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ABSTRACT

The foliar rust caused by *Melampsora larici-populina* (*Mlp*) is the main disease affecting poplar plantations in Europe. The biotrophic status of this fungus is a major limitation to address *in planta* transcripts profiling. Thus, identification of reference rust genes steadily expressed during plant tissue colonization is a crucial point. A quantitative PCR approach to assess fungal ITS amplification profile and Reverse Transcription quantitative-PCR was set to compare candidate reference genes amplification profiles in poplar infected tissues. We selected two *M. larici-populina* genes encoding an alpha-tubulin and the elongation factor-1-alpha that showed the highest expression stability across biological samples and for which transcript levels were correlated with fungal ITS amplification during time-course infection of poplar leaves. We report the use of these reference genes to assess *in planta* expression profiles of two genes involved in thiamine biosynthesis (*THI1* and *THI2*) for which specific haustorium expression was previously described in the bean rust fungus *Uromyces fabae*. *Mlp-THI1* and *Mlp-THI2* showed similar expression profiles. Trancripts were barely detectable in urediniospores as well as during the early stages of infection compared to those reported in the bean rust, whereas a strong induction was observed after haustorial formation after 24 hpi. These data are in frame with the results obtained in *U. fabae* and consistent with a metabolic reorientation that likely occurs after the fungus derived nutrients from its host in the haustorial structure essential for fungal biotrophy.

1. Introduction

Rust caused by *Melampsora larici-populina* is the main disease affecting poplar plantations in Europe with severe economic losses [1]. Epidemiology of the disease and poplar defence responses have been previously addressed [2,3] but still very little is known about fungal molecular mechanisms during the infection process. It is crucial to increase our understanding of the biology of the pathogen in order to develop strategies to control the disease. In the wake of the *Populus* genome sequencing [4], the ~100 Mb genome of *M. larici-populina* has been recently sequenced by the Joint Genome Institute (JGI; http://genome.jgi-psf.org/Mellp1/Mellp1.home.html) and the analysis of this genome is a great opportunity to identify effector genes expressed by the rust fungus to achieve plant infection [3,5]. Availability of the genome sequences of both the plant host and the rust fungus and recent updates on *Populus* defence reactions upon rust infection [6,7,8], make the *Populus/Melampsora* pathosystem an emerging model in forest pathology to study the molecular mechanisms developed by rust to infect plant tissues and to feed on the host [3].

The biotrophic status of rust fungi precludes some routine manipulations in the laboratory. Up to now, most of the knowledge gained on rust gene expression was obtained after sequencing of Expressed Sequence Tags (EST) from urediniospores, haustorial tissues or infected plant tissues [5,9,10,11]. A synthetic system has been established to study the development of *Uromyces fabae* infection structure after the growth of urediniospores on polyethylene membranes [12]. By this means, it is possible to follow transcripts accumulation in most fungal structures usually formed

in planta after penetration in the leaf tissue, i.e. germtubes, appressoria, primary infection hyphae and haustorial mother cells. Beside, semi-quantitative PCR was used to monitor *in planta* gene expression of *U. fabae* during bean infection [13,14]. Another method developed to specifically isolate haustoria from infected bean leaves also allowed measuring transcript levels in this structure [9,11,15]. Such approaches led to the description of specific haustorial expression profiles for several rust genes such as the thiamine biosynthesis genes *THI1*; *THI2*, the hexose transporter *HXT1* and the Rust Transferred Protein *RTP1* [15,16,17].

Great progresses have been made in epidemiology after the development of the quantitative-PCR (qPCR) technique that allows a better detection of fungal pathogens *in planta* [18,19,20,21]. Such technique was successfully applied to the poplar leaf rust disease and has allowed to assess *M. larici-populina* progression in the plant tissue through specific amplification of fungal internal transcribed spacer (ITS) by qPCR [7,19]. Due to its sensitivity and ease of use in routine in laboratory, qPCR is now a technique of choice to study gene expression, although it requires rigorous standardization to accurately interpret the data and generate reliable results [22]. Based on the specificity of primers to target fungal sequence and to its great sensitivity, qPCR is a perfect tool to follow-up accumulation of specific fungal transcripts *in planta*. However, in order to allow comparison of expression levels in time-course experiments to draw-up expression profiles, reference genes are required to take into account differences in fungal content between different time-points [23,24].

Here we present an expression assay of five reference fungal genes commonly used in molecular studies, by Reverse Transcription (RT) semi-quantitative-PCR (semi-qPCR) and qPCR during time-course infection of leaves from resistant and susceptible poplar cultivars challenged by *M. larici-populina* strain 98AG31. Based on their expression levels and stability and comparison to fungal ITS amplification profiles *in planta*, we selected alpha-tubulin (*Mlp-aTUB*) and elongation factor-1-alpha (*Mlp-ELF1a*) as candidate reference genes for *M. larici-populina* gene expression *in planta*. Finally, we report the use of these reference genes to derive expression profiles of genes for which specific haustorium expression was previously described in the bean rust fungus *U. fabae* during a compatible poplar-rust interaction.

