MATERIALS AND METHODS

Biological materials. Vegetative mycelia of *T. borchii* Vittad (isolate ATCC 96540) were grown for 30 days in the dark at 24°C without shaking. Modified

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Melin-Norkrans synthetic medium (MMN medium) or potato dextrose broth (Difco) were used as liquid nutrient solutions (23). Mycelium was harvested by filtration, fixed in liquid N₂, and stored at -80°C. Spore-bearing ascomata of *T. borchii* were collected under hazelnut trees from natural truffle grounds near Alba in Piedmont (Italy) during the December 1999-March 2000 production season. They were washed and brushed, and the peridium was peeled. Their degree of maturation was evaluated by determining the ratio of immature and mature ascospores as described previously (12). Two stages of maturation (i.e., 15 and 70% mature spores; referred to as CF15 and CF70, respectively) were selected by observing sections under a light microscope (magnification, $\times 10$), and the corresponding ascomata were fixed in liquid N₂ and stored at -80°C.

Isolation of total RNA and genomic DNA. Total RNA for cDNA hybridizations, RNA blot analyses, and reverse transcriptase (RT) PCR analyses was isolated from vegetative mycelium and ascomata according to Viotti et al. (39). Total DNA was isolated by using the phenol-chloroform method according to Garnero et al. (12).

RT PCR assays and RNA blot analyses. To assess the expression of isocitrate lyase (ICL) transcripts, reverse transcription assays were performed at 42°C for 50 min in 20 μ l of first-strand buffer of the Superscript II RNase H-RT (Life Technologies, Carlsbad, Calif.), supplemented with dithiothreitol as recommended by the manufacturer, 20 U of RnaseOUT recombinant RNase inhibitor (Life Technologies), 1 mM each deoxynucleoside triphosphate, 0.25 ng of oligo(dT) 18-mer, 0.1 to 0.5 μ g of total RNA, and 200 U of Superscript II. Five microliters of reverse transcription product was amplified in a 50- μ l PCR mixture containing 1 μ M specific primers, 6.5 U of REDTaq DNA polymerase (Sigma), and the buffer supplied by the manufacturer of the GeneAmp 9700 PCR system (PE Applied Biosystems, Foster City, Calif.). The PCR parameters were as follows: 94°C for 3 min; 94°C for 30 s, 55°C (annealing temperature) for 0.5 min, and 72°C for 1 min for 35 cycles; and a final cycle at 72°C for 10 min. For RNA blot analyses, electrophoresis under denaturing conditions was performed with 1.2% agarose containing 0.7 M formaldehyde (18). Gels were stained with ethidium bromide and blotted on nylon membranes (Hybond-N+; Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) as described by the manufacturer. Hybridization was carried out as recommended by Amersham Pharmacia Biotech.

cDNA library construction, sequencing, and analyses. Total RNA was extracted from 20-day-old mycelium grown on potato dextrose agar and 30-day-old mycelium grown in MMN medium. A unidirectional cDNA library was then constructed by using a UniZapXR cDNA library system construction kit (Stratagene) (5; B. Lazzari and A. Viotti, unpublished results). λ UniZapXR clones were converted to pBK-CMV phagemid clones by using *Escherichia coli* BM25.8 as the bacterial host. A total of 214 recombinant bacterial clones were picked at random, and plasmid DNA was purified by using a Concert rapid plasmid miniprep system (Life Technologies). Inserted cDNA fragments were amplified by PCR with universal T3 or T7 primers. Automated sequencing of amplified cDNA was performed by using a BigDye terminator cycle sequencing kit (PE Applied Biosystems) and Genome Express with universal T3 or T7 primers. Leading and trailing vector and polylinker sequences and sequences with more than 3% ambiguous base calls were removed. Nucleotide and protein searches were performed by batch processing with BLASTN and WU-BLASTX against the nonredundant nucleic acid sequence GenBank database at the Baylor College of Medicine World Wide Web server by using a MacPerl script Mac-search-launcher (version 2.6) (43). Sequences with an expected value of $1e^{-5}$ were considered to identify known genes or to have partial homology to known genes (2). Expressed sequence tags (ESTs) and homology comparisons were organized into an online database that is accessible via the World Wide Web at <http://mycor.nancy.inra.fr/TuberDB/index.html>.

cDNA array construction and hybridization. cDNA inserts of purified plasmids corresponding to 171 selected ESTs of vegetative mycelium were amplified by PCR with 1 μ M universal primers T3 and T7, 100 μ M each deoxynucleoside triphosphate, 2.5 U of REDTaq DNA polymerase, and the buffer supplied by the manufacturer of the GeneAmp 9700 PCR system. The PCR parameters were as follows: 94°C for 3 min; 94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1 min for 35 cycles; and a final cycle at 72°C for 10 min. Successful production of PCR products was confirmed by agarose gel electrophoresis. The amplified cDNAs (10 to 15 ng/ μ l) were placed in spotting solution (0.2 M NaOH in $10\times$ SSC [$1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) to a volume of 25 μ l and were spotted on Hybond-N+ nylon filters (8 by 12 cm) by using a Minifold spot blot system (Schleicher & Schuell). Processing of the filters was done as described by Voiblet et al. (40).

