***Melampsora larici-populina* Transcript Profiling During Germination and Timecourse Infection of Poplar Leaves Reveals Dynamic Expression Patterns Associated with Virulence and Biotrophy**

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Melampsora larici-populina is responsible for poplar leaf rust disease and causes severe epidemics in poplar plantations in Europe. The poplar rust genome has been recently sequenced and, in order to find the genetic determinants associated with its biotrophic lifestyle, we generated a whole-genome custom oligoarray and analyzed transcript profiles of *M. larici-populina* during the infection timecourse in poplar leaves. Different stages were investigated during the asexual development of the rust fungus, including resting and germinating urediniospores and seven in planta stages in the telial host. In total, 76% of the transcripts were detected during leaf infection as well as in urediniospores, whereas 20% were only detected in planta, including several transporters and many small secreted proteins (SSP). We focused our analysis on gene categories known to be related to plant colonization and biotrophic growth in rust pathogens, such as SSP, carbohydrate active enzymes (CAZymes), transporters, lipases, and proteases. Distinct sets of SSP transcripts were expressed all along the infection process, suggesting highly dynamic expression of candidate rust effectors. In contrast, transcripts encoding transporters and proteases were mostly expressed after 48 h postinoculation, when numerous haustoria are already formed in the leaf mesophyll until uredinia formation, supporting their role in nutrient acquisition during biotrophic growth. Finally, CAZymes and lipase transcripts were predominantly expressed at late stages of infection, highlighting their importance during sporulation.

Melampsora larici-populina (division Basidiomycota, order Pucciniales) is an obligate biotrophic fungus responsible for foliar rust disease in poplar. This is the most devastating and widespread pathogen for this tree, and it has limited the use of poplars for environmental and wood production goals in many parts of the world (Steenackers et al. 1996). In Europe, *Populus* × *euramericana* and *P.* × *interamericana* hybrids were selected in the mid-20th century for their complete resistance to

the rust fungus (Steenackers 1988). Cultivation of these hybrids was successful for many years but led to the emergence of new virulent fungal races and the breakdown of several major resistance genes (Frey et al. 2005; Pinon and Frey 2005). To date, most poplar cultivars are susceptible to *M. larici-populina*, and new virulent strains are continuously developing. Like other rust fungi, *M. larici-populina* has a complex life cycle. Five different spore types are successively produced and two distinct host plants (larch and poplar) are required to complete its cycle. In contrast to limited symptoms on larch, *M. larici-populina* causes severe epidemics during its asexual phase on poplar (the telial host). The infection process, previously described for poplar leaf colonization (Hacquard et al. 2010; Laurans and Pilate 1999; Rinaldi et al. 2007), starts when urediniospores land on the abaxial surface of poplar leaves (Fig. 1). A primary germ tube is formed within two hours and extends to allow fungal penetration through stomata. Infection hyphae are formed from substomatal vesicles followed by the differentiation of the first haustoria within 17 h postinoculation (hpi) (Laurans and Pilate 1999). Haustoria are essential for the uptake of host metabolites and the suppression of host defenses (Catanzariti et al. 2007; Voegelé et al. 2009), enabling proliferation of the rust fungus between 24 and 96 hpi (Hacquard et al. 2011; Rinaldi et al. 2007) (Fig. 1). By 96 hpi, infection structures have colonized the whole plant mesophyll and differentiations of the first sporogenous hyphae are observed (Hacquard et al. 2010). Seven days after infection, uredinia emerge on the leaf abaxial epidermis and newly formed dikaryotic spores are released (Fig. 1).

Molecular mechanisms underlying poplar defense reactions in response to *Melampsora* spp. infection have been addressed experimentally using transcriptomic approaches (Azaiez et al. 2009; Boyle et al. 2010; Miranda et al. 2007; Rinaldi et al. 2007); still however, little is known about the molecular mechanisms underlying successful invasion of the plant host by rust fungi. The recent sequencing of the *M. larici-populina* genome is a great opportunity to identify key genes involved in colonization and biotrophic growth (Duplessis et al. 2011). Recently, a late stage of rust infection (i.e., 168 hpi) was investigated in poplar after laser capture microdissection and microarray analysis led to the identification of distinct transcriptional programs associated with biotrophy in the palisade mesophyll and sporulation in the uredinia (Hacquard et al. 2010). However, the genetic programs controlling poplar rust germination and host colonization remain to be determined.

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Obligate biotrophic fungi such as *M. larici-populina* establish an intimate relationship with the host cell, essential for pathogen growth and spore production. Previous attempts to isolate determinants controlling biotrophic rust development were mostly based on the generation of expressed sequence tags (EST) from isolated rust haustoria or infected leaves (Broeker et al. 2006; Catanzariti et al. 2006; Feau et al. 2007; Hahn and Mendgen 1997; Joly et al. 2010; Puthoff et al. 2008; Thara et al. 2003; Yin et al. 2009). However, the development of DNA arrays offers the chance to analyze transcriptional changes of large numbers of genes. To date, DNA arrays have been used to study rust transcriptomes only in *Uromyces fabae* (Jakupovic et al. 2006) and *M. larici-populina* (Hacquard et al. 2010) at late stages of host infection or germinating urediniospores. Transcript profiling has been successfully used to identify candidate genes involved in fungal virulence as well as to monitor dynamic changes during in planta development of *Blumeria graminis*, the ascomycete biotroph responsible for powdery mildew of cereals (Both et al. 2005a and b). Similar studies were also conducted in *Magnaporthe oryzae*, the hemibiotroph pathogen responsible for rice blast (Dean et al. 2005; Oh et al. 2008), and the biotroph corn smut fungus *Ustilago maydis* during colonization of plant tissues (Kämper et al. 2006; Skibbe et al. 2010) and also in the basidiomycete symbionts *Pisolithus microcarpus* and *Paxillus involutus* during the development of the ectomycorrhizal symbiosis in their respective hosts (Duplessis et al. 2005; Le Quééré et al. 2005).

