Laser Capture Microdissection of Uredinia Formed by *Melampsora larici-populina* Revealed a Transcriptional Switch Between Biotrophy and Sporulation

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The foliar rust caused by the basidiomycete *Melampsora larici-populina* is the main disease affecting poplar plantations in Europe. The biotrophic status of rust fungi is a major limitation to study gene expression of cell or tissue types during host infection. At the uredinial stage, infected poplar leaves contain distinct rust tissues such as haustoria, infection hyphae, and uredinia with sporogenous hyphae and newly formed asexual urediniospores. Laser capture microdissection (LCM) was used to isolate three areas corresponding to uredinia and subjacent zones in the host mesophyll for expression analysis with *M. larici-populina* whole-genome exon oligoarrays. Optimization of tissue preparation prior to LCM allowed isolation of RNA of good integrity for genome-wide expression profiling. Our results indicate that the poplar rust uredinial stage is marked by distinct genetic programs related to biotrophy in the host palisade mesophyll and to sporulation in the uredinium. A strong induction of transcripts encoding small secreted proteins, likely containing rust effectors, is observed in the mesophyll, suggesting a late maintenance of suppression of host defense in the tissue containing haustoria and infection hyphae. On the other hand, cell cycle and cell defense rescue transcripts are strongly accumulated in the sporulation area. This combined LCM-transcriptomic approach brings new insights on the molecular mechanisms underlying urediniospore formation in rust fungi.

The foliar rust caused by the basidiomycete fungus *Melampsora larici-populina* is the main disease affecting poplar in Europe, causing severe damages in plantations (Gérard et al. 2006). At present, almost all planted poplar cultivars are susceptible to *M. larici-populina*, and sustainability of newly selected resistances requires a better understanding of the molecular mechanisms underlying *Populus-Melampsora* spp. interaction. The recent sequencing of the *M. larici-populina* genome is a great opportunity to identify loci coding for the arsenal developed by the rust fungus to penetrate and exploit its host (Duplessis et al. 2009). *M. larici-populina* has a complex bio-

logical lifecycle, performing sexual reproduction on an alternate host (*Larix* spp.), whereas polycyclic production of asexual urediniospores occurs during summer on poplar trees, leading to severe epidemics (Barrès et al. 2008). The fungus penetrates through stomata on the abaxial epidermis of poplar leaves between 2 and 6 h after a spore lands on a leaf surface (Rinaldi et al. 2007). A drastic increase in fungal biomass is observed at 96 h postinfection (hpi) inside parenchyma cells after the formation of haustoria (Rinaldi et al. 2007). This infection structure, involved in the redirection of host metabolism and the suppression of host defenses, allows the establishment of a successful biotrophic relationship (Heath 1997; Voegelé et al. 2009). By 168 hpi, orange pustules corresponding to uredinia release urediniospores on the leaf surface to achieve further infection of poplar leaves.

The obligate biotroph status of rust fungi is a limitation to the study of gene expression in structures involved in biotrophic growth or sporulation stage because rust cannot be cultivated outside of their hosts. Most of the knowledge gained on rust gene expression in planta derived from sequencing of expressed sequence tags (EST) from haustorial structures or infected plant tissues (Hahn and Mendgen 1997; Thara et al. 2003; Broeker et al. 2006; Catanzariti et al. 2006; Feau et al. 2007; Puthoff et al. 2008; Yin et al. 2009). Identification of genes specifically expressed during the biotrophic phase was reported in the bean rust *Uromyces fabae* such as the thiamine biosynthesis genes *THI1* and *THI2* (Sohn et al. 2000), the amino-acid transporter AAT2 and the sugar transporter HXT1 (Hahn et al. 1997; Voegelé et al. 2001), or the rust-transferred protein 1 *RTP1* (Kemen et al. 2005). In the flax rust *Melampsora lini*, haustorially expressed secreted proteins (HESP) were reported as highly enriched for avirulence elicitors (Dodds et al. 2004; Catanzariti et al. 2006, 2010). In *Uromyces appendiculatus*, a large number of EST confirmed the importance of secreted proteins, primary metabolism, and nutrient uptake in haustorial structures (Puthoff et al. 2008). In contrast to the knowledge of molecular events related to rust invasion in plant hosts, nothing is known about gene networks underlying spore formation in rust fungi. The complexity of fungal structures at late infection stages (i.e., host tissues containing haustoria, infection hyphae, sporogenous hyphae, and newly formed spores) is a major limitation to identifying genes specifically involved in sporulation.

Laser capture microdissection (LCM) is a powerful tool to isolate populations of cells from heterogeneous tissues and

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offers new possibilities to explore transcript expression analysis on specific cells or tissues. LCM has been successfully used to study gene expression in different plant tissues such as epidermal cells, shoot meristem tissues, and root cap tissues

Fig. 1. Development of uredinia from *Melampsora larici-populina* 98AG31 in the susceptible *Populus trichocarpa* × *P. deltoides* cv. Beaupré. **A** through **D**, Confocal laser scanning microscopy images of fungal structures in planta at uredinal stage using Uvitex 2B staining plant and fungal cell walls (green) and propidium iodide staining fungal material (red). **A**, Early and **B**, mature uredinium formed after 96 and 168 h postinfection (hpi). **C**, Infection hyphae and **D**, haustorium colonizing spongy and palisade mesophyll at 168 hpi. Pictures were taken using a ×100 objective lens; scale bar represents 5 µm. **E**, Macroscopic view of mature uredinia. Bar represents 1 mm; sh = sporogenous hyphae, sp = spores, ih = infection hyphae, ha = haustorium, sm = spongy mesophyll, pm = palisade mesophyll, ur = uredinium.

(Nakazono et al. 2003; Jiang et al. 2006; Brooks et al. 2009). So far, only a few studies have reported the use of LCM to study plant–microbe interactions (Balestrini et al. 2009) such as those between plants and nematodes (Ramsay et al. 2004; Klink et al. 2007; Ithal et al. 2007), arbuscular mycorrhizal fungi (Balestrini et al. 2007; Gomez and Harrison 2009), and pathogenic fungi (Tang et al. 2006; Tremblay et al. 2009; Chandran et al. 2010). An LCM-based approach allowed collecting maize stalk cells associated with *Colletotrichum graminicola* hyphae at early stage of infection and microarray analysis led to the identification of several transcripts highly accumulated and potentially involved in pathogenesis (Tang et al. 2006). In a study conducted on soybean infected by the rust fungus *Phakopsora pachyrhizi*, RNA isolation through LCM from uredinia allowed cDNA library construction and access to a few rust unisquences (Tremblay et al. 2009). More recently, microdissection of infection sites from *Arabidopsis* leaves challenged by the powdery mildew *Golovinomyces orontii* helped in identifying downstream elements of the plant defense response not previously detected through whole-leaf transcriptome analysis (Chandran et al. 2010).

In this study, we successfully used LCM to dissect three distinct areas from poplar leaves infected by *M. larici-populina* at the uredinal stage. Using this strategy, we were able to separate fungal sporulation and fungal infection tissues. High-quality total RNA were isolated and amplified from these areas to assess rust transcript profiles through transcriptome analysis using *M. larici-populina* whole-genome exon oligoarrays and reverse-transcription quantitative polymerase chain reaction (RT-qPCR).