2. Material and methods

2.1. Plant material, growth conditions and inoculation procedures

All experiments were performed on rooted cuttings of *Populus trichocarpa x Populus deltoides* 'Beaupré' (susceptible cultivar) and 'Hoogvorst' (resistant cultivar). For the analysis of incompatible and compatible poplar-rust interactions, plants were grown in greenhouse from dormant cuttings as previously described [7]. Isolate 98AG31 of *M. larici-populina* (pathotype 3-4-7, virulent on 'Beaupré', avirulent on 'Hoogvorst'), was used in this study and was grown on detached leaves of susceptible *P. deltoides* × *Populus nigra* 'Robusta' as previously reported [7]. Expanded leaves from leaf plastochrony index (LPI) 5 to 9 were detached from several 'Beaupré' and 'Hoogvorst' plants and were spray-inoculated on their abaxial surface with an urediniospore suspension (100,000 urediniospores/ml) or with water-agar as a control (mock-inoculated leaves) as described in Rinaldi et al. [7]. Inoculations were done by pooling leaves of different LPI from different plants for each treatment (i.e. compatible, incompatible and mock-inoculation) and each time point. The inoculated leaves were incubated with the abaxial surface uppermost, floating on deionized water in Petri dishes, at $19 \pm 1\ °C$ under continuous artificial illumination, for various durations. The material harvested at different time points in the different treatments consisted of 30 mm diameter leaf discs randomly sampled on the overall leaf surface. The leaf discs were immediately snap-frozen in liquid nitrogen and transferred to $-80\ °C$.

2.2. DNA and RNA extraction

Total DNA was extracted from leaf tissues with the DNeasy Plant Mini kit (Qiagen, Courtaboeuf, France) from 100 mg of frozen ($-80\ °C$) material. RNA was removed by the addition of ribonuclease A during extraction. DNA quality was verified by electrophoresis on agarose gel, and DNA quantity was measured by spectrophotometry [25].

Isolation of total RNA was performed with the RNeasy Plant Mini kit (Qiagen) from 100 mg of pooled ($-80\ °C$) foliar disks harvested from leaves of various LPI and various individual poplar plants for each treatment considered. Pooling of samples from different trees and LPI helped in minimizing the variations between individual RNA samples. A DNase I (Qiagen) treatment was included in the RNA isolation procedure according to the manufacturer's instructions to eliminate traces of genomic DNA. Quality and quantity of RNA samples were checked with the RNA analyzer Experion (Bio-Rad, Marnes la Coquette, France) following the manufacturer's recommendation.

2.3. ITS amplification by semi-quantitative and quantitative-PCR

In planta development of the rust fungus was followed in 'Beaupré' and 'Hoogvorst' by specific amplification of the nuclear ribosomal DNA (rDNA) ITS on total DNA isolated from inoculated leaf tissues [7,19]. Amounts of 100 ng and 10 ng DNA were used respectively for semi-qPCR and qPCR amplifications with ITS primers specific for poplar and for *M. larici-populina* (Table 1). Specificity of ITS primers was validated *in silico* with *M. larici-populina* ITS sequences available at the JGI *M. larici-populina* genome portal (http://genome.jgi-psf.org/Mellp1/Mellp1.home.html) and in international databases. In order to improve qPCR amplification efficiency, we designed a new set of *M. larici-populina* ITS primers amplifying a 169 bp fragment shorter than those previously described in [7,19]. These primers also matched other *Melampsora* spp. in the non-redundant nucleotide database at the National Center for Biotechnology Information (BLASTN), thus, although perfectly suited for molecular studies with controlled *M. larici-populina* inoculation, these primers should not be used with infected plants collected in field experiments due to the risk of cross-hybridization with other rust species. Semi-qPCR amplification was performed in 25 µL reaction volumes using 10 µM of primers with following amplification parameters: 95 °C for 5 min, then 95 °C for 1 min, 60 °C for 30 s, 72 °C for 1 min for a maximum of 35 cycles. For qPCR, amplifications were performed in 1X iQ SYBR Green Supermix (Bio-Rad) with 1.6 µM of specific 5′ and 3′ ITS primers with an MJ-opticon2 DNA engine (Bio-Rad). Assuming a signal intensity proportional to amplified ITS sequences, we considered the pathogen growth as the relative difference between cycle threshold or quantification cycle (Cq) of fungal ITS amplicons quantified in the compatible interaction compared to Cq of poplar ITS amplicons at 2, 6, 12, 24, 48, 96 and 168 h post-inoculation (hpi) ($2^{-\Delta Cq}$ calculation, [26]). PCR amplifications were carried out on two biological replicates and included two distinct technical replicates.

