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# Host Adaptation and Virulence in Heteroecious Rust Fungi

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Uredinales, life cycle, heteroecism, transcriptomics, pathogenesis, effectors

## Abstract

Rust fungi (Pucciniales, Basidiomycota) are obligate biotrophic pathogens that cause rust diseases in plants, inflicting severe damage to agricultural crops. Pucciniales possess the most complex life cycles known in fungi. These include an alternation of generations, the development of up to five different sporulating stages, and, for many species, the requirement of infecting two unrelated host plants during different parts of their life cycle, termed heteroecism. These fungi have been extensively studied in the past century through microscopy and inoculation studies, providing precise descriptions of their infection processes, although the molecular mechanisms underlying their unique biology are poorly understood. In this review, we cover recent genomic and life cycle transcriptomic studies in several heteroecious rust species, which provide insights into the genetic tool kits associated with host adaptation and virulence, opening new avenues for unraveling their unique evolution.

## 1. PUCCINIALES: NOTORIOUS FUNGAL PLANT PATHOGENS CAUSING RUST DISEASES

**Sporothallus:** dikaryotic thallus, produced from germinating dikaryotic mitospores (aeciospores), that produces uredinia and/or telia

**Gametothallus:** initially a monokaryotic thallus produced from germinating meiospores (basidiospores) on which spermogonia develop that becomes dikaryotinized via fertilization to produce aecia

Fungi of the order Pucciniales are plant pathogens responsible for rust diseases. Rust disease epidemics have accompanied the evolution of agriculture dating back to at least ancient Greek and Roman times (79, 104). Rusts still impact modern agriculture and threaten major crops such as wheat and soybean (35, 39, 83). Rust fungi are obligate plant biotrophs that cannot complete their life cycle outside of their host plants. In vitro culture of these organisms has been attempted for a small number of species but only with limited mycelium production; thus, host plant colonization remains a prerequisite for experimental work (90). Rust fungi possess the most complex life cycle in the Kingdom Fungi and probably one of the most complex in eukaryotes (4). They are one of the few fungal groups with known alternation of generations (termed the sporothallus and the gametothallus), produce as many as five different spore types at prescripted stages, and typically alternate between infection of two different host plants (termed heteroecism) to complete their life cycle (3). They are present worldwide in environments where vascular plants occur, and long-distance dispersal of rust spores (e.g., intercontinental movement) has been repeatedly documented (13). For instance, wheat stem rust (*Puccinia graminis* f. sp. *tritici*) isolates in Australia are closely related to South African isolates and are believed to have crossed the Indian Ocean on high-altitude winds (103).

Pucciniales is one of the largest orders in the Kingdom Fungi, with more than 7,000 species grouped into seven suborders, 18 families, and approximately 170 accepted genera (3). The twentieth century has witnessed intense study efforts focused on rust fungi using microscopy, biochemistry, and molecular and genetic approaches (14, 53, 59, 104, 110). The past decade has seen the dawn of rust fungal genome sequencing and associated large-scale omics approaches. The availability of this genomic information represents an unprecedented opportunity to explore host adaptation and virulence mechanisms (4, 8, 34).