In this paper, we report a near-comprehensive transcriptome analysis of the rust fungus *M. larici-populina* during germination and poplar leaf timecourse infection by using whole-genome custom oligoarrays. Several stages related to the asexual development in the telial host were analyzed, including resting and germinating urediniospores and in planta fungal infection structures from the early colonization stage (i.e., first haustoria) to the formation of mature uredinia. Transcript profiles were also assessed using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Our results indicate that plant infection is marked by dynamic and coordinated tempo-

ral patterns of rust gene expression. We identified gene families potentially associated with virulence, biotrophic growth, and uredinia formation. This genome-scale analysis of rust gene expression during host infection focuses new light into rust biology.

RESULTS

Experimental design and data normalization.

RNA was isolated from nine different stages covering the main developmental transitions of the poplar rust fungus during asexual development in its telial host (Fig. 1). This includes resting and germinating urediniospores (stages outside plant tissues) and seven timepoints in a timecourse infection of poplar 'Beaupré' leaves by *M. larici-populina* 98AG31 (in planta stages) from 2 hpi, when urediniospores are germinating on the poplar leaf abaxial epidermis, to 168 hpi, when new urediniospores are produced and released from uredinia (Hacquard et al. 2010; Rinaldi et al. 2007). A custom oligoarray was built to survey transcript expression of the poplar rust fungus, based on the initial annotation of the *M. larici-populina* genome (April 2008; 17,556 putative genes) performed by the Joint Genome Institute (JGI). Expert annotation of the genome identified dubious (i.e., transposable elements) or mispredicted gene calls and the curated gene catalog now contains 16,399 genes (Duplessis et al. 2011). Expression levels could be measured for 13,093 of the 16,399 genes (discussed below). Sufficient amounts of cDNA were recovered from all biological stages to perform hybridization onto oligoarrays. However, the overall expression levels (i.e., fluorescence levels) measured at early stages of the timecourse infection were too low for comparison with late stages. Less than 500 transcripts were detected above background fluorescence levels at 2, 6, and 12 hpi, and most transcripts corresponded to ribosomal protein encoding genes or hypothetical unknown genes (data not shown). Thus, these timepoints were not considered for comparison with other stages. Principal component analysis of expression levels measured at the six remaining stages showed good con-

Fig. 1. Schematic drawing presenting the major stages of *Melampsora larici-populina* uredinial development (top) and the transcript expression analyses performed in this study (bottom). **A**, Gene expression levels were measured using custom oligoarrays in resting (Usp) and germinating (UspG) urediniospores freshly isolated from the plant host and at 2, 6, 12, 24, 48, 96, and 168 h postinoculation (hpi) on poplar leaves to identify genes highly or specifically expressed in spores or in planta. **B**, Expression ratios were calculated between UspG and Usp, 48 and 24 hpi, 96 and 48 hpi, and 168 and 96 hpi in order to identify groups of genes significantly regulated during spore germination and during poplar leaf infection. **C**, A relative expression index (expression level at a given timepoint relative to the mean expression level across the timecourse) was calculated to derive gene expression patterns during the timecourse infection.

sistency between biological replicates (Fig. 2), particularly for resting and germinating urediniospores, and at 168 hpi, which corresponds to the in planta stage with the largest proportion of fungal structures (Hacquard et al. 2011). In addition, data dispersion indicates close profiles for the 24 and 48 hpi stages as well as for resting and germinating urediniospores. In order to avoid artifactual biases due to dilution of labeled fungal transcripts in fluorescent probes, in planta stages and urediniospores were normalized separately. Different means were used to analyze the data (Fig. 1). First, gene-expression levels were considered, to identify the most highly expressed genes at each timepoint and to highlight spore- or in planta-specific genes. Then, in order to detect genes significantly regulated during germination or plant infection, successive stages were compared by calculating direct ratios, i.e., UspG to Usp and 48 to 24, 96 to 48, and 168 to 96 hpi. Finally, to derive expression patterns of genes involved in plant colonization and fungal biotrophic growth, relative expression indexes (REI) were calculated as ratios between transcript expression level at a given in planta stage and the mean expression level calculated across the whole timecourse.

Urediniospores and in planta-expressed rust genes.