2.4. Transcript profiling through semi-quantitative and quantitative RT-PCR

To allow the amplification of specific transcripts by RT followed by either semi-qPCR and qPCR, we designed primers for the following *M. larici-populina* genes retrieved from the *M. larici-*

Table 1

Specific 5′ and 3′ primers designed for qPCR and semi-qPCR amplification of *Melampsora larici-populina* and *Populus* ITS and amplification of *Melampsora larici-populina* transcripts during controlled poplar-rust time-course infection. Primers of *Laccaria bicolor* MISSP8 gene used to test amplification inhibition are also detailed.

Accession no.	Target sequence	5′ primer	3′ primer	Amplicon length	Primer Efficiency (%)
NCBI[a]					
AJ006438	*Populus* ITS	GAAGCGCCAAGGAAATTGA	GCGTTCAAAGACTCGATGGT	192	84
AY375268	*Melampsora* ITS	TCGGCCCTCATTCTTTCTAA	GATAGTCGGAGCGACCAAAG	169	107
JGI proteinID[b]					
45600	Actin (ACT)	GGCACCTCTTAATCCAAAAGTC	GTGAGTAACACCGTCACCAGAA	163	91
73137	Alpha-tubulin (aTUB)	CAAGCGTACCATTCAGTTCGT	CACCCTCCTCCATACCTTCTC	239	94
73378	Elongation factor-1-alpha (ELF1a)	CGAGACTCCCAAATACTTCGTT	GTTCACGAGTTTGACCATCCTT	167	102
46086	Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)	CCATCAACGACCCCTTTATC	ATCGGCACCAGATTTACCC	185	92
40152	Histone H4 (HISTH4)	GAGGAAAATCCGGTGGAAAG	TGGCATGTTCGGTGTAGGTA	225	99
67013	Thiamine biosynthesis gene 1 (THI1)	GCTTCTGCTCCATCCTTTACAT	GATTTGGTTAAGTGGGCTGTTC	179	97
53832	Thiamine biosynthesis gene 2 (THI2)	CTCTCGTATCGAAGGCAACTCT	CTCCTCCATGTCGTTGAAGTATC	161	95
388224[c]	Mycorrhiza-induced small secreted protein MISSP8	AGCTGATCTCAACGGAGACGTTGAGGCC	TCAATCACTATCGCGCCTCCAGTCAC	118	121

populina JGI genome portal (Table 1): actin, alpha-tubulin, elongation factor-1-alpha, glyceraldehyde-3-phosphate-dehydrogenase, histone H4, thiamine 1 *THI1* (*U. fabae THI1* homologue previously described as *PIG1*; [9]) and thiamine 2 *THI2* (*U. fabae THI2* homologue previously described as *PIG4* [9]). The primers were designed in the coding sequence, and amplified fragments showed expected lengths ranging between 161 and 239 nucleotides (Table 1). Primers efficiency was assessed for each target sequence and ranged between 91 and 102%. A BLASTN search was performed against the *P. trichocarpa* genome sequence [4] with each primer sequence to verify the absence of cross annealing in the poplar genome sequence. First-strand cDNAs were synthesized by RT from 1 μg 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. For the semi-qPCR amplification, 1 μL of cDNA was amplified by PCR (95 °C for 5 min, then 95 °C for 1 min, 60 °C for 30 s, 72 °C for 1a min for a maximum of 35 cycles) in 25 μL reaction volume using 10 μM of primers. For the qPCR amplification, 2 μL of one-tenth diluted RT products were amplified in 1X iQ SYBR Green Supermix (Bio-Rad) with 1.6 μM of specific 5′- and 3′-primers with an MJ-opticon2 DNA engine (Bio-Rad). *M. larici-populina* gene expression was normalized to reference genes, a-tubulin (*Mlp-aTUB*) and elongation factor-1-alpha (*Mlp-ELF1a*) using the $2^{-\Delta Cq}$ calculation [26], and expressed relative to the highest expression level, set at 100%. The GeNorm VBA Applet for Microsoft Excel (http://medgen.ugent.be/~jvdesomp/genorm/) based on the principles and calculations described in Vandesompele et al. [27] helps to identify most stably expressed reference genes in a given panel of samples for qPCR normalization. This approach was used to compare the selected *M. larici-populina* candidate reference genes to select stably expressed housekeeping genes. In order to validate the absence of amplification inhibition in any of the rust infected plant samples tested in this study, we used the *Laccaria bicolor* gene encoding MISSP8, a protein specific of *L. bicolor* (http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html, protein ID 388224), as a spike control [28]. This gene was cloned in the plasmid TOPO-PCR2 (Invitrogen, Cergy-Pontoise, France) and primers were designed to specifically amplify a 118 bp region in the target gene (Table 1). Primers did not match any loci of the *P. trichocarpa* or the *M. larici-populina* genomes. An aliquot of 0.1 ng of the *pLbiMISSP8* plasmid was added to each biological sample of the time-course serie (three biological replicates) and amplification of *LbiMISSP8* was performed by qPCR as decribed above. *LbiMISSP8* Cq in poplar-rust samples were then compared to Cq obtained from a 0.1 ng control plasmid sample.