Complex cDNA probes were prepared from total RNA isolated from vegetative mycelium grown under the same conditions as those used to construct the cDNA libraries or from CF15 and CF70 ascomata by using oligo(dT)-primed

Superscript II RT and a SMART PCR cDNA synthesis kit (Clontech, Palo Alto, Calif.). Labeling of cDNA probes was carried out in the presence of 30 μ Ci of [³²P]dCTP, 30 μ Ci of [³²P]dATP, and random hexamers by using a Prime-A-Gene labeling system (Promega, Madison, Wis.) according to the manufacturer's instructions. Three copies of the EST blots were then hybridized in duplicate with the labeled cDNAs from the mycelium and the CF15 and CF70 ascomata essentially as described previously (40). Clones corresponding to transcripts showing the highest level of regulation in fruit body RNA compared to vegetative mycelium RNA were hybridized to RNA blots containing size-fractionated RNAs (12) isolated from ascomata and vegetative mycelium to confirm the induction or repression of the corresponding genes in fruit bodies. Briefly, 10 μ g of total RNA was loaded on a 1.2% (wt/vol) agarose gel under denaturation conditions, blotted onto a Hybond-N filter, and hybridized with the selected cDNA labeled with [³²P]dCTP by random priming. A stringent wash was performed at 65°C with $0.5\times$ SSC and 0.1% (wt/vol) sodium dodecyl sulfate. The filter was then dehybridized and probed with *T. borchii* 5.8S ribosomal DNA (rDNA) to estimate the level of total RNA loaded in each lane. The dry filter was then wrapped in a plastic bag and exposed to a phosphorimaging screen (Kodak) for various periods (1 h to 3 days), after which the imaging plate was scanned with Personal Molecular Imager FX (Bio-Rad Laboratories, Hercules, Calif.) at a maximum resolution of 50 μ m/pixel.

Data acquisition and analysis. The raw image data obtained with the phosphorimager system were imported into an Apple Macintosh G3 computer. Detection and quantification of the signals representing hybridized DNA were performed by using the volume quantitation method of Quantity One software (Bio-Rad). Each spot was defined by manual positioning of a grid of squares over the array image. For each image, the average pixel intensity within each square was determined. The local background value for each membrane was calculated on the basis of five positions with no DNA-spotted areas. The net signal was determined by subtraction of this mean background value from the intensity for each spot. Spots deemed unsuitable for accurate quantitation because of array artifacts were manually flagged and excluded from further analysis. The data table generated by Quantity One, containing the intensity for each spot, was then exported to the Excel 98 worksheet program (Microsoft Corporation, Redmond, Wash.) for further manipulation. The probe-to-probe variance was filtered out by using the signal intensities of human desmin spotted at six locations on the filters (i.e., interfilter normalization) (40). Eight cDNA clones (VL76, VL12, VA76, VL79, VA71, VA17, VL47, and VL70) coding for constitutively expressed transcripts in vegetative mycelium and ascomata were used to normalize the signal intensities (VL indicates clones derived from potato dextrose broth, and VA indicates clones derived from MMN medium). Only genes with reproducible expression differences of 2.5-fold or more were considered in our analysis. Hierarchical gene clustering was carried out by using average linkage (unweighted pair-group method with arithmetic averages) clustering based on the Euclidean distance of the log-transformed normalized transcript ratio (<http://ep.ebi.ac.uk/EP/EPCLUST>). This distance-based analysis allowed the grouping of genes sharing similar expression patterns during spore maturation (6).

Nucleotide sequence accession numbers. All of the ESTs have been deposited in dbEST at the National Center for Biotechnology Information under accession numbers BM266140 to BM266336.

RESULTS

ESTs of *T. borchii* vegetative mycelium. Up to 214 cDNAs were selected at random from two cDNA libraries of vegetative mycelium of *T. borchii* grown on potato dextrose agar or in MMN medium. Upon assembly of the readable sequences obtained from the 5' end, we were left with 183 nonredundant ESTs corresponding to different genes. Among them, 107 sequences (58%) were similar to known genes, including ascomycetous (79%), plant and animal (18%), and bacterial (3%) sequences. According to their putative biological roles, these homologs have been classified in seven groups (see <http://mycor.nancy.inra.fr/TuberDB.html>). The largest category (25%) of identified sequences corresponded to genes involved in gene or protein expression machinery, which includes transcripts such as those coding for ribosomal proteins, translational regulatory proteins, elongation factors, and the ubiquitin/proteasome pathway. A large proportion

of genes (22%) expressed in vegetative mycelium coded for enzymes of primary and secondary metabolism (e.g., phosphofructokinase [PFK], aldolase, citrate synthase, glutamine synthetase [GS], and ICL). Transcripts involved in stress responses (10%) (e.g., thioredoxin, glutaredoxin, and heat shock protein), cell signaling (7%) (e.g., GTPases, 14-3-3 protein, and calmodulin), and cell structure (7%) (e.g., actin) represented smaller proportions of the sequenced cDNAs.