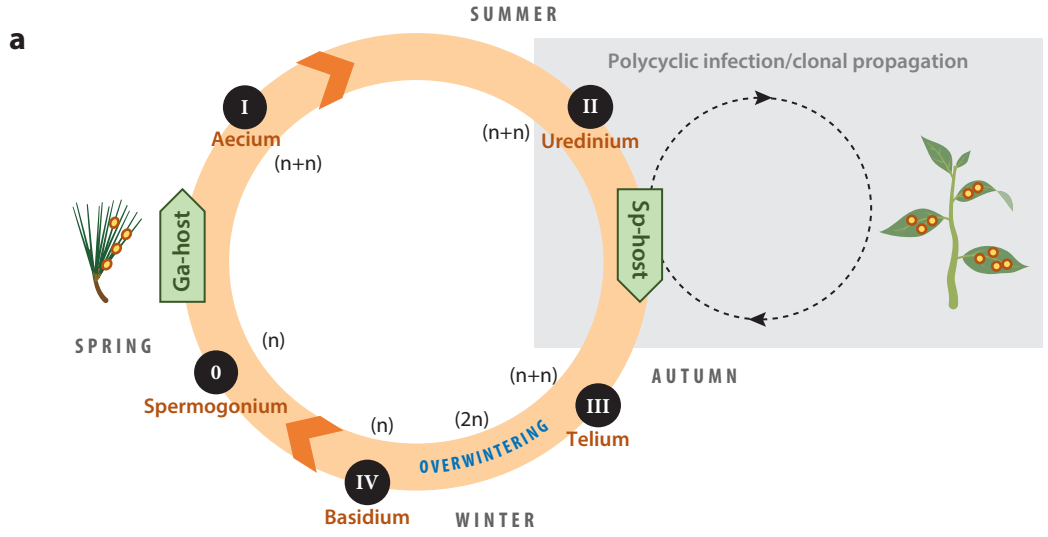
In this review, we first describe the typical life cycle of a temperate, heteroecious rust fungus, with an emphasis on the specific stages involved in infection of the two host plants, and we address evolutionary concepts that may explain specific adaptations toward the two alternate hosts of heteroecious rust fungi. We then report on the most recent advances made in the study of genes underlying host adaptation and virulence in Pucciniales. For this purpose, we consider transcriptomic profiling conducted at different stages of the life cycle for a handful of species.

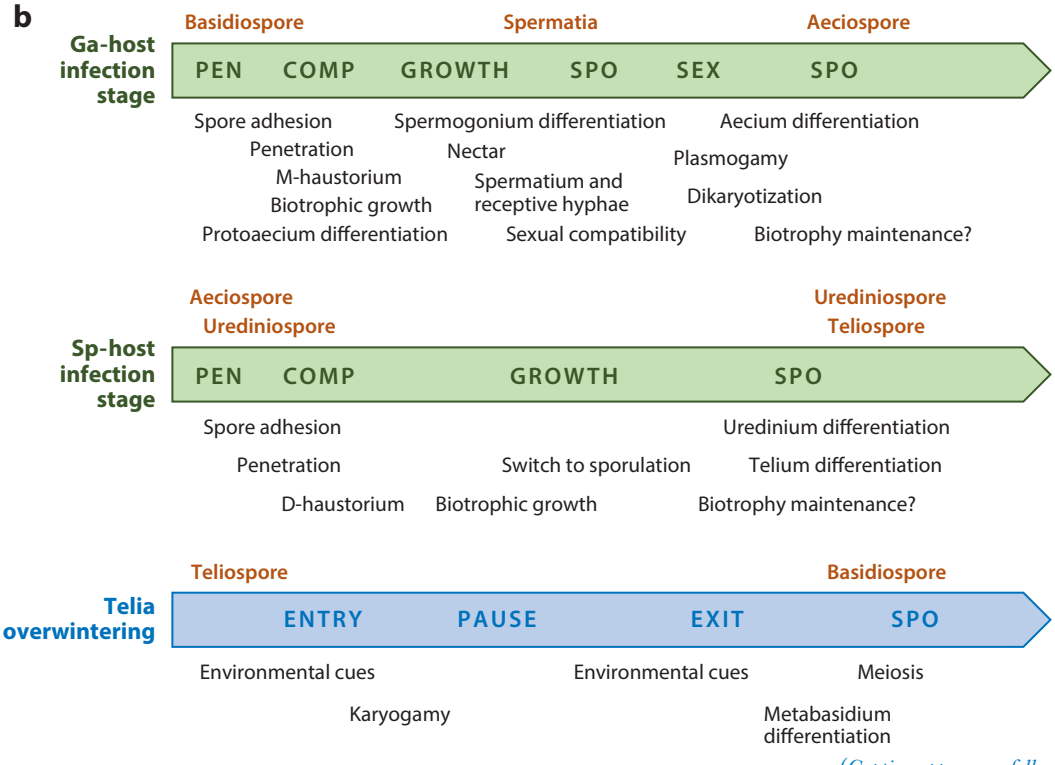
## 2. FROM A TYPICAL HETEROECIOUS CYCLE PERSPECTIVE: A JOURNEY INTO THE SUCCESSIVE COLONIZATION OF HOST PLANTS