3. Results

3.1. M. larici-populina growth in poplar leaves during compatible and incompatible interactions

In planta growth of *M. larici-populina* strain 98AG31 was limited during the early stages of 'Beaupré' leaves infection (2, 6 and 12 hpi; Fig. 1). A slow increase of fungal ITS amount was observed between 12 and 48 hpi (6-fold) while a drastic increase of about 70-fold was measured between 48 and 96 hpi, indicating a fast colonization of the plant mesophyll by the rust fungus during the compatible interaction. A fungal growth slackening was observed between 96 and 168 hpi, the later time-point corresponding to urediniospores formation. Interestingly, at the end of the time-course infection, fungal ITS amount in 'Beaupré' leaves was about 7000-fold higher than the amount measured at the earliest time-point (2 hpi). At the contrary, *M. larici-populina* strain 98AG31 growth was limited during the whole time-course infection of 'Hoogvorst' leaves, corresponding to an incompatible poplar-rust interaction (Fig. 1). These fungal growth profiles confirm and extend the results

Fig. 1. Time-course infection of poplar 'Beaupré' (susceptible cultivar, black dots) and 'Hoogvorst' (resistant cultivar, white dots) leaves by *M. larici-populina* strain 98AG31. Development of the rust strain was monitored by specific quantitative PCR amplification of the rDNA intergenic *ITS* region from total DNA extracted from inoculated leaf tissues at 2, 6, 12, 24, 48, 96, 168 h post-inoculation. Pathogen growth curves correspond to fungal ITS amplicons compared to poplar ITS amplicons ($2^{-\Delta Cq}$). Note the log-scale.

described by Rinaldi et al. [7] during the poplar-rust interaction. Whereas Rinaldi et al. [7] compared compatible and incompatible poplar-rust interactions based on the growth curves of two distinct *M. larici-populina* strains (i.e. virulent and avirulent) on the same poplar cultivar ('Beaupré'), the results presented here corresponded to the same *M. larici-populina* strain infecting two poplar cultivars with distinct genetic resistance background. Interestingly, similar fungal growth profiles were observed independently of the combination of cultivars/strains between 2 and 96 hpi. In the present study, the time-course was extended to the uredinia formation and urediniospores production, indicating that the uredinial stage is marked by a drastic increase in fungal mass *in planta*.

3.2. RT-semi-qPCR expression profiles of M. larici-populina reference genes

In order to accurately measure the relative abundance of a target transcript, normalization to stable reference transcripts is required [26,27,28,29]. Expression level of the typical fungal reference genes actin (*Mlp-ACT*), glyceraldehyde-3-phosphate-deshydrogenase (*Mlp-GAPDH*), alpha-tubulin (*Mlp-aTUB*), elongation factor-1-alpha (*Mlp-ELF1a*) and histone H4 (*Mlp-HISTH4*) in *M. larici-populina* was assessed by semi-qPCR during incompatible and compatible

Fig. 2. Semi-quantitative RT-PCR expression profiles for *M. larici-populina* transcripts encoding actin (*Mlp-ACT*), alpha-tubulin (*Mlp-aTUB*), elongation factor-1-alpha (*Mlp-ELF1a*), glyceraldehyde-3-phosphate-deshydrogenase (*Mlp-GAPDH*), histone H4 (*Mlp-HISTH4*) during time-course infection of *P. trichocarpa x P. deltoides* 'Beaupré' (compatible interaction, C) and 'Hoogvorst' (incompatible interaction, I) leaves at 2, 6, 12, 24, 48, 96 and 168 h post-inoculation (hpi) and non inoculated leaves (0 h). A, total RNA of 'Beaupré' or 'Hoogvorst' leaves inoculated with *M. larici-populina* strain 98AG31 was isolated and aliquots of 1 μg were used for first-strand cDNA synthesis. PCR was performed using 1 μl of first-strand cDNA. A control with no RT in the first-strand cDNA synthesis reaction mix was included to control for the lack of genomic DNA. B, PCR amplification of poplar and fungal ITS fragments from DNA samples are indicated as a reference of *in planta* fungal growth progression. In Fig. 2A and B, amplified DNA was sampled after a total of 35 cycles of PCR.

interactions (Fig. 2A) and compared to fungal ITS semi-qPCR amplification profiles (Fig. 2B). Transcripts of these genes were not detected or detected at lower levels during the incompatible interaction compared to the compatible one, consistent with the lower growth noticed by fungal ITS amplification (Figs. 1 and 2). During the compatible interaction, *Mlp-ACT* and *Mlp-GAPDH* were not or barely detectable at early stages of infection and thus might not be proper reference genes in the corresponding biological situations. In contrast, *Mlp-aTUB*, *Mlp-ELF1a* and *Mlp-HISTH4* were detected at all time-points of the infection process in the compatible interaction. Semi-qPCR profiles of these candidate genes were comparable to *M. larici-populina* ITS profile (Fig. 2) and might represent candidate reference genes. Accuracy of these *M. larici-populina* reference genes was further tested by RT-qPCR.