Gene expression profiles in vegetative mycelium and fruit bodies. A total of 171 cDNA clones from vegetative mycelium were differentially screened with probes from vegetative mycelium (stage 1), CF15 ascomata (stage 2), and CF70 ascomata (stage 3) by cDNA array hybridization. The aim of the experiment was to select clones corresponding to genes whose transcripts were regulated by fruit body formation in which karyogamy and meiosis are occurring. Nonregulated genes, which encode hypothetical proteins, were arrayed to normalize signal differences. Totals of 128 (75%) and 130 (76%) of the cDNA clones did not show significant differences (2.5-fold) in RNA expression levels (Fig. 1 and Table 1) between vegetative mycelium and CF15 and CF70 ascomata, respectively. On the other hand, 28 (16.5%) and 27 (16%) of the transcripts showed increased expression levels in CF15 and CF70 fruit bodies compared to mycelium, respectively. Fifteen (9%) and 14 (8%) of the genes showed decreased expression levels in CF15 and CF70 ascomata compared to mycelium, respectively (Table 1).

Genes that showed up-regulated expression in fruit bodies encoded proteins involved in nitrogen and carbon metabolism (e.g., GS, 5-aminolevulinic acid synthetase, PFK, succinate dehydrogenase [SDH], and ICL), antioxidative enzymes (thioredoxin and glutaredoxin), P_i and hexose transport, and cell wall synthesis (glucan 1,3- β -glucosidase and UDP-glucose:sterol glucosyl transferase) (Table 1). The highest differential expression (54-fold increase) was detected for the GS gene (clone VA113). The mRNA capping enzyme (clone VL29), 5-aminolevulinic acid synthetase (clone VL26), thioredoxin (clone VL19), and ICL (clone VL9) transcripts also showed a striking increase in expression (>10-fold) in fruit bodies. Of the genes that were up-regulated >2.5-fold, five had unknown functions; most genes whose expression was diminished >2.5-fold also had unknown functions.

Analysis of the expression profile (Fig. 2) by hierarchical clustering allowed us to define groups of coregulated genes among the 171 ESTs. Cluster A contained transcripts (e.g., GS, thioredoxin, and ICL) showing a high level of up-regulation and identical expression patterns in CF15 and CF70 ascomata. Transcripts in cluster B (e.g., UDP-glucose:sterol glucosyl transferase and glucan 1,3- β -glucosidase) and cluster E (e.g., glutaredoxin) exhibited a higher concentration in CF70 ascomata, whereas transcripts in cluster C (e.g., 5-aminolevulinic acid synthetase and mRNA capping enzyme) showed their highest expression levels in CF15 ascomata. All genes of clusters F and G were highly down-regulated (up to 50-fold) in CF15 and CF70 fruit bodies and can be considered mycelium specific.

To validate the cDNA array data, the expression of six moderately and strongly regulated genes (i.e., those for GS, ICL, thioredoxin, and three unknown proteins) was monitored by RNA blot analyses (Fig. 3). RNA blot analyses confirmed that the expression of all six vegetative mycelium ESTs was

responsive to fruiting even when quantitative differences due to the different hybridization techniques used (i.e., RNA blot analyses versus cDNA arrays) were observed. Thioredoxin transcripts were abundant in vegetative mycelium, but their concentrations were drastically increased in stage 2 and 3 ascomata. GS and ICL transcripts gave no signal or only a weak signal with the corresponding mycelium mRNA sequence. They were induced in stage 2, even though the band was lower and more diffuse in CF15 samples, probably due to some degradation events. Transcripts reached a higher level in the last phase of spore maturation (stage 3), i.e., CF70 ascomata. In contrast, clones VA65, VA115, and VL71, coding for hypothetical proteins, hybridized strongly to unique vegetative mycelium sequences and seemed to be absent from or else present at very low concentrations in ascomata (Fig. 2 and 3).

ICL expression was further characterized by monitoring transcript accumulation by using RT PCR analysis of vegetative mycelium grown with different media (Fig. 4). ICL transcripts were not detected in vegetative mycelium grown in MMN medium containing 4% glucose, whereas an intense band was detected when vegetative mycelium was grown in sugar-deprived MMN medium (Fig. 4).

DISCUSSION

An EST database containing more than 2,000 clones was recently set up for the widely cultivated edible mushroom *Pleurotus ostreatus* with the aim of providing information on differential gene expression during the transition of vegetative mycelium to spore-bearing structures (17). In other ascomycetes and basidiomycetes, the transition has already been demonstrated to be coupled to transcriptional changes in many genes which regulate reproductive development after induction (1, 8, 10, 34, 41, 44). With differential screening approaches, cDNAs from genes showing down- or up-regulation during the development of spore-bearing structures have been characterized for the ascomycetes *N. crassa* (26) and *A. nidulans* (38) and the basidiomycetes *Agrocybe aegerita* (32), *Lentinus edodes* (15), *Schizophyllum commune* (41), and *Agaricus bisporus* (8).