RESULTS

Development of *M. larici-populina* uredinia in poplar leaves.

M. larici-populina uredinia development was visualized in planta at 96 and 168 hpi using laser-scanning confocal microscopy. Primordia of uredinia containing initial sporogenous hyphae were observed at 96 hpi inside spongy mesophyll (Fig. 1A) whereas mature uredinia, from which newly formed dikaryotic urediniospores are released, were observed at 168 hpi (Fig. 1B). In addition, these stages were marked by a massive accumulation of fungal infection hyphae (Fig. 1C), and the presence of many haustoria was also noticeable (Fig. 1D) in both spongy and palisade mesophylls. At 168 hpi, uredinia emerged as yellow-orange pustules on the abaxial leaf surface

Fig. 2. Laser capture microdissection (LCM) of an uredinium formed by *Melampsora larici-populina* strain 98AG31 on the susceptible poplar cv. Beaupré. **A**, Transversal section of an uredinium; **B**, selection of areas for LCM; and **C**, section after laser capture of the three selected areas. Area 1, subepidermal *M. larici-populina* uredinium below poplar abaxial surface containing urediniospores and sporogenous hyphae; area 2, poplar leaf spongy mesophyll containing infection hyphae and haustoria; and area 3, poplar leaf palisade mesophyll containing *M. larici-populina* infection hyphae and haustoria. Pictures were taken using a ×40 objective lens; scale bar represents 150 µm; sh = sporogenous hyphae, sp = spores, sm = spongy mesophyll, pm = palisade mesophyll, le = lower leaf epidermis, ue = upper leaf epidermis.

of poplar and led to a massive production of asexual urediniospores (Fig. 1E).

Optimization of RNA isolation from LCM uredinia.

Ethanol-acetic acid (EAA) fixation has been reported to maintain histological integrity while enabling good RNA yield recovery (Kerk et al. 2003) and has been successfully used for LCM and microarray analysis of plant tissues (Nakazono et al. 2003; Casson et al. 2008). EAA-fixed and sectioned uredinia are shown before (Fig. 2A and B) and after (Fig. 2C) laser capture of area 1, 2, and 3 (i.e., spores and sporogenous hyphae, fungal infection tissues in spongy mesophyll, and fungal infection tissues in palisade mesophyll, respectively) (Fig. 2B). Good preservation of plant and fungal cell structures were obtained from EAA-fixed, paraffin-embedded sections but initial efforts to isolate intact RNA from laser-captured areas were unsuccessful. Using the conventional method of slide mounting, where paraffin ribbons were floated onto a bead of diethylpyrocarbonate (DEPC) water and mounted onto a slide, strong RNA degradation was observed for the different areas as revealed by the absence of ribosomal (r)RNA peaks on RNA electrophoresis profiles (Fig. 3A). When paraffin ribbons were directly transferred onto a microscope slide without water and slide drying, a drastic increase of RNA integrity was observed (Fig. 3B). A moderate degradation of RNA was still observed as revealed by the attenuated signal of the 28S ribosomal subunits of rRNA. In order to improve RNA integrity, we used a 37°C melting point paraffin instead of conventional paraffin at 58°C to prevent exposition of RNA molecules to high temperatures during paraffin impregnation of samples. Using this low-melting-point paraffin coupled with a direct transfer of paraffin ribbons onto microscope slides, two distinct peaks of rRNA were observed for each sampled area on electrophoresis profile, indicating improved RNA integrity after LCM (Fig. 3C; Supplementary Fig. 1A). Less than 5 ng of total RNA were isolated from the three LCM uredinia areas and more than 4 µg of amplified RNA (aRNA) were produced for all samples through linear amplification (Nakazono et al. 2003; Chandran et al. 2010), enabling proper synthesis of cDNA amounts to perform labeling and array hybridization.

Gene expression profiling of LCM fungal tissues.

M. larici-populina whole-genome exon oligoarrays were used to measure transcripts expression in the three areas isolated from uredinia. Among the 15,388 genes analyzed on the oligoarrays, 9,650 (63%) were expressed above background level in at least one of the different areas. In detail, 8,145, 7,786, and 7,288 transcripts were detected in area 1, 2, and 3, respectively. Few differentially expressed transcripts (*t* test, *P* value < 0.05, fold-change > 3) were detected between area 2 and 1 or area 2 and 3 (0 and 56 transcripts respectively; *data not shown*). In contrast, a strong proportion of transcripts were differentially accumulated between area 1 and 3, suggesting a continuum of transcript expression between the three sampled areas in uredinia. In total, 542 transcripts were induced in area 3 compared with area 1, whereas 762 were induced in area 1 compared with area 3. The comparison of transcript profiles between these two contrasted areas at the uredinial stage allowed us to gain insights into the genes associated with biotrophy (area 3) and sporulation (area 1).

Poplar rust biotrophy-related transcripts.

Among the 542 *M. larici-populina* transcripts upregulated in area 3 compared with area 1, 117 shared similarities with known proteins and were assigned to functional categories (Table 1) based on the Functional Catalogue (FunCat) scheme (Ruepp et al. 2004) to decipher molecular mechanisms under-

lying biotrophic growth. Six transcripts encoding *M. lini* HESP homologs previously identified in haustoria during flax infection (Catanzariti et al. 2006) (JGI [Joint Genome Institute] protein ID nos. 64764, 58916, 103805, 110232, 71126, and 93569) were gradually expressed in spongy mesophyll toward palisade mesophyll and showed inductions ranking between seven- and 90-fold compared with the sporulation area (Table 1). Two of them were identified among the 25 most highly induced *M. larici-populina* transcripts (>80-fold) in area 3 compared with area 1 (Table 2). Interestingly, 19 of these 25 most highly upregulated transcripts encoded secreted proteins of less than 300 amino acids (small secreted proteins [SSP]) and most of them were lineage specific but of unknown function. The most highly induced transcript (JGI protein ID no. 106078; 400-fold induction) encoded a 137-amino-acid cysteine-rich SSP with one ortholog in the wheat stem rust *Puccinia graminis* f. sp. *tritici* genome and no ortholog in other fungi (Table 2). Induction of expression of transcripts related to lipid or polysaccharide degradation such as cutinase (JGI protein ID no. 123735), lipases (JGI protein ID nos. 110949 and 106095), and β-glycosidase (JGI protein ID no. 91623) was observed in the biotrophic area (Table 1), indicating that the fungus might still degrade the host cell wall at the uredinial stage. Several enzymes involved in carbohydrate metabolism were also strongly induced in the biotrophic area (>10-fold) such as an invertase (JGI protein ID no. 44167), able to cleave sucrose from the host plant cell in glucose and fructose. Transcripts encoding sugar transporters (JGI protein ID nos. 38418 and 85306), an amino acid permease (JGI protein ID no. 34045), or an oligopeptide transporter (JGI protein ID no. 105630) were also highly induced in the biotrophic area (more than sixfold), highlighting the importance of nutrients uptake in the host mesophyll. Interestingly, the two most induced transporter transcripts (JGI protein ID nos. 38418 and 34045), with 31.1- and 12.1-fold changes, respectively, are homologs of the *U. fabae* hexose transporter HXT1 and amino acid permease AAT2 previously described for their expression restricted to haustoria (Hahn et al. 1997; Voegelé et al. 2001). In addition, exocytosis- and cellular-trafficking-related transcripts (JGI protein ID nos. 95032, 47066, and 103049) were also