3.3. qPCR amplification inhibition assay

The absence of amplification inhibition was verified in our experimental system using the SPUD assay described by Nolan et al. [28] with MISSP8 a specific gene of the symbiotic basidiomycete *L. bicolor* as a spike control gene. For this, 0.1 ng of plasmid *pLbiMISSP8* were added to cDNA and genomic DNA samples corresponding to the time-course infection series used in the study for semi-qPCR and qPCR, then qPCR amplification cycle Cq of the 118 bp MISSP8 cDNA were compared with the sole plasmid. Identical Cq values were recovered for the different biological situations and the control plasmid validating the absence of amplification inhibition in the poplar-rust interactions samples (Supplemental Fig. 1).

3.4. Validation of M. larici-populina reference genes expression profiles by RT-qPCR

To validate the use of *M. larici-populina* candidate reference genes as relative controls in expression studies of compatible poplar-rust interaction, we tested their amplification by RT-qPCR and compared the *M. larici-populina* ITS Cq to reference genes Cq during time-course infection of 'Beaupré' leaves. The low expression of *Mlp-GAPDH* observed by semi-qPCR was confirmed, indicating that this gene is not suitable for normalization during time-course infection. Remaining candidate reference genes were all detected and Cq could be determined and compared to fungal ITS Cq (Fig. 3A). Although all tested genes presented similar profiles at late stages of infection, differences were observed between 2 and 24 hpi. Indeed, *Mlp-HISTH4* and *Mlp-ACT* both showed a higher Cq at 24 hpi inconsistent with the ITS Cq (Fig. 3A). GeNorm [27] was used to define the qPCR expression stability of the four genes. *Mlp-aTub* and *Mlp-ELF1a* showed the lowest expression stability values (M) which correspond to the most stable expressed genes (Fig. 3B). Based on the qPCR profiles and the GeNorm assay, we picked-up the *Mlp-aTub* and the *Mlp-ELF1a* as the best candidate reference genes for RT-qPCR normalization. To further validate the use of those two reference genes, a total of five distinct technical replicates of *Mlp-aTUB* and *Mlp-ELF1a* RT-qPCR amplification (three biological replicates in each assay) were performed by two different manipulators in different laboratory conditions such as different laboratories, different days, different iQ SYBR Green Supermixes (Bio-Rad) and different pools of cDNA synthesized from the same RNA (technical replicates 1–5 in Supplemental Fig. 2).

At early stages of infection, *Mlp-aTUB* and *Mlp-ELF1a* could be detected with a Cq of 30 and 29 cycles respectively (Fig. 3A), although the *in planta* fungal biomass was rather low compared to later time-points. During the infection process, the Cq of those genes showed similar decreases to 17 and 16 cycles respectively, at 168 hpi. Cq profiles of reference genes and ITS were almost comparable during the overall time-course infection. The curves
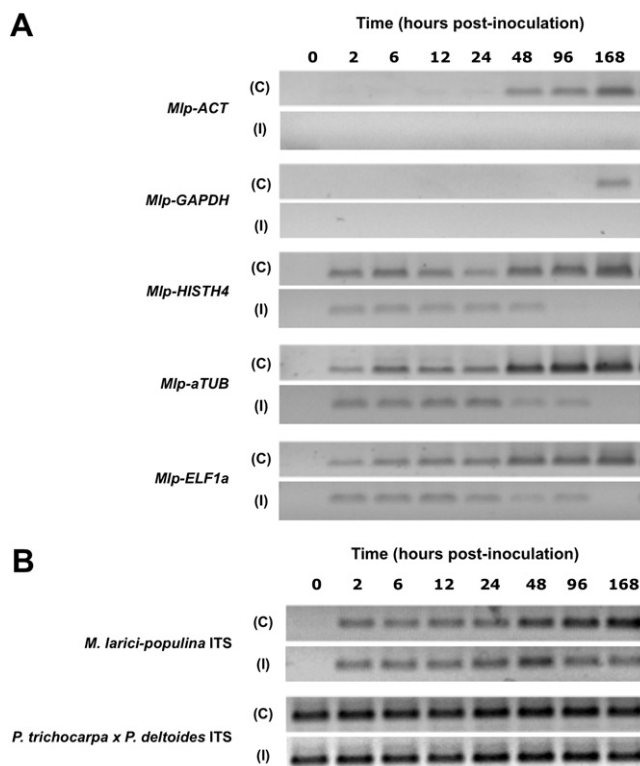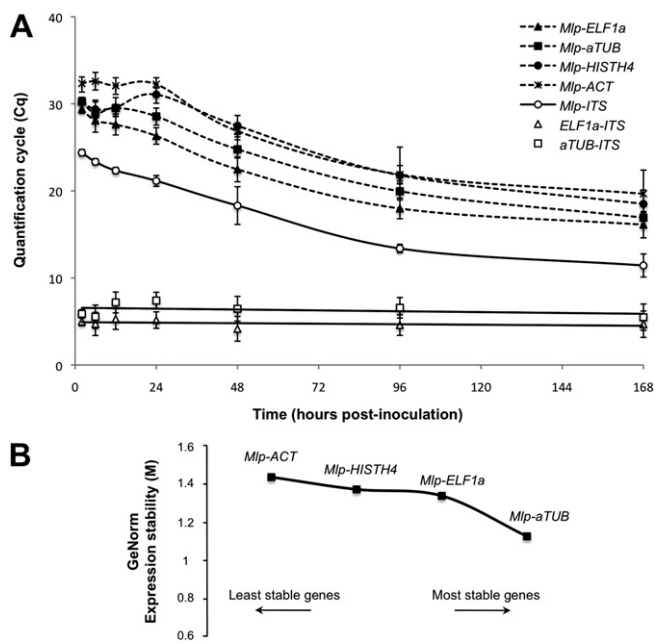
A