In the present study, we isolated from vegetative mycelium of *T. borchii* 57 genes having up- or down-regulated expression in fruit bodies. Fruit body formation and sporulation are therefore accompanied by the differential expression of about 33% of the investigated genes. Forty-one transcripts showed striking up-regulation at either of the developmental stages, suggesting that the corresponding proteins are directly implicated in the morphogenesis and functioning of fruit bodies during spore maturation. In *Lentinula edodes* (19), *A. bisporus* (8), and *A. aegerita* (32), similar numbers of fruit body-regulated genes were identified. Sixteen genes, mostly coding for unknown proteins, had lower transcript concentrations and probably represented vegetative mycelium-specific genes. The hierarchical clustering of ascoma-regulated transcripts indicated concomitant up- or down-regulated expression of groups of genes (Fig. 2), suggesting that common inducer signals may coordinate their expression.

Fruiting in truffles requires the establishment of a functional ectomycorrhizal symbiosis and is associated with the presence of soil microorganisms (13). Since truffles cannot fruit under axenic conditions, we compared gene expression in vegetative

FIG. 1. Expression profiles of 171 genes in vegetative mycelium and ascomata of *T. borchii*. For each gene, transcript levels were calculated for the free-living mycelium and CF15 or CF70 ascomata and are displayed on a scatter plot (see Materials and Methods). If the genes are not affected by fruit body development, then their transcript levels will fall between the lines labeled 2.5X. Solid lines indicate 2.5-fold expression differences between free-living partners and ascomata; broken lines indicate 10-fold expression differences.

TABLE 1. Differential gene expression in ascomata and vegetative mycelium from *T. borchii*^a

GenBank accession no.	cDNA		Best database match (corresponding species) determined with WU-BLASTX	%			Expected value	Transcript ratio (normalized hybridization)	
	Name	Size (bp)		Identity	Similarity	Overlap in aa		CF15/VM	CF70/VM
BM266151	VA113	698	GS (<i>A. nidulans</i>)	76	89	204	3e-98	11.1	55.0
BM266330	VL94	694	Glucan 1,3- β -glucosidase (<i>N. crassa</i>)	59	74	226	1e-77	2.4	20.0
BM266140	VA1	629	Hypothetical protein					3.8	10.7
BM266256	VL19	609	Thioredoxin (<i>Emericella nidulans</i>)	55	74	81	1e-20	11.5	17.3
BM266266	VL29	644	mRNA capping enzyme β subunit (<i>Schizo-</i> <i>saccharomyces pombe</i>)	31	46	192	3e-15	12.0	1.5
BM266325	VL9	611	ICL (<i>E. nidulans</i>)	74	77	154	1e-59	10.8	9.9
BM266263	VL26	605	5-Aminolevulinic acid synthetase amino- transferase (<i>Aspergillus oryzae</i>)	78	87	180	2e-80	11.1	3.1
BM266273	VL36	620	Hypothetical protein					5.2	9.1
BM266248	VL11	685	UDP-glucose:sterol glucosyl transferase (<i>Magnaporthe grisea</i>)	63	71	253	1e-91	5.0	7.8
BM266163	VA20	659	Regulator of Pho81 (phosphate transport regulation) (<i>Saccharomyces cerevisiae</i>)	54	66	79	5e-17	6.4	4.9
BM266252	VL15	680	Heat shock protein, 70 kDa (<i>E. nidulans</i>)	77	84	226	4e-93	5.0	3.8
BM266317	VL82	722	SDH (<i>Mycosphaerella graminicola</i>)	80	87	214	1e-103	4.7	2.2
BM266255	VL18	657	Cell cycle regulator p21 protein (<i>S. pombe</i>)	44	60	125	7e-24	4.1	2.2
BM266239	VA92	695	Glycine-rich protein (<i>Coccidioides immitis</i>)	84	93	93	2e-38	4.0	1.7
BM266329	VL93	693	UDP-galactose transporter (<i>S. pombe</i>)	45	63	155	4e-27	3.9	3.7
BM266155	VA12 ^b	551	Glutaredoxin (<i>N. crassa</i>)	47	65	109	1e-21	2.3	3.7
BM266324	VL89	574	Hypothetical protein					1.9	2.6
BM266272	VL34	639	Hypothetical protein					1.5	4.4
BM266210	VA65	948	Hypothetical protein					-3.9	-4.0
BM266156	VA13	421	Hypothetical protein					-5.3	-9.1
BM266192	VA48	666	Hypothetical protein					-7.7	-11.1
BM266171	VA28	393	Hypothetical protein					-12.5	-7.2
BM266240	VA93	969	Hypothetical protein					-20.0	-33.3
BM266153	VA115	644	Hypothetical protein					-33.3	-25.0
BM266305	VL71	907	Hypothetical protein					-33.3	-50.0
BM266295	VL60	630	Hypothetical protein					-50.0	-50.0

^a The 26 genes with the highest (up-regulation) and lowest (down-regulation) ascoma/vegetative mycelium (VM) expression ratios are listed. Signal ratios of <1.0 were inverted and multiplied by -1 to aid in their interpretation. aa, amino acids.