Fig. 3. Electrophoretic profiles of total RNA isolated from laser microdissected areas 1, 2, and 3 using different sample preparation methods. **A**, Sample impregnation using high-melting point paraffin and water mounted on the slide; **B**, sample impregnation using high-melting point paraffin and dry mounted on the slide; and **C**, sample impregnation using low-melting point paraffin and dry mounted on the slide. Stars indicate ribosomal RNA subunits.

upregulated, consistent with an intense cellular transport activity in the biotrophic area. Several transcripts involved in protein degradation were also differentially expressed in area 3 compared with area 1, such as an ubiquitin-conjugating enzyme (JGI protein ID no. 41986) and a RING-finger-containing ubiquitin ligase (JGI protein ID no. 118355) with 5.5- and fivefold regulation, respectively. Moreover, two proteases (JGI protein ID nos. 69042 and 66970) acting in peptide bonds hydrolysis were upregulated 13.5- and 9.1-fold, respectively, suggesting a strong activation of fungal proteasome and proteolysis inside palisade mesophyll at late uredinial stage.

Poplar rust sporulation-related transcripts.

Among the 762 transcripts upregulated in area 1 compared with area 3, 381 showed homologies with known proteins and were assigned to functional cellular categories in order to identify gene networks associated with urediniospore formation (Table 3). An important proportion of transcripts induced in the sporulation area encode detoxification enzymes such as cytosolic Cu/Zn superoxide dismutases (JGI protein ID nos. 72564 and

72563), peroxidases (JGI protein ID nos. 87334 and 94172), and a cytochrome P450 (JGI protein ID no. 76380), suggesting cellular protection against oxygen-derived free radicals during sporogenesis. Nine transcripts related to cell cycle control and encoding a centrin 3, septin, M-phase inducer phosphatase, and DNA replication initiation factor *cdc45* were also upregulated in area 1, highlighting the high rate of cell division in the sporulation area compared with the biotrophic area. In addition, DNA replication-, recombination-, and repair-related transcripts were strongly upregulated, consistent with cell division activity. Interestingly, most of the genes involved in cell division and detoxification were among the 25 most highly upregulated transcripts (>90-fold) in the sporulation area compared with the biotrophic area (Table 4). Transcripts encoding lipid metabolism enzymes, such as a squalene synthase (86.7-fold induction, JGI protein ID no. 94543) involved in sterol biosynthesis pathway, and three enzymes showing lipase activity, such as a phospholipase (60.4-fold induction, JGI protein ID no. 59333), an epoxide hydrolase (39.2-fold induction, JGI protein ID no. 123933), and a lipase (31.1-fold induction, JGI protein ID no. 63115), were observed

Table 1. Selected *Melampsora larici-populina* genes presenting transcript induction in the palisade mesophyll (area 3) versus sporogenous (area 1) microdissected structures, assessed by whole-genome expression oligoarrays and categorized according to their cellular category (Functional Catalogue)

Protein ID ^b	Putative function or feature	Uredinia laser capture microdissection ^a				
		Expression level			Expression ratio	
		Area1	Area2	Area3	Area3/Area1	P
	Subcellular localization					
64764	Small secreted protein (hesp-376 homology)	15	185	1,363	90.0	0.012
58916	Small secreted protein (hesp-C63 homology)	15	246	1,247	85.7	0.010
103805	Small secreted protein (hesp-270 homology)	109	2,994	4,509	41.5	0.010
110232	Small secreted protein (hesp-897 homology)	53	300	1,512	28.3	0.042
71126	Small secreted protein (hesp-C49 homology)	355	4,389	8,691	24.4	0.023
93569	Small secreted protein (hesp-417 homology)	5,665	18,108	40,009	7.0	0.033
	Metabolism					
	Lipid					
123735	Cutinase	88	2,311	6,193	70.2	0.011
110949	Lipase B	53	649	2,338	44.0	0.020
106095	Lysophospholipase A	88	1,161	2,659	30.0	0.025
87140	Polyprenyl synthetase	22	36	349	16.0	0.046
72337	α/β hydrolase	553	1,663	2,822	5.1	0.039
	Carbon					
53785	Ribokinase	24	263	1,020	42.7	0.018
44167	β -Fructosidase (invertase) (glycosyl hydrolase family 32)	417	1,377	4,421	10.6	0.023
29139	Phosphoribosylglycinamide formyltransferase	470	1,537	4,720	10.0	0.018
91623	β -Glycosidase (glycosyl hydrolase family 5)	30	54	205	6.8	0.034
	Vitamin					
79837	Riboflavin biosynthesis protein	155	490	874	5.6	0.044
	Redox					
86038	Oxidoreductase, 2OG-Fe(II) oxygenase family	408	4,197	10,964	26.8	0.039
90083	Indoleamine 2,3-dioxygenase	142	1,324	3,357	23.6	0.049
63255	NAD dependent epimerase/dehydratase	72	666	1,502	20.8	0.031
114173	Aldo/keto reductase	794	9,026	15,541	19.5	0.037
93258	Oxidoreductase 2OG-Fe(II) oxygenase family	131	837	1,273	9.7	0.047
33839	FAD dependent oxidoreductase	686	4,556	5,160	7.5	0.030
109018	Glutaredoxin	207	653	1,551	7.4	0.036
84159	NAD dependent epimerase/dehydratase	204	644	1,433	7.0	0.013
33377	Short-chain dehydrogenase/reductase	1,197	6,383	7,263	6.0	0.041
103191	Oxidoreductase, 2OG-Fe(II) oxygenase family	1,028	3,604	5,722	5.5	0.032
25325	D-arabinono-1,4-lactone oxidase	11	16	57	5.1	0.020
	Cellular transport, transport facilitation					
38418	HXT1p Sugar transporter	29	329	901	31.1	0.014
34045	Amino acid permease AAT2	34	90	415	12.1	0.039
85306	Sugar transporter-MFS monosaccharide transporter	213	313	2,315	10.8	0.016
95032	Exocyst complex component, sec3 subunit	13	17	112	8.6	0.036
47066	Clathrin assembly protein	178	168	1,179	6.6	0.029
105630	Oligopeptide transporter	121	458	792	6.5	0.040
103049	Got1-like vesicle transport protein	42	113	237	5.6	0.013

(continued on next page)

^a Normalized transcript levels in area 1, 2, and 3 (i.e., spores and sporogenous hyphae, fungal infection tissues in spongy mesophyll, and fungal infection tissues in palisade mesophyll, respectively), transcript expression ratio (area 3/area 1), and associated *t* test *P* value (<0.05).