B

Fig. 3. Comparison of quantitative PCR (qPCR) quantification cycle Cq between fungal ITS fragments and fungal transcripts encoding an actin (*Mlp-ACT*), an alpha-tubulin (*Mlp-aTUB*), an elongation factor-1-alpha (*Mlp-ELF1a*) and an histone H4 (*Mlp-HISTH4*) amplified from *P. trichocarpa x P. deltoides* 'Beaupré' infected leaves during time-course infection with *M. larici-populina* strain 98AG31. A, *M. larici-populina* ITS Cq were measured by qPCR from DNA isolated from inoculated leaves at 2, 6, 12, 24, 48, 96, 168 hpi. *Mlp-ACT*, *Mlp-aTUB*, *Mlp-ELF1a* and *Mlp-HISTH4* transcripts Cq were obtained by RT-qPCR from total RNA isolated from the same tissues at the same time-points. Direct subtraction of ITS Cq from *Mlp-aTUB* and *Mlp-ELF1a* transcripts Cq shows a linear correlation between Cq, respectively $y = 0.004\times + 6.58$ ($R^2 = 0.11$) and $y = 0.002\times + 4.92$ ($R^2 = 0.16$), during time-course infection of poplar leaves. B, Expression stability values (M) of *Mlp-ACT*, *Mlp-aTUB*, *Mlp-ELF1a* and *Mlp-HISTH4* candidate reference genes calculated with GeNorm. Lowest M value indicates most stable expressed genes.

Fig. 4. Expression patterns of *M. larici-populina* thiamine biosynthesis transcripts *Mlp-THI1* and *Mlp-THI2*. Total RNA of ungerminated urediniospores and inoculated *P. trichocarpa x P. deltoides* 'Beaupré' leaves with *M. larici-populina* strain 98AG31 at 2, 6, 12, 24, 48, 96 and 168 h post-inoculation were isolated and aliquots of 1 μg were used for first-strand cDNA synthesis. PCR was performed using 2 μl of one-tenth diluted first-strand cDNA. A control with no RT in the first-strand cDNA synthesis reaction mix was included to control the lack of genomic DNA. Both *Mlp-aTUB* and *Mlp-ELF1a* transcripts were considered as reference transcripts for the calculation of *Mlp-THI1 and Mlp-THI2* regulation levels ($2^{-\Delta Cq}$). For each transcript, expression regulation levels are presented as percentages of the highest regulation level measured (100%).

obtained with *Mlp-aTUB* and *Mlp-ELF1a* genes were parallel to that obtained with ITS, suggesting a strong correlation between reference genes expression and fungal biomass content in plant tissue during time-course infection. Subtraction of the ITS Cq from *Mlp-aTUB* and *Mlp-ELF1a* transcripts Cq shows parallel slopes (Fig. 3A), respectively of $y = 0.004\times + 6.58$ ($R^2 = 0.11$) and $y = 0.002\times + 4.92$ ($R^2 = 0.16$), indicating a good correlation between reference genes expression and fungal biomass content in plant tissue during time-course infection.