Table 1 presents the proportions of genes expressed in planta and in urediniospores and highlights the contrasted expression profiles between the surveyed stages. Indeed, in planta stages present a lower number of highly expressed genes (>5,000) compared with urediniospores. The largest number of expressed genes was detected in planta at 168 hpi (8,326 transcripts), and a large number of genes were already expressed at 96 hpi. In total, 10,236 transcripts (i.e., 78% of the 13,093 assessed) were expressed in at least one biological situation. Among these transcripts, 76% were both expressed in planta and in urediniospores (Fig. 3), whereas only 4 and 20%, respectively, were specifically detected in urediniospores (Supplementary Table 1) or in planta (Supplementary Table 2). A core set of 32.2% of the genes was always expressed in planta between 24 and 168 hpi. Smaller sets of genes were also expressed at each timepoint (Fig. 3). In detail, genes highly expressed in planta at early timepoints mostly corresponded to ribosomal proteins and hypothetical proteins of unknown function (Supplementary Table 3). Interestingly, many of the hypothetical proteins encode small secreted proteins (SSP) of unknown function that

Fig. 2. Principal component analysis (PCA) of *Melampsora larici-populina* transcript levels measured in infected poplar leaves at 24, 48, 96, and 168 h postinoculation (hpi), resting urediniospores (Usp), and germinating urediniospores (UspG) with custom oligoarrays. Three biological replicates per stage (R1, R2, and R3) were used for the PCA and the correlation circle is presented. Expression level of each gene assessed in a given biological situation and biological replicate was reported to the mean expression level calculated for the 18 hybridizations (six conditions $\times$ three replicates) and was $\log_{10}$ -normalized before proceeding with PCA. The PCA plot places biological conditions along the two axes (components 1 and 2) explaining 39.9 and 19.0% of the variance observed within samples.

Table 1. Number of *Melampsora larici-populina* genes expressed during timecourse infection of poplar leaves and in resting or germinating urediniospores

Expression level ^a	24 hpi ^b	48 hpi	96 hpi	168 hpi	Usp ^c	UspG ^c
Background to 1,000	2,587	3,798	4,113	4,266	1,726	1,845
1,001 to 5,000	1,366	1,921	2,832	3,040	2,014	2,104
5,001 to 10,000	210	314	590	693	1,322	1,346
10,001 to 20,000	101	144	243	257	1,405	1,345
>20,001	15	39	78	70	1,268	1,232
Total number of genes	13,093	13,093	13,093	13,093	13,093	13,093
Not detected (<background)	8,814	6,877	5,237	4,767	5,358	5,221
Expressed (>background)	4,279	6,216	7,856	8,326	7,735	7,872

^a Genes were classified according to their normalized expression levels measured with custom oligoarrays. A transcript was deemed expressed when its fluorescence signal intensity was threefold higher than the mean signal-to-noise threshold (cut-off background value) of 1,063 random oligonucleotide probes.

^b hpi = h postinoculation.

^c Usp = resting urediniospores and UspG = germinating urediniospores.

could represent putative rust effectors. Some SSP transcripts were highly expressed from 24 until 168 hpi (JGI protein ID numbers 74599 and 124067). Among the 2,078 genes specifically detected in planta (Fig. 3), almost 80% ($n = 1,617$) did not show homology with the nonredundant database at the National Center for Biotechnology Information (NCBI). Only 481 of these unknown proteins displayed similarities with genes predicted in the *Puccinia graminis* f. sp. *tritici* genome sequence (Duplessis et al. 2011). A total of 270 SSP genes reside in the specific in planta-expressed genes. Almost 70% (1,400 of 2,078) of the specific in planta expressed genes were presenting a higher level at early stages of rust infection (i.e., 24 and 48 hpi), whereas only 24% (502 of 2,078) were preferentially expressed after 48 hpi. Among the 400 transcripts expressed specifically in urediniospores (Fig. 3), 62% ($n = 248$) did not show homology in databases and 10% ($n = 42$) correspond to SSP genes expressed in resting ($n = 38$) or in germinating ($n = 28$) urediniospores.

Rust transcripts induced during germination and poplar leaf infection.

The numbers of rust genes significantly regulated during germination and between distinct stages of plant infection are detailed in Supplementary Table 4. A majority of genes encoding ribosomal proteins were accumulated in resting spores as compared with germination. Interestingly, among the transcripts

significantly and highly induced during germination were many hypothetical proteins, including three SSP (JGI protein ID numbers 123742, 123929, 62280), an oligopeptide transporter (JGI protein ID number 88574), and an auxin carrier protein (JGI protein ID number 63226). Strikingly, almost no genes were deemed as significantly regulated in planta between 24 and 48 hpi, consistent with the close profiles and the dispersion of replicates observed for these timepoints in the principal component analysis (Fig. 2). Although a higher number of regulated genes was found during germination, a larger proportion of strongly up-regulated genes was observed at 96 versus 48 hpi and 168 versus 96 hpi (>3-fold change) (Table 2). This highlights the idea that expression is highly dynamic during plant colonization. Among the 855 genes significantly induced at 96 hpi compared with 48 hpi, the most highly up-regulated transcript (191-fold induction) encodes a CFEM domain SSP (JGI protein ID number 114961). Notably, two genes related to mating and encoding a mating pheromone A receptor (JGI protein ID number 41282) and a mating factor A secretion protein (JGI protein ID number 493844) were also identified among the most highly up-regulated transcripts at 96 hpi (85-fold and 55-fold, respectively). Among the 1,649 genes significantly up-regulated at 168 hpi (uredinia formation) compared with 96 hpi, several of the most highly induced genes (Prot ID numbers 72264, 72999, 124001) displayed homologies to genes previously described in *Lentinula edodes*

Fig. 3. Venn diagram detailing the percentage of *Melampsora larici-populina* genes expressed during poplar leaf infection (in planta) or in urediniospores. Expression was assessed with custom oligoarrays at 24, 48, 96, and 168 h postinoculation (hpi) in infected poplar leaf infection and in resting and germinating urediniospores. The main Venn diagram compares genes expressed in planta (at any stage) and genes expressed in urediniospores (resting or germinating). Smaller Venn diagrams give the detail of all the genes expressed in each spore condition (left) or at each stage in the timecourse series (right).