^b Protein ID number of corresponding best gene model in the *M. larici-populina* JGI genome sequence.

in the sporulation area (Table 3). Other enzymes involved in carbohydrate degradation, such as α -glycosidase (JGI protein ID nos. 92210), β -glycosidases (JGI protein ID nos. 116147 and 55983), and α -L-arabinofuranosidase (JGI protein ID no. 107307), also showed transcript induction during sporogenesis. In contrast with the biotrophic area, genes involved in nucleotide catabolism, such as ribonucleases (JGI protein ID nos. 117154 and 54248) and an endonuclease (JGI protein ID no. 26899), were strongly induced in the sporulation area, indicating a strong turnover of nucleic acids. Several transporters were specifically detected during urediniospores formation. The most induced encoded a GDP-fucose transporter (JGI protein ID no. 49361) that showed a 93-fold induction. MFS permeases (JGI protein ID nos. 94938 and 25355), an amino acid permease (JGI protein ID no. 49500), and an oligopeptide transporter (JGI protein ID no. 94692) were among the highly induced transcripts (>30-fold), indicating strong metabolite trafficking concomitant with urediniospore synthesis. In contrast with the strong proportion of SSP identified among the most highly induced transcripts in the biotrophic area (Tables 1 and 2), one *M. lini* HESP homo-

log was induced in the sporulation tissues (Table 3) and only five SSP were identified among the 25 most highly induced transcripts (>80-fold) in the sporulation area (Table 4), suggesting the preferential role of SSP during the biotrophic growth phase.

Validation of differential gene expression profiles.

The expression level of 29 transcripts encoding *M. larici-populina* SSP and known rust protein homologs or that showed a preferential expression pattern in area 1 or 3 was assessed by RT-qPCR. A linear correlation ($R^2 = 0.96$) was observed between expression profiles measured by RT-qPCR and oligoarrays validating the transcriptomic approach (Fig. 4). The slope of the regression line was close to unity (0.86), indicating a minimal variation in expression data and consistency between both approaches.

DISCUSSION

As noncultivable organisms, obligate biotrophic fungi are dependent on their host to achieve their growth and develop-

Table 1. (continued from preceding page)

Protein ID ^b	Putative function or feature	Uredinia laser capture microdissection ^a				
		Expression level			Expression ratio	
		Area1	Area2	Area3	Area3/Area1	P
	Protein fate (folding, modification, destination)					
69042	Pepsin	741	9,008	10,207	13.5	0.011
66970	Cysteine proteases	24	64	228	9.1	0.048
41986	Ubiquitin-conjugating enzyme E2	990	2,606	5,531	5.5	0.035
118355	C3HC4 type RING finger containing ubiquitin ligase	1,193	3,193	6,042	5.0	0.013
	Transcription					
108564	GCN5-related N-acetyltransferase	20	73	575	28.7	0.025
107345	C2H2-type Zn finger protein	175	2,448	2,377	13.5	0.021
74108	Transcriptional regulator Prz1	1,147	4,880	11,866	10.3	0.016
94759	C2H2-type Zn finger protein 7	282	3,356	2,585	9.1	0.024
39532	Transcription elongation factor	627	1,579	3,293	5.2	0.027
	Cellular communication or signal transduction mechanism					
106239	NUDIX hydrolase	44	392	991	22.3	0.010
94327	Protein kinase AGC family	23	30	165	7.3	0.039
43665	Response regulator receiver	682	1,908	4,450	6.5	0.048
	Cell rescue, defense, and virulence					
40544	Survival protein SurE	844	1,810	13,302	15.7	0.010
106535	Heat shock protein	304	755	1,554	5.1	0.018
	Cell cycle and DNA processing					
60363	DNA topoisomerase I	27	69	349	13.1	0.034
47226	G1 Cyclin	472	2,140	3,863	8.1	0.028
	Protein synthesis					
30347	Ribosomal protein S8	30	62	418	13.8	0.038
37443	Diphthine synthase	618	531	3,279	5.3	0.020
	Biogenesis of cellular components					
27318	Cell wall protein	23	78	248	10.6	0.030
95757	Proteophosphoglycan 5	35	62	277	7.9	0.040
64466	Proteophosphoglycan ppg4	20	42	123	6.1	0.038
	Transposable elements, viral and plasmid proteins					
88937	Transposase	42	110	514	12.2	0.048
94469	Transposable element	14	22	75	5.2	0.048
	Energy					
93294	Malate dehydrogenase	485	986	3,429	7.0	0.042
95852	Malate dehydrogenase, NAD-dependent	40	73	235	5.9	0.014
65530	Electron transport complex	12	45	72	5.8	0.031
	Protein with binding function or cofactor requirement					
105154	Phosphatidylethanolamine-binding protein	200	2,567	13,666	68.4	0.013
66416	Zinc finger SWIM domain-containing protein	21	37	184	8.9	0.049
62481	Glycine-rich RNA binding protein	1,682	8,050	12,280	7.3	0.021
38287	Phosphatidylethanolamine-binding protein	34	44	234	6.8	0.046
	Unclassified proteins					
28849	Histidine-rich protein	21	49	280	13.5	0.039
93926	Sporozoite surface protein	22	62	294	13.2	0.043
61950	Glycoprotein	20	34	162	8.0	0.042
85862	CRKRS protein	14	19	84	6.0	0.027
87097	EPS15 homology (EH) domain-containing protein	20	31	105	5.3	0.018

ment. This is a major limitation to studying the biology of rust fungi and, thus far, there is a limited knowledge of molecular mechanisms at stake during rust proliferation in planta and, particularly, at the late stage of infection when sporulation occurs. In order to show that physiological heterogeneity and metabolic zonation occurred within fungal tissues (Cairney and Burke 1996), we used an LCM-based approach to study gene expression at the uredinal stage of rust infection in plant tissue (i.e., production of asexual urediniospores) targeting different leaf areas below uredinal structures. Using this strategy, we were able to separate two contrasted fungal tissues from poplar leaf tissues infected by *M. larici-populina*: a biotrophic area in the plant mesophyll and a sporulation area below the abaxial epidermis where the uredinal dome is formed and from which newly formed urediniospores are released.

Transcriptome analyses strongly depend on RNA quality (Copoio et al. 2007) and the use of LCM-collected samples to accurately determine integrity of RNA isolated from microdissected tissues. Low-volume automated electrophoresis bioanalyzers now allow assessment of RNA profiles at very low concentrations, down to 100 pg/μl. In microdissection studies, RNA quality is often based on RT-PCR transcript amplification (Kerk et al. 2003; Inada and Wildermuth 2005; Yu et al. 2007) and not on total RNA profiles. Tissue preparation prior to LCM and fixation methods, for instance, can strongly impact quality of RNA (Kerk et al. 2003). Farmer's fixation has been reported to maintain good histological and RNA integrity (Kerk et al. 2003; Ivashikina et al. 2003; Nakazono et al. 2003; Ramsay et al. 2004; Nakada et al. 2006). Loss of RNA integrity during slide mounting was previously pointed out and resolved by the

use of a tape transfer system (Cai and Lashbrook 2006). Here, we report that other factors impact RNA quality during tissue preparation, such as sample impregnation and slide mounting. Low-melting paraffin coupled with a direct transfer of paraffin ribbons onto microscope slides without water and slide drying led to a drastic increase of RNA integrity (Fig. 3).