The life cycles of rust fungi are diverse but largely represent variations on a common theme that involves a complex cycle with alternation of generations on two different host plants (4). In this section, we describe this typical heteroecious macrocyclic life cycle of a rust fungus in a temperate environment to highlight the sequential events that occur throughout the year (**Figure 1**). All the events hold true for rust fungi with autoecious and/or reduced life cycles, although most rust fungi adapted to tropical climates do not undergo an overwintering phase. For instance, macrocyclic autoecious rust fungi exhibit all five spore stages but on a single host, heteroecious demicyclic rust fungi lack a urediniospore stage, and microcyclic rusts produce only teliospores and basidiospores. For some rust fungi, only one of the alternate hosts and associated spore stages is known, although other hosts/stages may exist in nature or the alternate host may have become extinct (3, 4, 62).

The schematic in **Figure 1*a*** depicts the overall heteroecious macrocyclic rust life cycle; detailed illustrations of the infection stages and spore types of specific rust fungi already exist

(e.g., 1, 2, 5, 53, 54). Our goal is to describe precisely and sequentially the various events at each of the stages to provide context for unanswered questions that require further investigation at the molecular level. The events reported are based on more than a century of detailed observations by generations of uredinologists studying the stages of many different rust species by light, electronic transmission, and scanning microscopy (reviewed in 14, 47, 59). Rust identification keys are




(*Caption appears on following page*)

**Figure 1** (*Figure appears on preceding page*)

The heteroecious life cycle of rust fungi. (*a*) Schematic representation of a typical heteroecious macrocyclic life cycle of rust fungus over the course of a year. The five life stages are noted with their corresponding roman numerals in black circles. The corresponding nuclear state of the spores produced at each life stage is indicated inside the cycle: n (haploid); n+n (dikaryotic); and 2n (diploid). The transition between two unrelated host plants is represented by orange arrows on the cycle, host plant infection stages are represented by green arrows, and the overwintering stage is indicated in blue: Ga-host (gametothallus host) and Sp-host (sporothallus host). The squared uredinium stage and the polycyclic infection by urediniospores occurring on the Sp-host are absent in the life cycle of demicyclic rust fungi. All five stages are present for macrocyclic rust fungi. Seasons are noted for temperate rust species. (*b*) Schematic temporal representation of selected transitions occurring during the Ga-host and the Sp-host infection stages and during overwintering in temperate rusts. In each schematic, selected genetic programs are indicated: PEN (penetration); COMP (compatibility); GROWTH (biotrophic growth); SPO (sporulation); SEX (mating and fertilization); and ENTRY, PAUSE, and EXIT (overwintering transitions influenced by environmental signals). The spore types initiating infection or produced during or at the end of the infection units and during overwintering are noted at the top of each schematic. M-haustoria are monokaryotic and D-haustoria are dikaryotic.

also excellent modern resources to explore life cycles (99), and both sources have fueled the ideas presented in this section. The alternating hosts are generally known as the gametothallus host (Ga-host) and sporothallus host (Sp-host). Classically, a terminology was adopted for rust spore stages using Roman numerals, with the cycle starting at the spermogonial stage (designated 0) (47). However, as we focus here on molecular events accompanying the infection process as a whole on each host plant, our description starts from the basidiospore (stage IV) landing on the Ga-host in spring.