Table 2. The number of *Melampsora larici-populina* genes significantly regulated during timecourse infection of poplar leaves and during germination using custom oligoarrays

Regulated genes ^a	48 to 24 hpi ^b	96 to 48 hpi ^b	168 to 96 hpi ^b	USpG to Usp ^b
Significantly regulated genes	7	1,848	3,193	3,347
Up-regulated	0	855	1,649	1,737
>3-fold	0	783	1,100	641
>10-fold	0	330	499	177
>100-fold	0	3	65	19
Down-regulated	7	993	1,544	1,610
<3 fold	5	806	638	545
<10 fold	4	76	59	98
<100 fold	0	0	0	1

^a Down- and up-regulated genes were classified according to their fold-change ratio. Transcripts with a significant *P* value (<0.05) and more than a threefold change in transcript level were considered as significantly differentially expressed.

^b hpi = h postinoculation, UspG = urediniospores germinating on agar, and UspG = resting urediniospores.

(shiitake mushroom) and *Agaricus bisporus* fruiting-body formation, including the developmentally regulated *priA* gene (Kajiwarra et al. 1992), the riboflavin-aldehyde-forming enzyme (Sreenivasaprasad et al. 2006), or the Drmip_hesp family proteins (Szeto et al. 2007). Several transcripts encoding a beta mannanase (JGI protein ID number 60617), a lysophospholipase (JGI protein ID number 94935), and tripeptidyl peptidases (JGI protein ID numbers 86030 and 45226) also showed drastic inductions between 225- to 730-fold, suggesting a possible involvement in poplar rust sporulation.

Dynamic rust gene expression during plant colonization and biotrophic growth.

We focused our analysis on gene categories commonly associated with plant colonization and biotrophic growth in rust fungi (Dodds et al. 2009; Voegelé et al. 2009), encoding SSP that likely contain candidate rust effectors, transporters, CAZymes, proteases, and lipases. Within these gene categories, a total of 1,058 genes were significantly regulated at least once during the infection process and were considered. In planta gene expression profiles of selected genes were derived using the k-means partitioning method implemented in the Genesis expression analysis package (Sturn et al. 2002), based on REI values (Fig. 4). A total of 30 k-means were derived to extract clusters of genes with distinct expression profiles. K-means were grouped in eight classes of expression patterns by

considering the REI peaks (Fig. 4, classes I to VIII). Surveyed genes showed a preferential expression at 168 hpi (classes I, VII, and VIII) when the fungus is highly abundant in the plant tissue and more than 25% of the genes were preferentially early expressed at 24 and 48 hpi (classes I, II, III, and IV). Strikingly, among the investigated categories, distinct and important groups of genes encoding SSP were clearly expressed in all classes, i.e., all along the infection process (Fig. 5). This indicates that expression of putative rust effectors was not restricted to the earliest stages of colonization but was highly dynamic and maintained all along plant infection. Several homologs of genes encoding haustorially expressed secreted proteins (HESP) and avirulence factors identified in the flax rust *M. lini* (JGI protein ID numbers 33341, 36204, 71404, 93569, 74496, 39396, 37347, 58916) were preferentially expressed between 48 and 96 hpi (classes IV, V, and VI). In contrast, transporter transcripts were mostly expressed when numerous haustoria are already formed in the leaf mesophyll (48 to 168 hpi), supporting their role in the biotrophic growth; many of them were preferentially expressed between 96 and 168 hpi (classes VI and VII), including several ammonium (protein ID numbers 48332, 24225, 42854), amino acid (JGI protein ID numbers 49500, 90273, 72195), oligopeptide (JGI protein ID numbers 93456, 88746, 77624, 118568, 34758, 85440), and sugar (JGI protein ID numbers 38418, 40410, 40379) transporters. Contrary, CAZymes and lipases showed higher ex-

Fig. 4. Expression patterns of 1,058 *Melampsora larici-populina* genes significantly regulated during poplar leaf infection encoding small secreted proteins (SSP), transporters, proteases, carbohydrate active enzymes, and lipases. Clusters of genes were derived by partitioning $\log_2$ -relative expression index (REI) values calculated at 24, 48, 96, and 168 h postinoculation (hpi) in 30 k-means profiles. REI of a gene corresponds to the ratio between the mean expression level measured at a given timepoint and the mean expression level calculated across the timecourse. The 30 k-means profiles were grouped in eight classes according to their peak of expression (classes I to VIII). Class I = genes preferentially expressed at 24 and 168 hpi, class II = 24 hpi, class III = 24 and 48 hpi, class IV = 48 hpi, class V = 48 and 96 hpi, class VI = 96 hpi, class VII = 96 and 168 hpi, and class VIII = 168 hpi.

pression levels at later stages of rust infection and, particularly, during sporulation (168 hpi, class VIII), although distinct genes were also highly expressed as early as 24 hpi. Interestingly, several CAZymes previously identified as strongly induced in the sporulation area, as compared with the biotrophic area of the poplar rust uredinal stage, using laser capture microdissection (Hacquard et al. 2010), were also strongly accumulated at 168 hpi, such as an alpha-glycosidase (GH71, JGI protein ID number 92210), a beta-glycosidase (GH16, JGI protein ID number 55983), an alpha-L-arabinofuranosidase (GH51, JGI protein ID number 107307), and a chitin deacetylase (JGI protein ID number 87698). The strong induction of other genes encoding alpha-glycosidases (JGI protein ID numbers 73317 [GH47], 104873 [GH71]), beta glycosidases (JGI protein ID numbers 51995 [GH26], 86044 [GH5], 90252 [GH5]) and chitinases (JGI protein ID numbers 46588, 90663) was observed during sporulation. Lipid metabolism also showed drastic changes during fungal sporulation. For example, several genes encoding an epoxide hydrolase (JGI protein ID number 115158), cutinases (JGI Protein ID numbers 86941, 89616), and lipases (JGI protein ID numbers 117051, 78777) were preferentially expressed at 168 hpi (class VIII), suggesting a metabolic switch between biotrophy and sporulation.