^b Full-length clone.

mycelium grown under axenic conditions and in fruit bodies collected from soil. Therefore, we cannot exclude the possibility that part of the difference in transcript levels observed for fruit body-regulated genes is a metabolic difference as a result of different genetic backgrounds, growth conditions (i.e., agar medium versus soil), and/or environmental cues. However, in order to minimize individual differences, different fruit bodies were investigated in separate experiments.

The spore contains surface structures which are lacking in vegetative mycelium, including an outer, proteinaceous lipid layer and a constant chitin layer (4). Changes in cell wall metabolism during fruit body morphogenesis have been observed for many fungal species (37, 41). The hydrophobin family has been investigated in detail during the morphogenesis of several ascomycetes and basidiomycetes (37, 41), but several additional proteins are probably involved in fruit body and spore formation. The *Tbf-1* gene from *T. borchii* codes for a structural cell wall protein specifically expressed in fruit bodies (7). In *T. magnatum* and *T. borchii*, chitin synthase genes are differentially expressed in fruit bodies in a maturation stage-dependent manner (5, 12). *Tbchs3*, identified among the EST clones of *T. borchii* as VA116, appears to be involved in spore maturation, whereas *Tbchs4* may play a role in ascoma enlargement. The hierarchical clustering of ascoma-regulated transcripts confirms that VA116 showed up-regulated expres-

sion in fruit bodies (data not shown). Together with chitin polymers, β -1,3-glucans are important components of *T. borchii* and *T. magnatum* hyphae as well as ascus walls (3, 5). Wessels and Sietsma (42) showed how cell wall components are continuously recycled during fungal morphogenesis. The induction of an exo-1,3- β -glucosidase (clone VL94) and UDP-glucose:sterol glucosyl transferase (clone VL11) in CF15 and CF70 ascomata of *T. borchii* (Table 1) is possibly related to the degradation of sterile hyphae located around the ascus during the ascoma maturation process or spore cell wall expansion (25).

A number of *T. borchii* genes encoding key enzymes of carbon and nitrogen metabolism (e.g., GS, PFK, ICL, and SDH) have been cloned from vegetative mycelium. Analysis of CF15 and CF70 ascomata showed increased expression of several of these genes. This result suggests that hyphae involved in spore production and maturation are metabolically very active. Glycolysis and the pentose phosphate pathway are down-regulated in mature *T. borchii* fruit bodies (31), suggesting that the availability of external hexose and oxygen is limited in these hypogeous tissues. Nutrient deprivation is probably a primary stress in fruiting hyphae, and catabolism of lipids accumulated in vegetative mycelium probably sustains the constant carbon flux needed for fruit body construction and maturation. Black Sudan staining indicated that lipid globules are abundant in *T.*

FIG. 2. Changes in transcript levels during *T. borchii* fruit body formation. Shown is the hierarchical clustering of ratios of the transcript levels of selected genes in CF15 (stage 2) or CF70 (stage 3) ascocata versus vegetative mycelium. Distance-based clustering (6) allowed definition of a subset of genes sharing similar expression profiles (A to G). Each gene is represented by a row of colored boxes, and each stage is represented by a single column (left, CF15/VM; right, CF70/VM). Regulation levels ($\log_2$ transformed for hierarchical analysis) range from pale to saturated colors (red for induction; green for repression). Black indicates no change in gene expression.

FIG. 3. Hybridization of six vegetative mycelium cDNAs strongly regulated during fruit body formation to total RNAs from vegetative mycelium (VM) and CF15 (stage 2) or CF70 (stage 3) ascomata. Total RNAs, isolated at the three stages in the course of fruit body formation, were hybridized with 32 P-labeled cDNA inserts of clones VL9, VL19, VL71, VA65, VA113, and VA115 and the nonregulated rDNA internal transcribed spacer. The 5.8S rDNA signal intensity was used to normalize RNA loading, and then the expression ratios were calculated as CF15/VM and CF70/VM. Ratios of less than 1 (repression) were multiplied by -1 to allow a direct comparison between up- and down-regulated genes.

borchii fruit bodies. Lipid accumulation was observed initially in vegetative hyphae of CF15 fruit bodies and then in mature spores of CF70 fruit bodies (I. Lacourt and P. Bonfante, unpublished data). The *TbSP1* phospholipase gene of *T. borchii* is strongly up-regulated in response to carbon and nitrogen deprivation (35). The *TbSP1* phospholipase was localized in hyphae and ascus cell walls of fruit bodies, where this enzyme could participate in the generation of free fatty acids. Two-carbon compounds resulting from this fatty acid degradation are probably assimilated into the tricarboxylic acid cycle through the glyoxylate cycle steps catalyzed by ICL and malate synthase. This anaplerotic metabolic pathway is operative in microorganisms experiencing nutritional deprivation (20, 22), including mycorrhizal fungi (16). The increased concentrations of ICL and SDH transcripts during *T. borchii* fruiting are consistent with active gluconeogenesis. Studies with isotope labeling should confirm the activities of these pathways (16). The glyoxylate/tricarboxylic acid/gluconeogenesis pathways are probably used to sustain the dramatic carbon drain accompanying fruit body enlargement and spore maturation (i.e., lipid and glycogen stores and chitin synthesis). The down-regulation

of ICL transcripts in vegetative mycelium grown in glucose-containing medium is in agreement with the catabolite repression of the glyoxylate cycle (14).