## 2.1. Infection of the Gametothallus Host

Infection of the Ga-host is marked by many morphological transitions, chronologically detailed in **Figure 1*b***. Completion of the infection on the Ga-host requires compatibility both with the host plant to establish a successful infection (governed by the basidiospore) and between fungal partners of different mating types for fertilization (governed by the spermatia). Overall, the Ga-host infection stage is defined by the expression of a predetermined genetic program that leads to spore release and mating. After landing, the monokaryotic (haploid) basidiospore adheres to the surface through modifications of its cell wall, and a germ tube emerges and elongates (59). Penetration at this stage is usually achieved directly through the host cuticle, followed by the formation of a vesicle in the invaded cell and then the production of intercellular infection hyphae and haustoria inside host cell cavities (45). The monokaryotic haustorium exhibits a different shape than the dikaryotic one (see section 2.2) but most likely sustains a similar role with the release of effector proteins to modulate host cell structures and functions to support nutrient acquisition and hyphal growth within the host tissue. Leaves or needles are commonly infected at this stage, but fungal colonization can also progress through vascular tissues to the stem or bark of woody plants (52). In the case of foliar infection, a haploid spermogonium (0) is formed on one side of the leaf by proliferation of the germinated basidiospore hyphae, whereas a protoaecium is formed below the epidermis on the other side of the leaf after fertilization from the products of two mating-compatible spermogonia (as detailed below). The spermogonium comprises different cell types that vary in shape and composition from one rust species to another (46). The spermogonium contains receptive flexuous hyphae in its core, which are connected to the protoaecium. Other cell types include paraphyses and spermatosporophores. The latter cell type mediates spermatization (21), which produces spermatia (0) in nectar droplets. Fertilization occurs through several different means. Spermatia may be exchanged via wind or by insects going from one nectar droplet to another. When a spermatium meets a compatible receptive hypha of opposite mating type, the haploid cells fuse to form a dikaryotic cell (plasmogamy). The nuclei divide and migrate along the receptive hyphae to the protoaecium where an aecium (I) differentiates and dikaryotic aeciospores are formed, typically in catenulate chains, and released (45). Different types of aecia have been

described for a variety of rust species with their own organization and cell types (59, 78). Note that in heteroecious rust species the basidiospores initiating infection on the Ga-host cannot infect the Sp-host and that the aeciospores produced by the aecial infection stage cannot reinfect the Ga-host. The overall infection of the Ga-host in springtime usually occurs within weeks.

## 2.2. Infection of the Sporothallus Host

Infection of the Sp-host has been the most studied process in macrocyclic rust fungi because this stage is usually responsible for disease epidemics in crops. The infection stage on the Sp-host is detailed in **Figure 1*b***. Aeciospores carried by the wind and/or rain land on the appropriate alternate Sp-host. Infection, development of the sporothallus, and production and release of urediniospores (II) can occur within days (91). Urediniospores are the only spore stage capable of reinfecting the Sp-host on which they are formed, which can lead to the production of a new generation of urediniospores within days. This asexual multiplication continues in a polycyclic mode during spring and summer and can trigger rapid development of epidemics. In some cases, when the life cycle cannot be completed on a Ga-host because of environmental circumstances or the absence of a Ga-host, asexual populations of urediniospores can survive by clonal multiplication if susceptible Sp-hosts are present and environmental conditions are favorable (9). For instance, one clonal lineage of *P. graminis* f. sp. *tritici* has been prevalent in southern Africa for at least one hundred years (55, 74, 103).