Validation of temporal expression patterns by RT-qPCR.

A total of 26 genes with distinct in planta expression patterns were selected to assess their transcript profiles by RT-qPCR. Transcript levels were normalized to alpha-tubulin and elongation factor 1-alpha reference genes for RT-qPCR (Hacquard et al. 2011), whereas expression levels were normalized through quantile normalization for oligoarrays. Thus, expression levels measured with oligoarrays and RT-qPCR were converted to REI to allow direct comparison of expression profiles for the selected genes (Fig. 6). Most timecourse expression patterns were confirmed by the two techniques. However, half of the genes presented a different profile at 24 hpi, with a higher level measured by RT-qPCR than with oligoarrays. The higher sensitivity of the RT-qPCR technique is well known, and such differences might reflect the impaired detection of fungal transcripts on oligoarrays at 24 hpi compared with RT-qPCR. Overall, the expression profiles were consistent and assessed the quality of the transcriptome analysis performed with a *M. larici-populina* custom oligoarray. The RT-qPCR profiles confirmed distinct profiles for several genes belonging to a same gene family, such as chitin deacetylases (JGI protein ID numbers 73400, 74070) and SSP HESP-417 homologs (JGI protein ID numbers 71402, 71399, 93569, 74496).

DISCUSSION

In this study, we analyzed the transcriptome of the rust fungus *M. larici-populina* during the asexual phase of its biological cycle, when the fungus colonizes its main host, poplar, and produces new asexual urediniospores in a matter of 7 days (Frey et al. 2005). We covered stages such as germination by comparing resting and germinating urediniospores and in planta biotrophic growth and sporulation in a timecourse experiment. The fungus must produce specialized structures, the so-called haustoria, to derive nutrients from the host in order to achieve spore production and sporulation (Voegelé et al. 2009). As an obligate biotroph fungus, *M. larici-populina* must overcome plant surveillance systems (i.e., pattern-recognition receptor and effector-triggered immunity), likely by secreting a large array of effectors in order to manipulate plant defense system (Dodds and Rathjen 2010). Thus, studying the rust fungus transcriptome during host infection could unravel the ge-

netic programs required to properly achieve these crucial steps. In planta-expressed rust genes have been investigated so far by studying transcripts expressed in resting and germinating urediniospores or in isolated haustorial structures (Catanzariti et al. 2006; Hahn and Mendgen 1997; Jakupovic et al. 2006; Joly et al. 2010; Zhang et al. 2008). A major limitation in the study of fungal expression in planta is the amount of fungal RNA recovered from host tissues. The purification of haustoria from rust-infected plants by Hahn and Mendgen (1992) using affin-

Fig. 5. Number of *Melampsora larici-populina* genes identified in eight clustered expression classes for the following selected categories: small secreted proteins (SSP), transporters, proteases, carbohydrate active enzymes (CAZymes), and lipases. Cluster classes indicate rust genes preferentially expressed at distinct stages of poplar leaf infection: class I = 24 and 168 hpi, class II = 24 hpi, class III = 24 and 48 hpi, class IV = 48 hpi, class V = 48 and 96 hpi, class VI = 96 hpi, class VII = 96 and 168 hpi, and class VIII = 168 hpi.

ity chromatography columns was a revolution for the study of rust biology. This approach was used to produce cDNA libraries and EST of *U. fabae* (Hahn and Mendgen 1997), *U. appendiculatus* (Puthoff et al. 2008), *Puccinia striiformis* (Yin et al. 2009), *M. lini* (Catanzariti et al. 2006), and *M. larici-populina* (Joly et al. 2010). These studies greatly helped in the identification of rust virulence determinants (Dodds et al. 2009; Voegelé et al. 2009). In the ascomycete *Blumeria graminis* f. sp. *hordei*, an obligate biotroph responsible for powdery mildew of cereals, gene-expression profiling was achieved during plant colonization using microarrays and helped in identifying genes related to virulence (Both et al. 2005a) and specific expression of metabolic pathways before and after the penetration of barley leaves (Both et al. 2005b). By analyzing the timecourse infection of poplar leaves by *M. larici-populina*, our study opens the possibility to identify rust functions associated with distinct developmental stages of the biotrophic growth. To our knowledge, this is the first genome-scale expression study conducted for a rust fungus during timecourse infection.