GS is involved in nitrogen assimilation and amino acid biosynthesis in ectomycorrhizal fungi (21). The isolation of a GS cDNA thus enabled study of the regulation of these key aspects of primary metabolism. The GS gene displayed the highest up-regulation of the investigated genes. Its transcript concentrations increased 11-fold in CF15 ascomata and 55-fold in CF70 ascomata. RNase protection experiments confirmed these increased levels of GS transcripts during spore maturation (S. Ottonello, personal communication), irrespective of the technique and the individual samples. A striking increase in GS activity was also reported during the maturation of *Coprinus cinereus* basidiomata (9). This up-regulation was correlated with the accumulation of urea and arginine. These nitrogen compounds are probably involved in cellular expansion through increased osmotic pressure, and the increased GS activity led to a lower level of the ammonium ion, a powerful inhibitor of meiosis (24). In *T. borchii*, the up-regulation of GS expression at the CF15 and CF70 stages may play a similar role

FIG. 4. Changes in the presence of the isocitrate lyase (VL9) transcript in *T. borchii* mycelium grown with various carbon sources, as determined by RT-PCR. Total RNAs were isolated from vegetative mycelium grown in MMN medium containing 4% glucose or no carbohydrate. Lane 1, lambda DNA digested with *Hind*III and *Eco*RI. Lanes 2 and 4, RT-PCR of RNAs extracted from mycelium grown in MMN medium with glucose and without glucose, respectively. Lanes 3 and 5, PCR of the same RNAs without RT (negative control). Lane 6, PCR of the ICL cDNA clone VL9. Lane 7, PCR of genomic DNA of *T. borchii*. The larger size of the genomic amplification product suggests the presence of an intervening intron(s) in the ICL gene (cf. lane 6).

by participating in ascoma enlargement and allowing meiosis during spore formation.

The concentrations of transcripts of the antioxidant enzymes thioredoxin and glutaredoxin were strikingly increased in CF15 and CF70 ascomata, suggesting that cell differentiation and nutrient deprivation in hyphae led to the production of active oxygen species. Similar genes were found to be up-regulated by dehydration in *Arabidopsis thaliana* (33). It is commonly accepted that hypogeous fruit body formation in *Tuber* may be a response to dehydration (28). It is therefore tempting to speculate that a similar mechanism of protection against water loss and based on the up-regulation of genes coding for antioxidant enzymes also operates in truffles. Such a mechanism would be active not only during the formation of truffle fruit bodies but also during the establishment of *Tuber* ectomycorrhizae. Polidori et al. (29) also found a plant glutaredoxin homolog to be up-regulated during the establishment of symbiosis.

Fifteen transcripts coding for unknown proteins were strongly regulated in *T. borchii* fruit bodies. Additionally, four differentially expressed genes were identified by mRNA display (45). These transcripts could be essential for functions required during ascoma morphogenesis. Further investigations (in situ hybridization and immunolocalization of the corresponding proteins) will concentrate on the elucidation of the functions of these genes.

The cDNA library used in this study was constructed by using mRNA expressed during the vegetative phase of the life cycle of *T. borchii* grown on agar media. The identification of genes specifically expressed during fruiting is hampered by

the fact that ascomata have not yet been produced under axenic conditions, but the construction of cDNA libraries from mRNAs isolated from fruit bodies collected in the field is under way. These libraries will allow the identification of a larger number of fruit body-regulated genes to decipher the complex networks of developmental and metabolic changes taking place during ascoma formation.