The events that occur during infection of the Sp-host are similar whether initiated from an aeciospore or a urediniospore (91). After spore adhesion on the host, a germ tube emerges and elongates on the plant surface. These initial events are often marked by changes in the composition of the fungal cell wall (104). In contrast to initiation of gametothallus infections, penetration of the Sp-host usually occurs through stomata after the formation of an appressorium rather than by direct penetration through the cuticle. However, there are exceptions. For example, urediniospores of the Asian soybean rust fungus *Phakopsora pachyrhizi* penetrate through the cuticle, relying on the formation of high turgor pressure inside the appressorium, in a manner similar to that of the ascomycete *Magnaporthe oryzae* (61, 76). Topological signals on the Sp-host surface serve as cues for the germ tube in locating stomata. Once the appressorium forms, it separates from the germ tube by a septum and nuclear division occurs prior to host penetration. A substomatal vesicle forms inside the host below the appressorium, from which infection hyphae progress (91). Hyphal progression stops when a haustorium is formed in a host cell cavity and secondary hyphae elongate from previous fungal cells by branching. The haustorium differentiation from a haustorial mother cell has been well described (59, 105). The plant cell wall is penetrated at a focal point from which the haustorium differentiates inside the host cell cavity by invaginating the host plasmalemma. The structure is surrounded by a specific interface called the extrahaustorial matrix (105). This matrix is separated from the rest of the apoplast by a dense electron-opaque neckband at the neck of the structure right after the point of entry into the plant cell wall (59). The haustorium expresses ATPase energy pumps, sugar and amino acid transporters, consistent with a role in nutrient acquisition from the host cell (92, 104). Haustoria show high expression of secreted proteins, some of which have been shown to play a role in virulence (17, 38, 44, 58, 104). Altogether, this demonstrates the dual role of this structure in establishing host compatibility by interfering with immune responses and acquiring nutrients to support biotrophic growth (62). The number of haustoria increases in the next few days before switching to the next developmental stage, which involves the differentiation of a uredinium (II). The signals or genetic controls underlying progression into spore production at this stage remain unknown. Primordia are formed below the host surface and sporogenous hyphae develop to form a cohesive structure, which often breaks through the host epidermis to release large numbers of urediniospores (91). In a few rust fungi,

such as the causal agent of coffee leaf rust, *Hemileia vastatrix*, urediniospores are released through the host stomata. Although spore release on the Sp-host surface damages plant tissues, no specific plant response is known, suggesting that compatibility and biotrophy are maintained at late stages of infection.

**Biological specialization:** specialization to a host or host lineage that may result in coevolution or codiversification between host and pathogen

### 2.3. Overwintering Before Switching Host Plants

The final life stage does not involve a new host infection but constitutes a crucial link between the Sp-host and Ga-host infection stages and encompasses meiotic production of haploid basidiospores. As shown in **Figure 1*b***, telia (III) form, either separately or as differentiated spores from uredinia in the Sp-host (59), upon specific environmental cues and possibly undefined host signals (66). Teliospores form before winter within foliar tissues, inside or below the host bark, or in specialized galls (e.g., *Gymnosporangium* spp.). Teliospores possess thick multilayered cell walls with characteristic ornamentation in comparison to other spore types and are the easiest spore stage to identify (66). Teliospores have reduced vacuoles, and they accumulate numerous lipid droplets and glycogen-like material. Depending on the rust species, nuclear fusion (karyogamy) can occur before or after the structure enters the dormant overwintering state (40, 66). The triggers that break this dormant stage in the life cycle are not known; however, light, water, and temperature influence this process (66). Under favorable conditions, the life cycle resumes with the rapid germination of a metabasidium (IV) from the teliospore once dormancy is broken. Meiosis takes place in the metabasidium, from which haploid basidiospores are produced. Basidiospores can contain one or two haploid nuclei, depending on the species (66). Recent molecular studies in both macrocyclic and demicyclic rust fungi have shown that meiosis-related genes are expressed early in telia development, suggesting a tight control of karyogamy and meiosis at this stage (40, 96). Interestingly, late teliospores of *Gymnosporangium* spp. accumulate candidate virulence-related transcripts (96, 97), and it remains to be determined whether these relate to the preparation of the Ga-host infection by short-lived basidiospores produced immediately from telia. Teliospore production and germination in tropical rust fungi are not well-studied, but in most cases, they lack a dormant period and teliospores may germinate immediately without release from the telium.