Transcriptome analysis of LCM-sampled tissues revealed gene networks of *M. larici-populina* associated with particular biological features of this rust fungus such as biotrophy and sporulation. Many transcripts were strongly induced in the biotrophic tissues compared with the sporulation tissues, suggesting that the corresponding proteins might be specifically involved in the parasitic growth (Tables 1 and 2). A striking result is the massive induction of genes encoding SSP in the palisade mesophyll, including several homologs of *M. lini* haustorially expressed secreted protein (Catanzariti et al. 2006). These SSP represent a reservoir of putative effectors that might be required to alter the host defense system (Ellis et al. 2007). Thus, it is tempting to link the induction of *M. larici-populina* SSP to the maintenance of the biotrophic interaction (i.e. through suppression of host defense) at the uredinal stage. Concomitantly with this massive induction of transcripts encoding SSP, the sec3 subunit of the exocyst complex, involved in the tethering, docking, and fusion of post-Golgi vesicles with the plasma membrane (Wu et al. 2008) as well as the Got1-like vesicle transport protein, showed consistent inductions in the fungal tissue (Table 1), suggesting that the exocytosis mechanism, one of the suggested routes for effector secretion (Voegelé et al. 2009), might be required for SSP release by rust infection structures. Several transcripts related to car-

Table 2. Most highly upregulated *Melampsora larici-populina* transcripts in the palisade mesophyll (area 3) versus sporogenous (area 1) microdissected structures identified using whole-genome expression oligoarrays and ranked by decreasing fold changes

Protein ID ^b	SP ^c	Length	Cysteine ^d	Putative function or features	Blastp ^e	Uredinia laser capture microdissection ^a				
						Expression level		Expression ratio		P
						Area1	Area2	Area3	Area3/Area1	
106078	Y	137	11	Rust-specific SSP	PGTG_15874	23	4,804	9,025	397.5	0.013
Mlp0002_0042	Y	76	1	Mlp-specific SSP	No significant hit	11	517	3,802	340.4	0.012
106150	Y	118	1	Mlp-specific SSP	No hits found	28	3,457	7,441	262.1	0.012
104758	–	136	0	Mlp-specific protein	No hits found	19	804	4,909	255.8	0.013
123218	Y	210	6	SSP	PGTG_02151	68	7,025	17,338	254.9	0.011
105547	Y	206	1	Mlp-specific SSP	No hits found	28	1,349	6,440	231.8	0.013
108588	–	109	4	Mlp-specific protein	No hits found	23	3,575	5,265	225.6	0.014
109323	–	142	3	Mlp-specific protein	No hits found	32	2,668	6,840	215.0	0.013
123731	Y	184	2	Mlp-specific SSP	No significant hit	27	1,417	5,769	212.8	0.011
Mlp0016_0150	Y	98	7	Mlp-specific SSP	No significant hit	18	820	3,662	202.1	0.011
112715	Y	142	9	Mlp-specific SSP	No hits found	33	2,788	6,355	190.9	0.021
56132	–	155	4	Rust-specific protein	PGTG_05628	27	130	5,186	189.5	0.018
123531	Y	103	9	Mlp-specific SSP	No significant hit	41	2,966	7,457	179.8	0.040
60448	Y	156	3	Mlp-specific SSP	No hits found	48	978	8,533	179.4	0.013
72983	Y	220	8	SSP	PGTG_06969	102	10,925	16,923	166.1	0.027
102036	Y	107	1	Mlp-specific SSP	No hits found	18	927	2,928	165.3	0.011
86274	Y	244	10	SSP	PGTG_04481	16	763	2,523	162.3	0.007
106925	–	105	10	Mlp-specific protein	No hits found	21	1,668	3,022	144.1	0.010
Mlp0032_0018	Y	76	2	Mlp-specific SSP	No significant hit	52	3,818	7,088	136.8	0.006
66715	Y	459	2	Mlp-specific secreted protein	No hits found	14	521	1,718	119.8	0.014
123438	Y	180	11	Rust-specific SSP	PGTG_08705	223	15,297	25,016	112.1	0.021
109567	Y	156	2	Mlp-specific SSP	No hits found	48	976	5,287	109.1	0.046
64764	Y	154	1	SSP (hesp-376 homology)	No hits found	15	185	1,363	90.0	0.012
68912	Y	144	2	Mlp-specific SSP	No hits found	38	1,255	3,390	89.7	0.014
58916	Y	219	4	SSP (hesp-C63 homology)	No hits found	15	246	1,247	85.7	0.010

^a Normalized transcript levels in area 1, 2, and 3 (i.e., spores and sporogenous hyphae, fungal infection tissues in spongy mesophyll, and fungal infection tissues in palisade mesophyll, respectively), transcript expression ratio (area 3/area 1), and associated *t* test *P* value (<0.05).

^b Protein ID number of corresponding best predicted gene model in the *M. larici-populina* JGI genome sequence; Mlp0002_0042, Mlp0016_0150, and Mlp0032_0018 correspond to additional predicted gene models not described on the *M. larici-populina* genome portal.

^c Signal peptide (SP) prediction. Presence (Y) or absence (–) of a signal peptide is indicated.

^d Number of cysteines.

SSP = small secreted protein.

^e Best blast hit against the genome sequence of *Puccinia graminis* f. sp. *tritici* deposited at the Broad Institute genome portal.

bohydrate transport and metabolism and expressed in haustoria of *U. fabae* were also detected in the biotrophic area of *M. larici-populina*. The strong expression of a secreted invertase, a β -glycosidase, and an hexose transporter (*U. fabae* HXT1 homolog) is consistent with the hypothesis that fungal enzymes should be present in the haustorial matrix to provide the substrate for the hexose transporter (Voegelé et al. 2001, 2006, 2009). In addition, transcripts encoding an amino acid permease showing homology with the haustoria-specific Uf-AAT2 permease (Hahn et al. 1997) were also strongly induced, suggesting a conserved role for rust transporters in nutrient uptake from infected host cells.

Most recent biochemical and molecular studies of rust fungi focused on the parasitic growth and, more precisely, the haustorial structure. In addition, the molecular basis underlying the sporulation process in rust is poorly known. This step is essential for rust development and leads to huge production of asexual urediniospores disseminated by winds over very large distances (Nagarajan and Singh 1990; Barrès et al. 2008). In ascomycetes, molecular genetic approaches have been successfully applied to dissect the genetic mechanisms controlling conidia morphogenesis in model fungal species (Adams et al. 1998). In rust fungi, LCM and EST sequencing of uredinia formed by *Phakopsora pachyrhizi* identified a limited number of unisquences displaying similarities to known proteins in databases (Tremblay et al. 2009). Among them, transcripts encoding ribosomal protein and serine/threonine-protein phosphatase were also strongly expressed in *M. larici-populina* uredinia. Our LCM-based transcriptome study allowed the unraveling of essential functions of rust sporulation in the basidiomycete *M. larici-populina* (Tables 3 and 4). Asexual urediniospores are the result of cell division occurring via mitosis. Several genes preferentially expressed in the sporulation area are involved in the cell cycle, such as the M phase inducer phosphatase *cdc25*, as well as *cdc45* required for initiation of DNA replication in *Saccharomyces cerevisiae* (Saha et al. 1998). This mitotic activity during sporogenesis is also supported by transcript induction of a septin involved in cytokinesis. Although cytokinesis mechanisms have been well described during yeast division and sporulation (Wolfe and Gould 2005; Diamond et al. 2009) and hyphae septation (Walther and Wendland 2003), their role in sporogenesis of basidiomycetes remains unclear. The transport mechanisms used to translocate metabolites from haustoria to urediniospores also remains to be determined. We report the

strong induction of several transporters that likely play an important role in metabolite trafficking toward the newly formed urediniospores. Peroxidases and superoxide dismutases are involved in the prevention of oxidative damages (Alscher et al. 2002). At later stages of compatible interaction between poplar leaves and *Melampsora* rust fungi, previous studies have shown the late expression of plant defense-related proteins and the accumulation of reactive oxygen species (Duplessis et al. 2009; Boyle et al. 2010). High levels of detoxification enzyme-related transcripts in the sporulation area could indicate that the fungus is setting protective mechanisms against late plant host defenses. In contrast with the biotrophic area, several transcripts involved in the degradation of plant cell wall polysaccharides were expressed in the sporulation area. The release of a cocktail of degrading enzymes by *M. larici-populina* during sporulation might be required to alter the host cell wall in order to break the epidermis cell layer and release the urediniospores.