3.5. RT-qPCR expression assays

RT-qPCR analysis was used to investigate the relative expression of *Mlp-THI1* and *Mlp-THI2*, two homologs of thiamine biosynthesis genes highly expressed in haustoria as previously reported in *U. fabae* [9,16]. cDNA templates derived from *M. larici-populina* ungerminated urediniospores and infected 'Beaupré' leaves (2, 6, 12, 24, 48, 96, 168 hpi) were used to follow *in planta* transcript profiles during compatible poplar-rust interaction. *Mlp-aTUB* and *Mlp-ELF1a* were used as constitutively expressed endogenous controls for normalization of gene expression. Mean Cq of the two genes was considered at each time-points for $2^{-\Delta Cq}$ calculations. Calculations performed separately with either *Mlp-aTUB* or *Mlp-ELF1a* as a unique reference gene, did not show significant differences (data not shown). Thus, the mean Cq of the two reference genes was used rather than comparison to a single reference gene. *Mlp-THI1* and *Mlp-THI2* were both expressed in *M. larici-populina* during plant infection after 48 hpi and 24 hpi respectively (Fig. 4).
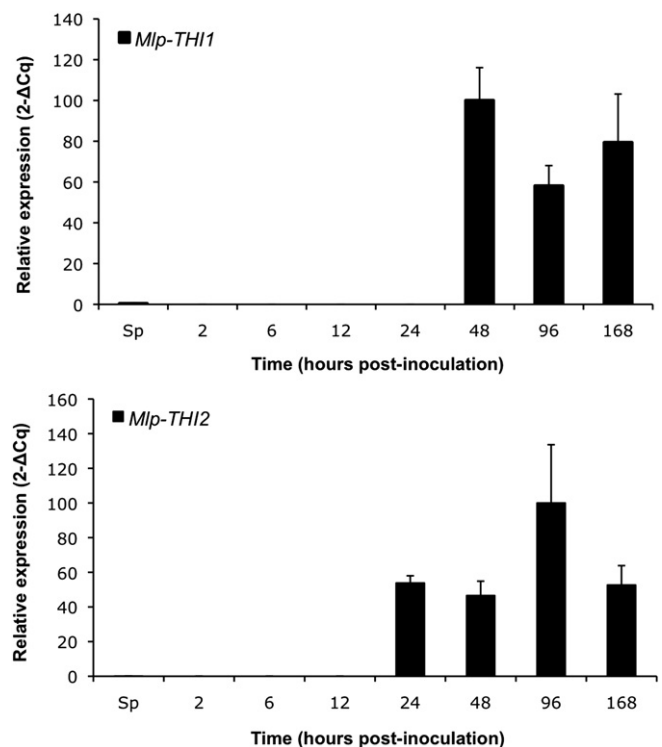
Expression was not or barely detectable at early stages of 'Beaupré' leaves infection or in ungerminated urediniospores. Both transcripts showed at least a 200-fold induction in infected leaves compared to ungerminated urediniospores suggesting their important role during the biotrophic growth phase.

4. Discussion

Expression profiling is an approach of choice to have a global view and a better understanding of the biological processes that control plant−pathogen interactions. Attempts to study *in planta* development of the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* had been made possible through the use of transcriptome profiling [30]. Similar attempts had been also conducted in hemi-biotroph and biotroph fungal pathogens including *Magnaporthe oryzae* [31,32], *Ustilago maydis* [33] and *U. fabae* [34] although only few studies addressed gene expression *in planta*.

Most of our knowledge on expressed genes in rust fungi mostly derived from the sequencing of expressed sequence tags (ESTs) from infected leaves or isolated haustoria [5,9,10]. In the bean rust *U. fabae*, such approaches led to the identification of *in planta* induced genes (PIGs) expressed after the host plant invasion [9,34]. Transcript profiling were analysed in isolated haustoria, in infected leaves at late time-points of interaction, and in fungal structures grown *in vitro* in a synthetic culture system which permits to produce *ex planta* early infection structures similar to those produced in the plant by the rust fungus. This useful system allows

to harvest synchronized structures such as germtubes, substomatal vesicles or infection hyphae [9]. Northern blot analyses of leaf infection by *U. fabae* helped in identifying transcripts highly or specifically expressed in the haustorial structure such as the secreted rust transferred protein 1 [17], the hexose transporter *HXT1* [15] or genes involved in thiamine biosynthesis such as *THI1* and *THI2* [9,16]. Specific *U. fabae* gene expression was further validated *in planta* after haustorium formation using cDNA-array [34], unfortunately, very little is known about *in planta* expression of rust genes during the early stages of the plant−fungus interaction.

The qPCR technique is very sensitive and had been successfully applied to pathogen detection in field samples for various pathosystems [35,36,37]. Particularly, this technique has allowed the detection and quantification of rust fungi belonging to *Melampsora* spp. in poplar leaves [7,19]. The RT-qPCR technique is now commonly used in molecular studies replacing Northern blotting as a routine tool to quantify transcripts. This approach should be an excellent mean to assess gene expression in biotroph pathogens infecting their hosts. To date, only few studies reported the application of real time RT-PCR to study the relative quantification of plant pathogen gene expression *in planta* [38,39,40,41]. However, there are serious pitfalls to avoid when conducting such quantification, and it is now required to comply with simple but necessary guidelines prior using the qPCR technique for gene expression [22].