Heteroecious rust species must be molecularly equipped to colonize two host species (i.e., to ensure penetration and establishment of a compatible interaction, bypass immunity, and subvert defense responses). The infected host species are most often phylogenetically unrelated and can also differ in terms of composition of the infected tissues (e.g., from annual to woody dicot or from gymnosperm to fern). The requirement for alternation on unrelated hosts means that rust fungus evolution must be driven by coevolutionary constraints imposed by both hosts. Recent studies of the phylogeny of host plants and rust fungi have helped in addressing such questions (2, 65). A cophylogeny and molecular dating analysis has timed the diversification of the order Pucciniales to some 215–230 million years ago, which matches the time of diversification of the corresponding gymnosperm and angiosperm host plants (2). From this study, it appears that the gametothallus and sporothallus stages that occur on different host plants are under differing evolutionary pressures, which would explain the current host–rust fungi associations, with the gametothallus being key to the biological specialization of the Pucciniales (2).

## 3. VIRULENCE: FEEDING UNBEKNOWNST TO THE HOST

### 3.1. Avirulence Genes of Rust Fungi: Pioneering Advances and Emerging Research

Understanding molecular mechanisms underlying virulence is necessary to define new strategies for fighting rust diseases. Pioneering work on rust fungi led to the concept of pathogen avirulence

## PLANT IMMUNITY: FROM MODEL TO MODEL

The current paradigm of plant immunity mechanisms is that pathogen recognition occurs through two layers of specific plant receptors present at the surface and inside the cytosol of plant cells (11). Pathogen molecules detected by such receptors are either molecular patterns present in the host cell surroundings (the apoplastic space) or effector proteins delivered inside the host cells. The seminal zig-zag model proposed by Jones & Dangl (49) brought together the successive recognition events in a unified scheme with two levels of immunity and susceptibility when the pathogen delivers effector molecules inside the host cell to impair the immune response in the absence of receptors. Long before this molecular model, the gene-for-gene relationship established by Harold Henry Flor (37) paved the way and determined that both a resistance gene in the host plant and an avirulence gene from the pathogen are required to trigger resistance to a pathogen. In the absence of the resistance gene or the avirulence gene, successful infection occurs, resulting in disease. This gene-for-gene interaction model was established with the flax–flax rust pathosystem and is foundational to progress in identifying resistance genes (27, 32).

determinants that drive incompatibility in the presence of corresponding host resistance (*R*) genes (see the sidebar titled Plant Immunity: From Model to Model). Previous studies of avirulence (*Avr*) genes in rust fungi and other pathosystems have demonstrated that they encode effector proteins that are released into host cells (27, 71). The molecular basis of virulence can be viewed as the sum of the concerted actions of effectors working in synergy to interfere with defense functions and establish a compatible interaction with the host. Most knowledge about fungal effector biology (i.e., their localization, target, and function) comes from model pathosystems (7, 11). Knowledge regarding the isolation and characterization of Avr effectors from rust fungi still lags behind because of the lack of in vitro culture and transformation capabilities as well as technical difficulties in generating high-quality reference genomes (reviewed in 8, 36, 62, 71). However, major advances have been achieved in the autoecious flax rust fungus *Melampsora lini*, for which six avirulence genes (*AvrL567*, *AvrL2-A*, *AvrM*, *AvrM14*, *AvrP123*, and *AvrP4*) have been identified and studied along with their *R* gene counterparts (*L2*, *L5*, *L6*, *L7*, *M*, *M1*, *M4*, *P*, *P1*, *P2*, *P3*, and *P4*) in the flax host *Linum usitatissimum* (6, 10, 17, 25). The study of specific molecular interactions between *Avr* and *R* gene products is instrumental to further our understanding of the plant immune system (11, 27, 32). The recognition of *M. lini* proteins in the host cytosol by R proteins evidences their delivery from the fungus, although their molecular function or pathogenicity targets in the host cell are yet to be determined (56, 71). In several cases, R proteins directly recognize Avr proteins (18, 26), and sequence polymorphism analysis shows evidence of diversifying selection in *M. lini Avr* genes, suggesting evolution shaped by the pressure to escape recognition by host immune receptors (10, 32). Recently, AvrSr35 and AvrSr50, two Avr proteins directly recognized by the wheat R proteins Sr35 and Sr50, respectively, have been identified in the wheat rust fungus *P. graminis* f. sp. *tritici* (19, 77). Isolation of a third *P. graminis* Avr gene, *AvrSr27*, and expression profiling analysis has revealed a common expression pattern exhibited by these Avr effectors, typified by high expression in haustoria and early induction during uredinial infection (102). A similar pattern was observed for *M. lini Avr* genes (107) and may be a hallmark of host-delivered rust effectors.