In summary, this LCM-transcriptomic-coupled study allowed us to distinguish the metabolic and developmental functions of two contrasted rust tissues at the uredinial stage: a biotrophy-related tissue in the host palisade mesophyll and a sporulation-related tissue below the abaxial leaf epidermis. Optimization of LCM technical conditions allowed us to isolate RNA of good integrity to perform an array-based expression analysis. This approach provides insights on distinct genetic programs activated by rust at the uredinial stage. Particularly, a massive induction of transcripts encoding putative effectors, such as SSP, support a late maintenance of biotrophy at the late stage of rust infection, when urediniospores are released. In contrast, cell cycle and cell defense rescue are important components of the rust sporulation process.

MATERIALS AND METHODS

Growth conditions and inoculation procedures.

M. larici-populina isolate 98AG31 (pathotype 3-4-7) was used in this study. Urediniospore multiplication and plant inoculation procedures were performed as previously described using the same inoculum of 100,000 urediniospores/ml, leaf plastochrony index for detached *Populus trichocarpa* $\times$ *P. deltoides* 'Beaupré' leaves, and strictly identical culture conditions (Rinaldi et al. 2007). For LCM analysis, leaves were harvested after 168 hpi when uredinial pustules were visible on the leaf abaxial epidermis.

Fig. 4. Validation of *Melampsora larici-populina* whole genome exon oligoarrays data using reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Log2 transcript fold changes measured on NimbleGen oligoarrays and by RT-qPCR are plotted for a set of 29 differentially-expressed genes between area 3 and area 1.

Laser-scanning confocal microscopy.

Infected leaves showing the first fungal decoloration and mature uredinia were harvested at 96 and 168 hpi, respectively, and were directly cut and fixed in 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS, pH 7) for 3 h at 4°C. Fixed tissues were washed in PBS buffer (1×) and embedded in 6% agarose (wt/vol). Sections of 20 µm were prepared using a VT1000S Leica vibratome (Leica Microsystems, Nanterre, France). Sections were dual stained with 1% (wt/vol) Uvitex 2B (Polysciences, Eppelheim, Germany) in PBS for 2 min and washed with propidium iodide (PI; 10 µg/ml) (Invitrogen, Cergy Pontoise, France). PI was used to stain fungal infection structures (Koh et al. 2005) and Uvitex was used to stain plant and fungal cell walls. Transversal sections were observed using a NIKON-BIORAD Radiance 2100 Rainbow confocal scanning laser microscope. Sections were excited with blue laser beams at 405 nm, scanned with a filter setting at 500 to 560 nm for Uvitex, excited with argon laser beams at 515 nm, and scanned with filter settings at 550 to 650 nm for PI. Merged images

were obtained using the LaserSharp 2000 program (Bio-Rad, Marnes la Coquette, France).

Tissue preparation for LCM.

Ten leaf pieces of 0.4 cm², each containing a single uredinium, were randomly sampled on the overall leaf surface and immediately fixed on ice in Farmer's solution (EAA) containing 75% (vol/vol) ethanol and 25% (vol/vol) acetic acid (Kerk et al. 2003). This fixative was first vacuum infiltrated into the section (400 mm of Hg) for 15 min and then left in contact with leaf pieces overnight at 4°C. Farmer's solution was removed and samples were dehydrated in a graded series of ethanol (45 min each on ice [vol/vol] at 75, 80, and 90% and three times in absolute ethanol), followed by an ethanol:otix+ (Microm Microtech, Francheville, France) series (45 min each on ice [vol/vol] at 75:25, 50:50, and 25:75% and three times in 100% Otix+). After Otix+ removal, two paraffins were used for sample impregnation. The first was a diawax paraffin (Diapath, Martinengo, Italy) with a 58°C melting point and the sec-

Table 3. Selected *Melampsora larici-populina* genes presenting transcript induction in sporogenous (area 1) versus palisade mesophyll (area 3) micro-dissected structures, assessed by whole-genome expression oligoarrays and categorized according to their cellular category (Functional Catalogue)

Protein ID ^b	Putative function or features	Uredinia laser capture microdissection ^a				
		Expression level			Expression ratio	
		Area1	Area2	Area3	Area1/Area3	P
	Cell rescue, defense and virulence					
72564	Cytosolic Cu/Zn superoxide dismutase	6,548	173	33	196.8	0.020
87334	Peroxidase, family 2	7,075	487	39	181.6	0.034
72563	Cytosolic Cu/Zn superoxide dismutase	5,662	1,102	59	96.4	0.046
94172	Peroxidase	6,961	3,624	81	86.2	0.046
76380	Cytochrome P450	401	126	11	37.3	0.013
	Metabolism					
	Lipid					
94543	Squalene synthase	985	348	11	86.7	0.022
59333	Phospholipase/lecithinase/hemolysin	2,393	645	40	60.4	0.045
123933	Epoxide hydrolase	3,162	2,553	81	39.2	0.016
123555	Coesterase/carboxylesterase	646	472	14	45.7	0.007
35173	Sphingomyelin phosphodiesterase	554	372	17	32.3	0.025
63115	Lipase	608	120	20	31.1	0.014
	Carbon					
92210	α-Glycosidase (glycoside hydrolase family 71)	700	101	13	55.7	0.019
116147	β-Glycosidase (glycoside hydrolase family 5)	2,919	1,197	55	52.8	0.044
55983	β-Glycosidase (glycoside hydrolase family 16)	2,246	717	48	47.1	0.046
107307	α-L-arabinofuranosidases (glycoside hydrolase family 51)	1,197	1,125	35	33.9	0.021
87698	Chitin deacetylase (carbohydrate esterase family 4)	907	112	29	31.1	0.010
	Nucleotide					
117154	Ribonuclease P protein subunit	2,665	1,312	12	214.4	0.010
88931	Adenosine deaminase-like protein	629	23	13	49.1	0.010
26899	Endonuclease III	393	105	9	45.9	0.009
54248	Ribonuclease	831	189	19	44.4	0.032
93485	Spermidine synthase	471	467	11	41.4	0.013
	Redox					
38530	Oxidoreductase, short chain dehydrogenase	3,257	336	13	252.9	0.010
	Cell cycle and DNA processing					
40080	Centrin 3	2,308	1,741	22	103.1	0.023
87088	Septin	3,232	119	35	92.5	0.013
110193	6-O-methylguanine-DNA methyltransferase	2,253	623	25	89.1	0.024
91152	20S cyclosome subunit	857	168	11	75.5	0.009
76389	Meiotic chromosome segregation protein	1,067	284	23	46.1	0.014
71714	Nuclear transcription factor	512	640	14	35.5	0.022
85106	Cell division control protein 45	312	98	9	33.8	0.026
30176	M-phase inducer phosphatase	638	42	19	33.0	0.047
89448	Transcription-associated recombination protein	397	91	12	31.8	0.038
	Cellular transport, transport facilitation					
49361	GDP-fucose transporter	1,119	21	12	93.0	0.024
79553	ER-Golgi vesicle-tethering protein	2,865	527	33	86.9	0.021
39514	Synaptobrevin	625	73	10	64.4	0.013

(continued on next page)

^a Normalized transcript levels in area 1, 2, and 3 (i.e., spores and sporogenous hyphae, fungal infection tissues in spongy mesophyll, and fungal infection tissues in palisade mesophyll, respectively), transcript expression ratio (area 1/area 3), and associated *t* test *P* value (<0.05).