As stressed above, analysis of in planta fungal expression could be impaired by low amounts of fungal biomass in plant tissues at early stages of infection. Sensitive methods such as RT-qPCR can detect transcripts at very low concentrations; however, DNA array-based expression studies are limited in

that way. Indeed, RNA isolated from infected plant tissues could contain limited amounts of fungal transcripts, so plant cDNA will capture most of the labeling during probe synthesis, leading to the detection of lower expression levels for fungal transcripts. In the poplar-rust pathosystem, the rust fungus penetrates the plant mesophyll through stomata and forms its first haustoria in the spongy mesophyll between 12 and 24 hpi (Laurans and Pilate 1999). In a first attempt to study early stages of poplar leaf infection, we included 2, 6, and 12 hpi timepoints (Fig. 1) that encompass germination on leaf epidermis, formation of appressoria, penetration through stomata, and the differentiation of substomatal vesicles (Rinaldi et al. 2007). Unfortunately, fluorescence levels measured on oligoarrays were very low and could not be compared with those of later stages. When the 12- and 24-hpi timepoints were normalized together, less than 4% of the oligoprobes had a signal higher than the background level and only 14 genes were expressed at 12 hpi (data not shown). Among the 14 genes, two encode Rho GTPases and two encode SSP containing CFEM (IPR008427) or SCP_CRISP (CDD_cl00133) domains. Thus, we focused the timecourse expression analysis to the frame 24 to 168 hpi, and the germination process was examined by comparing resting and germinating urediniospores outside the host, after a separate normalization due to differences in fluorescence levels compared with the timecourse experiment (Table 1).

Fig. 6. Comparison of expression profiles obtained by reverse transcription quantitative polymerase chain reaction and with custom oligoarrays for 26 selected *Melampsora larici-populina* genes. Expression levels at 24, 48, 96, and 168 h postinoculation (hpi) were converted to relative expression index (REI = expression level at a given timepoint divided by the mean level across the timecourse series) to help direct comparison of expression profiles. Log₂-REI were used for clustering. REI levels range from pale to saturated colors (yellow for negative values and blue for positive value).

The development of *M. larici-populina* is rather synchronous in planta (Laurans and Pilate 1999; Rinaldi et al. 2007). The number of haustoria formed in the plant mesophyll increases from 12 to 48 hpi and remains stable until sporulation at 7 days postinoculation, indicating that haustorium formation mostly occurs at early stages of the biotrophic growth (Laurans and Pilate 1999). However, at late stages of infection, the plant mesophyll contains distinct fungal structures that include numerous infection hyphae and haustoria, sporogenous hyphae, and urediniospores (Hacquard et al. 2010). Thus, fungal expression at these late stages cannot relate to a particular rust cell type and reflects concomitant biotrophic growth and sporulation at 168 hpi. Although fewer genes were expressed at 24 hpi (Table 1) and almost no difference in transcript accumulation was found when compared with 48 hpi (Table 2), we were able to detect sets of genes showing a higher expression at this early stage than at any other timepoints (Fig. 4, classes II and III). Expression of selected genes that exhibited different expression patterns during timecourse infection (i.e., belonging to different classes [Fig. 4]) as assessed by RT-qPCR showed consistency with expression profiles derived from oligoarrays analysis, except at 24 hpi. This suggests that rust transcripts expressed at this early timepoints might be underestimated.

Direct comparison of rust transcript levels between timepoints revealed time-regulated functions during the infection process. Among the genes significantly regulated at 96 and 168 hpi were many genes expressed only in planta and not detected in urediniospores or during germination. A strikingly important number of these genes did not present any homology to other fungi, including the rust *Puccinia graminis* f. sp. *tritici*. This result indicates that a specific genetic program that comprises several unknown genetic determinants is used by *M. larici-populina* to achieve its growth in poplar leaves. Many were specific to *M. larici-populina* and might underlie host specialization.

REI profiles greatly help to compare overall patterns of expression during timecourse series (Both et al. 2005a and b). Based on REI, informative patterns of expression were ex-

tracted from the dataset and eight classes of genes associated with early, intermediate, or late infection stages were derived. Our results showed that the rust transcriptome is marked by the dynamic expression of different sets of SSP (Figs. 5 and 7). SSP are candidate virulence effectors previously described in pathogenic fungi and oomycetes (Schornack et al. 2009; Stergiopoulos and de Wit 2009), including rust fungi (RTP1 [Kemen et al. 2005], AvrL567 [Dodds et al. 2004], AvrM [Catanzariti et al. 2006; Rafiqi et al. 2010]). Those effectors were particularly identified in rust EST libraries constructed from purified haustoria (Catanzariti et al. 2006; Hahn and Mendgen 1997). The number of poplar rust SSP expressed and the dynamic expression observed during leaf infection are striking and suggest that hundreds of distinct SSP are expressed during the seven days from germination until sporulation. Almost all these SSP genes have no known function. Many SSP transcripts were still expressed at 168 hpi when uredinia are formed, which suggests that the rust fungus could exert late suppression of host defense, in accordance with previous observations in microdissected poplar rust infection structures (Hacquard et al. 2010). Among the SSP transcripts showing homology to previously described *M. lini* HESP or avirulence genes, several were specifically detected in planta and some belonged to multigene families such as the HESP-417 SSP homologs. Distinct expression profiles were confirmed by RT-qPCR for four genes in this family (Fig. 6), leading to interesting questions regarding the dynamic expression of SSP during host infection. Several SSP genes were also specifically expressed in urediniospores and, particularly, during germination, which suggests that SSP expression could occur at early stages of colonization between germination and 24 hpi. The phosphorylation of the resistance protein RPG1 is elicited in barley after 5 min of exposure to spores of the stem rust *Puccinia graminis* f. sp. *tritici*, far before penetration in the host, likely in reaction to an unknown effector already present in or on the rust spore (Nirmala et al. 2010). SSP genes expressed by *M. larici-populina* urediniospores could represent interesting targets for early detection by the plant host. The poplar rust