As non-cultivable organisms, biotrophic pathogens such as rust fungi require to set compatible systems with their host plant. Thus, a major concern to perform gene expression study by RT-qPCR *in planta* is the increase of fungal biomass during the time-course of interaction with the host. Considering this limitation, identification of genes showing a constant expression related to fungal growth is a crucial point in order to have a common reference to derive expression profiles and set comparison between genes at different time-points. To validate the use of RT-qPCR for *in planta* gene expression of the poplar-rust *M. larici-populina*, five candidate reference genes typically used in plant pathogen expression studies [23,42] were selected and primers were defined to perform their amplification. Time-course infections have been established between *M. larici-populina* strain 98AG31 and *P. trichocarpa x P. deltoides* 'Beaupré' (compatible interaction) and 'Hoogvorst' (incompatible interaction) leaves. Fungal growth was monitored *in planta* by qPCR using quantification of the ITS of fungal nuclear rDNA [19]. Fungal ITS amplification by qPCR during time-course infection of *P. trichocarpa* × *P. deltoides* 'Beaupré' leaves was considered as an *in planta* growth curve reference for the rust fungus (Fig. 1). The assessment of the fungal growth during the compatible poplar−rust interaction is consistent with our knowledge of the progression of rust infection structures in poplar leaves as described using histology and microscopy approaches [7,41,43,44]. Fungal growth was limited to a basal level during the time-course incompatible interaction, which is consistent with the early and strong defense response mounted by poplar to constrain rust progression in the mesophyll [3,7]. RT Semi-qPCR profiles of rust genes during the incompatible interaction indicated lower levels of expression compared to the compatible interaction. However, as the plant express a strong defense reaction during this type of interaction (i.e. hypersensitive response, secreted pathogenesis-related proteins and proteases) [3,7], we considered that potential degradation of fungal nucleic acids might interfere with proper quantification of fungal material. Thus, we focused our analysis to the compatible interaction. The *Mlp-aTUB* and *Mlp-ELF1a* transcripts encoding respectively an alpha-tubulin and an elongation factor-1-alpha of *M. larici-populina*, presented RT-qPCR Cq profiles consistent with ITS qPCR amplification curve, i.e. fungal increase during poplar leaf colonization (Fig. 3), during the compatible interaction. GeNorm is an application designed to

identify the most stable genes within a selection of candidate reference genes across biological samples [27]. Both *Mlp-aTUB* and *Mlp-ELF1a* were considered by GeNorm as more stable than *Mlp-ACT* and *Mlp-HISTH4*. *Mlp-GAPDH* was also tested in our experimental set-up but not detected. Finally, we considered that *Mlp-aTUB* and *Mlp-ELF1a* represent appropriate and reliable candidate reference genes to perform *in planta* rust transcript profiling by RT-qPCR during compatible poplar-rust interaction.

Both *Mlp-aTUB* and *Mlp-ELF1a* transcripts were used to normalize the *in planta* expression profiles of the thiamine biosynthesis genes *THI1* and *THI2* for which a specific expression was previously described in *U. fabae* haustoria [9,16]. In rust infected bean leaves, similar expression patterns were observed for *Uf-THI1* and *Uf-THI2*, with no transcript detection in urediniospores and *in vitro*-differentiated infection structures up to 18 h after germination. A few transcripts were detected in 24 h-old *in vitro* infection structures whereas a high mRNA accumulation was described in haustoria and rust infected leaves at 5 dpi [16]. In poplar leaves infected by *M. larici-populina*, RT-qPCR assays showed *THI* transcript profiles similar to those decribed for *U. fabae THI* genes. In the time-course infection of 'Beaupré' leaves, transcripts were barely detected in ungerminated urediniospores or at early stages of infection (i.e., 2, 6 and 12 hpi). *Mlp-THI2* transcripts were detected after 24 hpi and *Mlp-THI1* after 48 hpi (Fig. 4). In *M. larici-populina*-infected poplar leaves, first haustoria have been reported at 17 hpi and their number consistently increase at later stages until uredinia formation [43]. The results described in the present study suggest that thiamine biosynthesis likely occurs in *M. larici-populina* after haustorium formation. Theses results are in frame with the hypothesis of Sohn et al. [16] that haustorium is an essential structure of the biotrophic rust mycelium not only for nutrient uptake from the plant host cell but also for metabolites biosynthesis such as thiamine. This cofactor, required for the activity of several enzymes of the central carbon metabolism is strongly accumulated in *U. fabae* only during parasitic growth, suggesting that thiamine is growth limiting and not available from the host plant [16]. We can postulate that thiamine biosynthesis also plays an important role for *M. larici-populina* and more generally that it has a key role in rust fungi biology during host infection.

Here we demonstrated that the high sensitivity of real time PCR can help in determining pathogen gene expression *in planta*, even at early stages of infection when the pathogen biomass is very limited in the host. Such approach is crucial to monitor *in planta* transcript profiles of biotrophic fungi. Rust genomic data recently made available to the community with the sequencing of two Pucciniales genomes (*M. larici-populina* at the Joint Genome Institute; *Puccinia graminis* f. sp. *tritici* at the Broad Institute) are valuable resources to design specific primers in order to address expression of rust transcripts during compatible interaction with their respective hosts and to get a better understanding of rust biology.

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Appendix. Supplementary material

Supplementary material associated with this paper can be found, in the online version, at doi:10.1016/j.pmpp.2010.10.003.

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