^b Protein ID number of corresponding best gene model in the *M. larici-populina* JGI genome sequence.

ond had a 37°C melting point (VWR, Fontenay sous bois, France). Samples were transferred to an oven at 37 or 58°C and approximately 10 ml of liquefied paraffin (VWR) was added to each vial and left at 37 or 58°C overnight. The paraffin was replaced five times at 1.5-h intervals. Then, samples were embedded using the conventional diawax paraffin with the 58°C melting point. This high-melting-point paraffin was retained for tissue embedding in order to realize proper sections. Sections (10 µm each) were cut on a rotary microtome and transferred to microscope membrane slide (Zeiss, Bernried, Germany) with or without water bath impregnation of paraffin ribbons. In order to improve adhesion between microscope slide and ribbons, slides were kept at 45°C during 15 min to spread out ribbons and stored at -20°C in darkness under dehydrating conditions. Just before LCM, slides were deparaffinized twice in Otx+ for 10 min and then in otx+:ethanol series (3 min each [vol/vol] at 67:33, 50:50, and 33:67% and three times in absolute ethanol).

LCM.

For each biological replicate, 100 sites of uredinium formation were isolated after sectioning of 10 uredinia randomly sampled from a single infected poplar leaf (10 serial sections per uredinium). For each of the 100 uredinia sections, three distinct areas were collected by LCM at each uredinial site corresponding to spores and sporogenous hyphae, fungal infection tissues in spongy mesophyll, and fungal infection tissues in palisade mesophyll (Fig. 2). A PALM Robot-Microbeam system (Carl Zeiss MicroImaging, Munich, Germany) was used to microdissect cells from deparaffinized and dried tissue sections. Laser parameters for dissection performed with the ×40 objective lens were energy, 52; delta, 12; and focus, 68 using the RoboLPC cutting and catapulting function. The catapulted material was collected in an adhesive cap of a 500-µl tube (Zeiss). Three biological replicates were analyzed in total (i.e., three leaves from different poplar plants independently infected by rust urediniospores) and three areas were LCM collected from each replicate.

Table 3. (continued from preceding page)

Protein ID ^b	Putative function or features	Uredinia laser capture microdissection ^a				
		Expression level			Expression ratio	
		Area1	Area2	Area3	Area1/Area3	P
	Cellular transport, transport facilitation (<i>continued</i>)					
71469	Aquaporin-1	8,125	1,313	143	56.6	0.049
94938	Permease-MFS transporter	710	461	15	48.2	0.015
46095	Endosomal membrane protein	811	113	20	40.6	0.044
32963	Vacuolar assembly/sorting protein	1,578	865	39	40.2	0.023
33903	Translocation protein	1,263	273	34	37.3	0.027
49500	Amino acid permease	585	129	16	35.7	0.031
94692	Oligopeptide transporter 4	312	28	10	31.8	0.041
25355	Permease-MFS transporter	410	34	13	31.2	0.020
	Transcription					
32431	Methyltransferase	1,211	774	16	74.7	0.011
48934	Ribosomal RNA assembly protein	2,092	1,298	30	70.5	0.010
50186	Elongation factor-type GTP-binding protein	3,359	2,787	51	65.7	0.021
25365	Glutamyl-tRNA amidotransferase	728	393	14	52.0	0.030
117808	tRNA methyltransferase	1,222	177	29	41.7	0.014
27169	tRNA methyltransferase complex subunit Cpd1	612	762	17	35.2	0.008
38981	Transcription factor C2H2 type	1,059	35	32	33.3	0.028
75483	ATP-dependent RNA helicase	1,441	209	48	30.3	0.013
	Cell fate					
73052	Expansin/rare lipoprotein A domain protein	2,384	166	41	58.1	0.023
55582	Riboflavin aldehyde-forming enzyme/Rare lipoprotein A domain protein	3,761	308	67	56.5	0.037
70937	Expansin/rare lipoprotein A domain protein	3,015	1,735	81	37.4	0.035
76387	Expansin/rare lipoprotein A domain protein	6,249	518	207	30.1	0.019
	Protein fate (folding, modification, destination)					
123605	Carboxypeptidase S1	2,270	698	31	73.5	0.029
92176	COP9 signalosome complex subunit	1,003	165	21	48.7	0.036
76645	TPR repeat-containing protein	1,327	324	41	31.3	0.038
	Protein synthesis					
71776	Ribosomal protein	1,042	98	12	84.0	0.010
96447	Capsular associated protein	1,538	796	34	45.2	0.040
	Energy					
110853	NADH-ubiquinone oxidoreductase	5,248	1,767	74	70.7	0.023
38407	FAD monooxygenase	384	340	12	31.9	0.027
	Subcellular localization					
33341	Small secreted protein containing CFEM domain (hesp-178 homology)	2,558	328	58	44.3	0.039
117611	GPI transamidase subunit	319	369	9	36.5	0.010
	Cellular communication/signal transduction mechanism					
117612	Ankyrin repeat protein	1,579	521	38	41.0	0.026
40575	Ras-related GTPase	551	350	18	31.3	0.011
	Transposable elements, viral and plasmid proteins					
72990	Transposase	1,280	705	32	40.4	0.012
	Protein with binding function or cofactor requirement					
104398	Cold-shock DNA/RNA-binding protein	881	625	23	38.6	0.040
77832	Nuclear cap-binding protein subunit	477	241	15	31.7	0.020
	Unclassified proteins					
117548	fmHP	1,950	575	24	80.3	0.018
85991	Pentatricopeptide (PPR) repeat-containing protein	2,097	109	35	60.4	0.016
95993	Major allergen TRI14	954	197	20	47.5	0.032

RNA extraction and amplification.

For the LCM experiment, total RNA (<5 ng) was isolated from all samples using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA, U.S.A.). The RNase-Free DNase Set kit (Qiagen, Courtaboeuf, France) was used during the RNA isolation procedure according to the manufacturer's instructions to remove traces of genomic DNA. Elution was performed with 12 µl of prewarmed DEPC water (65°C). Quality and quantity of RNA samples were assessed using the Bio-Rad Experion analyzer and Experion RNA high-sense analysis kit (Bio-Rad). Total RNA from each sample (<5 ng) was subjected to two rounds of amplification using the MessageAmp II aRNA amplification kit (Ambion, Austin, TX, U.S.A.) following the manufacturer's instructions. RNA amplification generated more than 4 µg of aRNA for each sample, and aRNA profiles were verified using the Experion analyzer and Experion RNA standard-sense analysis kit (Bio-Rad).

Exon oligoarray analyses.