Population genetics approaches have been used to identify candidate rust *Avr* genes based on specific DNA variants in populations (e.g., point mutation, deletion, insertion, epigenetic mark affecting gene expression, chromosomal rearrangements) that correlate with avirulent phenotypes (8, 26, 36). In recent years, studies have reported genes or loci that may underlie avirulence in a variety of species such as *Melampsora larici-populina*, *Puccinia hordei*, *Puccinia coronata* f. sp. *avenae*, *Puccinia striiformis* f. sp. *tritici*, and *P. graminis* f. sp. *tritici* (16, 20, 57, 67, 70, 85, 101, 106, 108). A

comparative genomics study of the broadly virulent Ug99 isolate of the wheat stem rust has elegantly shown that the Ug99 lineage results from a somatic hybridization between two parental dikaryotic isolates, including a member of a common South African lineage (34, 55). For decades, somatic hybridization was postulated to occur (69); however, the work by Li et al. (55) provided the first evidence that this mechanism can mediate emergence of new virulence profiles in rust fungi. Importantly, combining long-read DNA sequencing (PacBio) and chromatin proximity analysis (Hi-C) techniques in *P. graminis* f. sp. *tritici* (Australian isolate 21–0) generated the first chromosome-level assembly for a rust fungus. The availability of this resource has brought new opportunities for effector discovery, easing gene identification and detection of allele variation. The characterization of spontaneous and induced mutations that confer gain-of-virulence phenotypes on plant genotypes carrying specific *R* genes can be powerful in isolating *Avr* genes (19, 50, 77, 102).

**CAZymes (carbohydrate-active enzymes):** all enzymes involved in modification of polysaccharides, including plant cell wall–degrading enzymes and fungal cell wall–remodeling enzymes

### 3.2. Cataloging Virulence Potential Through Effector Prediction in the Genomics Era

In less than ten years, the genomes of nearly a dozen rust species have been made available to the scientific community (4, 8, 28, 34). Considering the obligate biotrophic nature of rust fungi, documenting their whole gene complement provides a means to unravel their biology at the molecular level. Rust *Avr* genes reported so far encode small secreted proteins of unknown function. These typical features are now commonly used to predict putative virulence factors in rust fungi (62, 64, 89). The repertoire of candidate secreted effector proteins (CSEPs) can be established using bioinformatic pipelines to predict secretion and common effector features from the protein sequence or by studying expression profiles during interaction with host plants. Repertoires can be derived from RNA-sequencing (RNA-seq) transcriptome studies when no reference genome is available. Machine learning effector prediction tools have been designed and applied to rust fungi and used to predict putative subcellular localization for these proteins (86–88). Long lists of CSEPs have been established in rust fungi, most of which are of unknown function and show high specificity at the order, family, or species rank, suggesting potential adaptation to their host plants (4, 62). A striking feature of rust genomes is the relative abundance of multigene families and the genome plasticity driven by repeat invasion, which may mediate diversification of virulence genes (4). Beyond CSEPs, homology-based annotation of rust fungal genomes provides extensive information about cellular categories that may be involved in pathogenesis and more particularly CAZymes (carbohydrate-active enzymes). Such enzymes are essential during the early stages of infection, i.e., adhesion, penetration, or haustoria formation (104). Considering the specific composition of each host plant, the diversification of genes falling into this category may reflect adaptation to distinct host plants. Gene loss and reduction of the CAZyme repertoires may also reflect specific adaptation, as shown for biotrophic mutualistic fungi (51, 68).