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REFERENCES

- Adams, T. H., J. K. Wieser, and J. H. Yu. 1998. Asexual sporulation in *Aspergillus nidulans*. *Microbiol. Mol. Biol. Rev.* **62**:35–54.
- Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **17**:3389–3402.
- Ambra, R., and G. Macino. 2000. Cloning and characterization of PKC-homologous genes in the truffle species *Tuber borchii* and *Tuber magnatum*. *FEMS Microbiol. Lett.* **189**:45–53.
- Balestrini, R., M. G. Hahn, and P. Bonfante. 1996. Location of cell-wall components in ectomycorrhizae of *Corylus avellana* and *Tuber magnatum*. *Protoplasma* **191**:55–69.
- Balestrini, R., D. Mainieri, E. Soragni, L. Garnero, S. Rollino, A. Viotti, S. Ottonello, and P. Bonfante. 2000. Differential expression of chitin synthase III and IV mRNAs in ascomata of *Tuber borchii* Vittad. *Fungal Genet. Biol.* **31**:219–232.
- Brazma, A., and J. Vilo. 2000. Gene expression data analysis. *FEBS Lett.* **480**:17–24.
- De Bellis, R., D. Agostini, G. Piccoli, L. Vallorani, L. Potenza, E. Polidori, D. Sisti, A. Amoresano, P. Pucci, G. Arpaia, G. Macino, R. Balestrini, P. Bonfante, and V. Stocchi. 1998. The *Tbf-1* gene from the white truffle *Tuber borchii* codes for a structural cell wall protein specifically expressed in fruit-body. *Fungal Genet. Biol.* **25**:87–99.
- De Groot, P. W. J., P. J. Schaap, L. J. L. D. Van Griensven, and J. Visser. 1997. Isolation of developmentally regulated genes from the edible mushroom *Agaricus bisporus*. *Microbiology* **143**:1993–2001.
- Ewaze, J. O., D. Moore, and G. R. Stewart. 1978. Coordinate regulation of enzymes involved in ornithine metabolism and its relation to sporophore morphogenesis in *Coprinus cinereus*. *J. Gen. Microbiol.* **107**:343–357.
- Fernandez-Espinar, M. T., and J. Labarère. 1997. Cloning and sequencing of the *Aa-Pr1* gene specifically expressed during fruiting initiation in the edible mushroom *Agrocybe aegerita* and analysis of the predicted amino-acid sequence. *Curr. Genet.* **32**:420–424.
- Garnero, L., and P. Bonfante. 2000. *TMpcp*: a *Tuber magnatum* gene which encodes a putative mitochondrial phosphate carrier. *DNA Seq.* **10**:407–410.
- Garnero, L., B. Lazzari, D. Mainieri, A. Viotti, and P. Bonfante. 2000. *TMchs4*, a class IV chitin synthase gene from the ectomycorrhizal *Tuber magnatum*. *Mycol. Res.* **6**:703–707.
- Gazzanelli, G., M. Malatesta, A. Pianetti, W. Baffone, V. Stocchi, and B. Citterio. 1999. Bacteria associated to fruit bodies of the ectomycorrhizal fungi *Tuber borchii* Vittad. *Symbiosis* **26**:211–222.
- Jennings, D. H. 1995. The physiology of fungal nutrition. Cambridge University Press, Cambridge, England.
- Kondoh, O., A. Muto, S. Kajiwara, J. Takagi, Y. Saito, and K. Shishido. 1995. Fruiting body-specific cDNA, *mfbAc*, from the mushroom *Lentinus edodes* encodes a high-molecular-weight cell-adhesion protein containing an Arg-Gly-Asp motif. *Gene* **154**:31–37.
- Lammers, P. J., J. Jun, J. Abubaker, R. Arreola, A. Gopalan, B. Bago, C. Hernandez-Sebastia, J. W. Allen, D. D. Douds, P. Pfeffer, and Y. Shachar-Hill. 2001. The glyoxylate cycle in an arbuscular mycorrhizal fungus. Carbon flux and gene expression. *Plant Physiol.* **127**:1287–1298.
- Lee, S. H., B. G. Kim, K. J. Kim, J. S. Lee, D. W. Yun, J. H. Hahn, G. H. Kim, K. H. Lee, D. S. Suh, S. T. Kwon, C. S. Lee, and Y. B. Yoo. 2002. Comparative analysis of sequences expressed during the liquid-cultured mycelia and fruit body stages of *Pleurotus ostreatus*. *Fungal Genet. Biol.* **35**:115–134.

18. Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker. 1977. RNA molecular weight determination by gel electrophoresis under denaturing conditions: a critical reexamination. *Biochemistry* **16**:4743–4751.
19. Leung, G. S., M. Zhang, W. J. Xie, and H. S. Kwan. 2000. Identification by RNA fingerprinting of genes differentially expressed during the development of the basidiomycete *Lentinula edodes*. *Mol. Gen. Genet.* **262**:977–990.
20. Lorenz, M. C., and G. R. Fink. 2001. The glyoxylate cycle is required for fungal virulence. *Nature* **412**:83–86.
21. Martin, F., J. B. Cliquet, and G. Stewart. 2001. Nitrogen acquisition and assimilation in mycorrhizal symbioses, p. 147–166. In P. Lea and J.-F. Morot-Gaudry (ed.), *The assimilation of nitrogen by plants*. Springer-Verlag KG, Berlin, Germany.
22. McKinney, J. D., K. Höner zu Bentrup, E. J. Muñoz-Elias, A. Miczak, B. Chen, W. Chan, D. Swenson, J. D. Sacchetti, W. R. Jacobs, and D. Russell. 2000. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* **406**:735–738.
23. Mischiati, P., and A. Fontana. 1993. *In vitro* culture of *Tuber magnatum* mycelium isolated from mycorrhizas. *Mycol. Res.* **97**:40–44.
24. Moore, D. 1998. *Fungal morphogenesis*. Cambridge University Press, Cambridge, England.
25. Muthukumar, G., S. H. Susung, P. T. Magee, R. D. Jewell, and D. A. Primarano. 1993. The *Saccharomyces cerevisiae* *SPR1* gene encodes a sporulation-specific exo-1,3- β -glucanase which contributes to ascospore thermoresistance. *J. Bacteriol.* **175**:386–394.
26. Nelson, M. A., S. Kang, E. L. Braun, M. E. Crawford, P. L. Dolan, P. M. Leonard, J. Mitchell, A. M. Armijo, L. Bean, E. Blueyes, T. Cushing, A. Errett, M. Fleharty, M. Gorman, K. Judson, R. Miller, J. Ortega, I. Pavlova, J. Perea, S. Todisco, R. Trujillo, J. Valentine, A. Wells, M. Werner-Washburne, S. Yazzie, and D. O. Natvig. 1997. Expressed sequences from conidial, mycelial, and sexual stages of *Neurospora crassa*. *Fungal Genet. Biol.* **21**:348–363.
27. Parguey-Leduc, A., M. C. Janex-Favre, C. Montant, and M. Kulifaj. 1989. Ontogénie et structure de l'ascocarpe du *Tuber melanosporum* Vitt. (Truffe noire du Périgord, Discomycètes). *Bull. Soc. Mycol. France* **3**:227–246.
28. Pegler, D. N., B. M. Spooner, and T. W. K. Young. 1993. British truffles. A revision of British hypogeous fungi. Royal Botanic Gardens, Kew, United Kingdom.
29. Polidori, E., D. Agostini, S. Zeppa, L. Potenza, F. Palma, D. Sisti, and V. Stocchi. 2002. Identification of differentially expressed cDNA clones in *Tilia platyphyllos*-*Tuber borchii* ectomycorrhizae using a differential screening approach. *Mol. Genet. Genomics* **266**:858–864.
30. Read, N. D., and A. Beckett. 1996. Ascus and ascospore morphogenesis. *Mycol. Res.* **100**:1281–1314.
31. Saltarelli, R., P. Ceccaroli, L. Vallorani, A. Zambonelli, B. Citterio, M. Malatesta, and V. Stocchi. 1998. Biochemical and morphological modifications during the growth of *Tuber borchii* mycelium. *Mycol. Res.* **102**:403–409.
32. Salvado, J. C., and J. Labarère. 1991. Isolation of transcripts preferentially expressed during fruit body primordium differentiation in the basidiomycete *Agrocybe aegerita*. *Curr. Genet.* **20**:205–210.
33. Seki, M., M. Narusaka, H. Abe, M. Kasuga, K. Yamaguchi-Shinozaki, P. Carnici, Y. Hayashizaki, and K. Shinozaki. 2001. Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell* **13**:61–72.
34. Sonnenberg, A. S. M., P. W. J. De Groot, P. J. Schaap, J. J. P. Baars, and J. Van Visser. 1996. Isolation of expressed sequence tags of *Agaricus bisporus* and their assignment to chromosomes. *Appl. Environ. Microbiol.* **62**:4542–4547.
35. Soragni, E., A. Bolchi, R. Balestrini, C. Gambaretto, R. Percudani, P. Bonfante, and S. Ottonello. 2001. A nutrient-regulated, dual localization phospholipase A2 in the symbiotic fungus *Tuber borchii*. *EMBO J.* **20**:5079–5090.
36. Stocchi, V., R. De Bellis, L. Potenza, S. Zeppa, F. Bernardini, L. Vallorani, R. Saltarelli, G. Piccoli, and D. Agostini. 2000. The truffle life cycle: a biochemical and molecular characterization, p. 101–109. In G. K. Podila and D. D. Douds, Jr. (ed.), *Current advances in mycorrhiza research*. The American Phytopathological Society, Saint Paul, Minn.
37. Stringer, M. A., and W. E. Timberlake. 1995. *dewA* encodes a fungal hydrophobin component of the *Aspergillus* spore wall. *Mol. Microbiol.* **16**:33–44.
38. Timberlake, W. E. 1990. Molecular genetics of *Aspergillus* development. *Annu. Rev. Genet.* **24**:5–36.
39. Viotti, A., D. Abildsten, N. Pogna, E. Sala, and V. Pirrotta. 1982. Multiplicity and diversity of cloned cDNA sequences and their chromosomal localization. *EMBO J.* **1**:53–58.
40. Voiblet, C., S. Duplessis, N. Encelot, and F. Martin. 2001. Identification of symbiosis-regulated genes in *Eucalyptus globulus*-*Pisolithus tinctorius* ectomycorrhiza by differential hybridization of arrayed cDNAs. *Plant J.* **25**:181–191.
41. Wessels, J. G. H. 1994. Developmental regulation of fungal cell wall formation. *Annu. Rev. Phytopathol.* **32**:413–437.
42. Wessels, J. G. H., and J. H. Sietsma. 1979. Wall structure and growth in *Schizophyllum commune*, p. 29–48. In J. H. Burnett and A. P. J. Trinci (ed.), *Fungal wall and hyphal growth*. Cambridge University Press, Cambridge, England.
43. Worley, K. C., P. Culpepper, B. A. Wiese, and R. F. Smith. 1998. BEAUTY-X: enhanced BLAST searches for DNA queries. *Bioinformatics* **14**:890–891.
44. Yashar, P. J., and B. M. Pukkila. 1985. Analysis of meiotic development in *Coprinus cinereus*. *Symp. Soc. Exp. Biol.* **38**:177–194.
45. Zeppa, S., M. Guescini, L. Potenza, D. Agostini, E. Polidori, and V. Stocchi. 2000. Analysis of gene expression in the vegetative and fructification phases of the white truffle, *Tuber borchii* Vittad, by mRNA differential display. *Biotechnol. Lett.* **22**:307–312.