Fig. 7. Major patterns of expression of *Melampsora larici-populina* genes encoding small secreted proteins (SSP), transporters, proteases, carbohydrate active enzymes (CAZymes), and lipases during poplar leaf infection (24, 48, 96, and 168 hpi). Expression profiles correspond to the different classes (classes I to VIII) and the percentage of genes in each class is indicated for each gene category. Boxes indicate a class containing more than 5% of the regulated genes. Percentages in bold highlight classes with the largest numbers of genes in gene categories.

urediniospores were propagated in the 'Robusta' cultivar susceptible to all *M. larici-populina* isolates. It might be interesting to determine whether the expression of SSP transcripts in resting and germinating urediniospores could be affected by the poplar cultivar used for spore collection. About 20% of the poplar rust genes (3,306) could not be assessed with the oligo-arrays used in this study, including 3,220 genes of unknown function, of which 247 encode SSP. Transcriptome analysis with next-generation sequencing technologies (RNA-Seq) might help to complete the present picture by addressing the expression of these SSP genes in planta.

Among the in planta-induced genes highly expressed in *U. fabae* haustoria, several encode amino acid (AAT2p [Hahn et al. 1997]; AAT1p [Struck et al. 2002]; AAT3p [Struck et al. 2004]) and sugar transporters (HXT1 [Voegelé et al. 2001]). During poplar-leaf infection, an *U. fabae* AAT3 homolog was specifically detected in planta (protein ID number 36162), whereas the closest homologs of *U. fabae* AAT2 (JGI protein ID number 37139) and *U. fabae* HXT1 (JGI protein ID number 38418) were preferentially expressed after haustoria formation, i.e., 48 and 96 hpi, respectively (Fig. 7). As previously described in *U. fabae*, those transporters could be essential for the uptake of host metabolites (Voegelé and Mendgen 2003; Voegelé et al. 2009). Voegelé and associates (2001) showed that the bean rust *U. fabae* acquires hexoses through the monosaccharide transporter HXT1. Here, several hexose transporter genes were induced in planta at 96 and 168 hpi (JGI protein ID numbers 38418, 40410, 40379), whereas an invertase transcript was preferentially accumulated after 48 hpi (GH32, JGI protein ID number 44167). Concomitant expression of genes encoding an invertase and hexose transporters strongly support carbon acquisition from the host after the action of invertases.

In the bean rust *U. fabae*, many lytic enzymes such as chitin deacetylase, proteases, and cellulases were identified concomitantly with appressorium formation at early stages of infection (Voegelé et al. 2009). A large proportion of CAZymes, proteases, and lipases were preferentially expressed at late stages of poplar rust infection, particularly when sporulation occurs (Figs. 5 and 7) compared with earlier stages (i.e., 24 hpi), which indicates that these gene categories are likely involved in the uredinia formation process and sporulation. Lipids are important storage forms in fungal spores (Weete 1989) and could play an important role in differentiation of infection structures (Wang et al. 2007). The late expression of rust CAZymes and lipases in poplar leaves could mark a shift in lipid metabolism during formation of urediniospores. This is strengthened by the high expression of lipase transcripts observed in resting and germinating urediniospores. Compatible poplar-rust interactions are marked by the late induction of defense-related transcripts such as pathogenesis-related proteins compared with early induction in incompatible interactions (Duplessis et al. 2009). Induction of *M. larici-populina* protease transcripts at 168 hpi could reflect an active defense of the rust fungus against host reactions.

Microdissection of rust-infected poplar leaves at 168 hpi revealed genetic programs related to sporulation and biotrophy in uredinia and infected mesophyll, respectively (Hacquard et al. 2010). Profiles of highly induced *M. larici-populina* transcripts at 168 hpi in the present timecourse study were predominantly detected in microdissected uredinia (Supplementary Table 5). On the other hand, most highly induced transcripts encoding SSP detected in microdissected infected mesophyll at 168 hpi (i.e., containing infection hyphae and haustoria [Hacquard et al. 2010]) were induced at 96 hpi in the timecourse study. This result strengthens the concomitant expression of different transcriptional programs at 168 hpi and demonstrates that the program expressed in uredinia predominates.

MATERIALS AND METHODS

Growth conditions of plants and fungi and inoculation procedures.

Experiments were performed using strain 98AG31 (pathotype 3-4-7) of *M. larici-populina* on *P. trichocarpa* × *P. deltoides* 'Beaupré' leaves (compatible interaction). Resting urediniospores of *M. larici-populina* were collected from leaves of susceptible *P. deltoides* × *P. nigra* 'Robusta' as previously reported (Rinaldi et al. 2007). Germlings were obtained from 1 mg of urediniospores grown on water agar medium (2%) in petri dishes for 3 h at 19 ± 1°C. Plant inoculation procedures were performed as previously described, using the same inoculum dose of 100,000 urediniospores per milliliter and strictly identical culture conditions (Rinaldi et al. 2007). The samples harvested at different timepoints (2, 6, 12, 24, 48, 96, and 168 hpi) in the different treatments were immediately snap-frozen in liquid nitrogen and kept at -80°C for further nucleic acid isolation.

RNA extraction and cDNA synthesis.