The *M. larici-populina* custom-exon expression array (4 × 72 K) manufactured by Roche NimbleGen Systems Limited (Madison, WI, U.S.A.) contained four independent, nonidentical, 60-mer probes per gene model coding sequence. In total, 17,556 predicted genes from *M. larici-populina* gene catalog (JGI Melampsora portal) and 1,063 random 60-mer control probes and labeling controls were included on the array (GEO platform no. GPL10350). Double-stranded cDNA were synthesized from 2.5 µg of aRNA using the Superscript Double-stranded cDNA synthesis kit (Invitrogen) according to the NimbleGen user protocol. Single-dye labeling of samples, hybridization procedures, and data acquisition were performed at the NimbleGen facilities (NimbleGen Systems, Reykjavik,

Iceland) following their standard protocol. Microarray probe intensities were quantile normalized across chips and average expression levels were calculated for each gene from the independent probes on the array and were used for further analyses. A transcript was deemed expressed when its signal intensity was threefold higher than the mean signal-to-noise threshold (cut-off value) of 1,063 random oligonucleotide probes present on the array. A Student *t* test with false discovery rate (Benjamini-Hochberg) multiple testing correction was applied to the data using the ARRAYSTAR software (DNASTAR, Inc. Madison, WI, U.S.A.). Transcripts presenting a threefold change in transcript level (*P* value < 0.05) were considered to be significantly differentially expressed. Data filtering, normalization process, and complete expression datasets are available at the Gene Expression Omnibus (National Center for Biotechnology Information) as series no. GSE21487.

RT-qPCR.

In order to assess transcript levels by RT-qPCR, specific primers of 29 genes were designed with the Primer 3 and Amplify 3X programs based on the *M. larici-populina* genome sequence (Supplementary Table 1). Amplified fragments showed a length ranging between 121 and 262 nucleotides. Homology searches against *M. larici-populina* and *P. trichocarpa* genome sequences were performed using the blastn algorithm to verify absence of cross annealing with other *M. larici-populina* transcripts as well as with host plant transcripts. First-strand cDNA was synthesized from 150 ng of aRNA using SuperScript II reverse transcriptase (Bio-Rad) with random primers in a final volume of 20 µl. cDNA derived from aRNA (1.5 ng) was amplified by qPCR in iQ SYBR Green supermix (Bio-Rad) with 0.3 µM specific 5' and 3' primers. Amplifications were

Table 4. Most highly upregulated *Melampsora larici-populina* transcripts in sporogenous (area 1) versus palisade mesophyll (area 3) microdissected structures using whole-genome expression oligoarrays and ranked by decreasing fold changes

Protein ID ^b	SP ^c	Length	Cysteine ^d	Putative function or features	Blastp ^e	Uredinia laser capture microdissection ^a				
						Expression level		Expression ratio		<i>P</i>
						Area1	Area2	Area3	Area1/Area3	
38530	–	240	4	Oxidoreductase, short chain dehydrogenase	PGTG_01366	3,257	336	13	252.9	0.010
85149	–	131	4	Mlp-specific protein	No hits found	5,230	6,754	23	222.6	0.032
112748	–	245	4	Mlp-specific protein	No hits found	5,637	2,493	26	218.9	0.022
117154	–	422	5	Ribonuclease P protein subunit	PGTG_13270	2,665	1,312	12	214.4	0.010
72564	–	122	4	Cytosolic Cu/Zn superoxide dismutase	PGTG_05675	6,548	173	33	196.8	0.020
87334	–	323	5	Peroxidase, family 2	PGTG_00594	7,075	487	39	181.6	0.034
114474	–	371	11	Rust-specific protein	PGTG_07899	1,568	1,170	11	149.2	0.009
73728	–	81	1	Mlp-specific protein	No hits found	1,557	347	11	145.5	0.009
76897	Y	185	10	Mlp-specific protein	No hits found	5,523	3,451	39	140.0	0.030
104937	Y	183	9	Mlp-specific protein	No hits found	2,650	1,282	21	128.9	0.011
112967	Y	185	6	Mlp-specific protein	No hits found	4,569	2,810	44	103.4	0.044
40080	–	166	0	Centrin 3	PGTG_06324	2,308	1,741	22	103.1	0.023
73760	–	92	1	Mlp-specific protein	No hits found	1,154	712	11	102.0	0.010
55089	–	74	0	Mlp-specific protein	No hits found	4,608	478	46	100.8	0.022
105220	Y	160	4	Mlp-specific protein	No hits found	1,105	22	11	100.0	0.026
34854	–	105	4	Hypothetical protein	PGTG_12203	4,042	363	40	100.0	0.031
72563	Y	237	5	Cytosolic Cu-Zn superoxide dismutase	PGTG_05675	5,662	1,102	59	96.4	0.046
63262	Y	572	3	Mlp-specific protein	No hits found	1,041	982	11	93.8	0.007
103398	–	116	3	Mlp-specific protein	No hits found	939	39	10	93.3	0.030
71121	–	113	3	Mlp-specific protein	No hits found	1,177	1,358	13	93.2	0.014
67049	–	376	7	Rust-specific protein	PGTG_15359	995	57	11	93.0	0.007
49361	–	486	6	GDP-fucose transporter	PGTG_17642	1,119	21	12	93.0	0.024
87088	–	369	2	Septin	PGTG_13103	3,232	119	35	92.5	0.013
110193	–	183	1	6- <i>O</i> -methylguanine-DNA methyltransferase	No hits found	2,253	623	25	89.1	0.024
57035	–	69	1	Mlp-specific protein	No hits found	1,082	2,251	12	89.1	0.008

^a Normalized transcript levels in area 1, 2 and 3 (i.e., spores and sporogenous hyphae, fungal infection tissues in spongy mesophyll, and fungal infection tissues in palisade mesophyll, respectively), transcript expression ratio (area 1/area 3), and associated *t* test *P* value (<0.05).

^b Protein ID number of corresponding best gene model in the *M. larici-populina* JGI genome sequence.

^c Signal peptide (SP) prediction. Presence (Y) or absence (–) of a signal peptide is indicated.

^d Number of cysteines.

^e Best blast hit against the genome sequence of *Puccinia graminis* f. sp. *tritici* deposited at the Broad Institute genome portal.

carried out in triplicate with an MJ-opticon2 DNA engine (Bio-Rad). After 3 min of denaturation, 40 cycles (95°C for 15 s and 60°C for 1 min) were performed and a melting curve was determined to verify absence of multiple amplifications. *M. larici-populina* transcript expression was normalized to the transcript level of α -tubulin (*Mlp- α TUB*, JGI protein ID no. 73137) and elongation factor (*Mlp-ELF1a*, JGI protein ID no. 73378) reference genes using the Pfaffl calculation (Pfaffl 2001).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Broad Institute Puccinia Group database:
www.broadinstitute.org/annotation/genome/puccinia_group/MultiHome.html
- Munich Information Center for Protein sequence (MIPS) functional catalogue: mips.helmholtz-muenchen.de/proj/funcatDB
- National Center for Biotechnology Information Gene Expression Omnibus database: www.ncbi.nlm.nih.gov/geo
- NimbleGen website: www.nimblegen.com
- U.S. Department of Energy JGI genome database queries:
genome.jgi-psf.org/Mellp1/Mellp1.home.html and
genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html