Isolation of total RNA was performed with the RNeasy plant mini kit (Qiagen, Courtaboeuf, France) from 1 mg of resting and germinating spores and from 100 mg of infected leaf tissues during the compatible poplar-rust interaction (2, 6, 12, 24, 48, 96, and 168 hpi), including a DNase I (Qiagen) treatment, according to the manufacturer's instructions, to eliminate traces of genomic DNA. Electrophoretic RNA profiles were assessed with an Experion analyzer using the Experion RNA Standard-Sens analysis kit (Bio-Rad, Marnes la Coquette, France). For microarray experiments, total RNA from each sample (resting and germinating urediniospores and 2 to 168 hpi) was subjected to a single round of amplification, using the MessageAmp II aRNA amplification kit (Ambion, Austin, TX, U.S.A.), following manufacturer's instructions. RNA amplification generated more than 12 µg of amplified RNA (aRNA) for each sample, and aRNA profiles were verified using the Experion analyzer and Experion RNA Standard-Sens analysis kit (Bio-Rad). Double-stranded cDNA was synthesized from 2.5 µg of aRNA, using the Superscript double-stranded cDNA synthesis kit (Invitrogen, Cergy Pontoise, France), according to the NimbleGen user protocol. Single-dye labeling of samples, hybridization procedures, and data acquisition were performed in the NimbleGen facilities (NimbleGen Systems, Reykjavik, Iceland), following their standard protocol. For qPCR, first-strand cDNA was synthesized by RT from 1 µg of DNase-treated total RNA, using the iScript cDNA synthesis kit (Bio-Rad) in a total volume of 20 µl, according to the manufacturer's instructions.

Construction of *M. larici-populina* exon oligoarray.

The *M. larici-populina* custom exon-expression oligoarray (4 × 72,000) manufactured by Roche NimbleGen Systems Ltd. (Madison, WI, U.S.A.) contained four independent, nonidentical, 60-mer probes per gene model coding sequence (NCBI Gene Expression Omnibus [GEO] platform GPL10350). Included in the oligoarray were 17,556 coding sequences, 1,063 random 60-mer control probes, and labeling controls (Hacquard et al. 2010). The 17,556 coding sequences correspond to the initial *M. larici-populina* gene annotation set at the JGI (April 2008). The actual *M. larici-populina* gene complement is of 16,399 genes (April 2011 [Duplessis et al. 2011]), of which 13,093 (80%) were analyzed on the array used in the study. Oligonucleotide probes that presented a risk of cross-hybridization with poplar transcripts (i.e., fluorescence signal over the background level when arrays were hybridized with noninoculated poplar leaf cDNA; 281 genes) or between transcript spe-

cies expressed by different genes from a same gene family (i.e., probes with more than 90% homology between two transcripts; 859 genes) were not considered in our analysis. Among the 3,306 genes that could not be assessed with the *M. larici-populina* oligoarrays, only 247 encode SSP (of 1,184 SSP genes annotated in the genome [Duplessis et al. 2011]), and only a few genes encode CAZymes, lipases, or transporters. The SSP genes not assessed in our study correspond to genes not identified in the initial genome annotation or belong to gene families and cannot be distinguished by the oligonucleotide probes.

Microarray data analysis.

Microarray probe intensities were quantile normalized across chips. Average expression levels were calculated for each gene from the independent probes on the array and were used for further analysis. Raw array data were then renormalized using ARRAYSTAR software (DNASTAR, Inc., Madison, WI, U.S.A.). 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. All expression assays were conducted on three independent biological replicates. A student *t*-test with false discovery rate (Benjamini-Hochberg) multiple testing correction was applied to the data, using ARRAYSTAR software (DNASTAR). Transcripts with a significant *P* value (<0.05) and more than a threefold change in transcript level were considered as differentially expressed. The complete expression datasets are available at the NCBI GEO website as series number GSE21624. REI were calculated to derive rust gene expression profiles along the timecourse series. For each gene, a mean expression level was calculated along the timecourse series, and the REI corresponds to the ratio between the expression level measured at a given timepoint and the mean reference. K-means partitioning of expression data was performed on log₂-normalized REI with the Genesis software (Sturn et al. 2002).

RT-qPCR.

Specific primers amplifying fragments ranging from 102 to 248 nucleotides were designed with the Primer 3 and Amplify 3× programs (Supplementary Table 6). Primer efficiency was assessed for each target sequence and ranged between 75 and 100%. A BLASTN search was performed against the *P. trichocarpa* genome sequence (Tuskan et al. 2006) with each primer sequence, to verify the absence of cross-annealing in the poplar genome. For amplification, 2 µl of 1/10-diluted RT products were amplified in 1× iQ SYBR Green Supermix (Bio-Rad) with 1.6 µM of specific 5' and 3' primers, using a MJ-opticon2 DNA engine (Bio-Rad). Normalization of gene expression was assessed using *M. larici-populina* alpha-tubulin and elongation factor-1-alpha reference genes (Hacquard et al. 2011). Expression of the *M. larici-populina* candidate genes was determined using the 2^{-ΔCt} calculation (Livak and Schmittgen 2001). Expression levels of selected genes were converted to REI in order to compare expression measured with oligoarrays and by RTqPCR.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- NCBI GEO database, series number GSE21624:
www.ncbi.nlm.nih.gov/geo
 Roche NimbleGen Systems Limited database:
www.nimblegen.com/products/exp/index.html