## 4. UNRAVELING HOST ADAPTATION IN HETEROECIOUS RUST FUNGI THROUGH COMPARATIVE TRANSCRIPTOMICS

Transcriptomics only requires the isolation of nondegraded RNA molecules from biological samples and is thus an approach of choice for investigating rust fungi. Transcriptomics has been applied to a variety of rust fungi, with most studies focusing on uredinial infections of the Sp-host plant (reviewed in 28, 31, 62). Time-course studies are reported, although low levels of fungal material in the samples preclude expression profiling at early stages of infection (73). Isolation of mature haustoria from infected tissues for transcriptomics and proteomics helped in capturing specific infection-related expression profiles (reviewed in 8, 62). However, characterizations of early

molecular events in haustoria formation are still lacking. At late stages of infection, many fungal cell types are produced inside the host tissues and a mixture of cells expressing different genetic programs are captured in the transcriptome profile (e.g., biotrophic growth, haustoria formation, nutrient acquisition, growth switching to spore-forming tissues, newly formed spores). Microdissection has been a useful approach to separate these tissues and has revealed the expression of a specific genetic program marked by high expression of CSEP transcripts in the plant palisade mesophyll enriched in haustoria compared with regions enriched in spore-producing cells (41, 100).

**Unigene:** unique genes reconstructed from redundant expressed sequence tags

Thus far, only a limited number of studies has addressed transcriptomics of heteroecious rust fungi on their different host plants or at different spore stages (**Table 1**). In this section, we report major results of studies in five rust species.

### 4.1. *Puccinia triticina*

*Puccinia triticina* is a heteroecious and macrocyclic rust fungus, the sporothalli of which infect wheat and cause severe damage; the gametothalli infect *Thalictrum* species, such as *Thalictrum speciosissimum* (meadow rue) (12). An early transcriptome study covered most stages of the life cycle with expressed sequence tags (ESTs) using the Sanger sequencing method to provide support for gene prediction (109). Thousands of ESTs were produced from germinated urediniospores, isolated haustoria, and dormant teliospores from the Sp-host; aeciospores and spermatia were isolated from the Ga-host. Wheat-infected ESTs from previous studies were added to enrich the data set. A total of 4,639 *P. triticina* unigenes were defined, of which 3,770 were stage specific. Overall, expressed genes fall into the metabolism, energy production and conversion, translation, and protein turnover categories. Some stages (e.g., telia and spermogonia) exhibited higher expression of ribosomal proteins indicative of intense cellular activity, whereas haustoria were marked by the expression of nutrient transport genes and the presence of many species-specific genes. CSEP genes were expressed at different stages and more than a hundred were specifically expressed in haustoria (109). The genome sequence of *P. triticina* was released several years later (22). The genome contains 14,880 genes, among which more than 5,000 are specific to *P. triticina* with no homology to genes in other sequenced *Puccinia* spp. and of which 1,358 are predicted to encode CSEPs. Interestingly, CSEPs represent approximately 17% of the species-specific genes but only 9% of all genes. The study also included an RNAseq expression survey at the following stages: dormant and germinated urediniospores, infected wheat tissues (Sp-host), spermatia, and a mixture of spermatia and aecia at a later stage on the Ga-host. In total, 784 CSEPs are deemed expressed in at least one life cycle stage. The top highly induced genes include host-specific CSEP genes. Expression of several glycosyl hydrolase (GH) genes is induced on one host or the other with stage-specific profiles for GH from different families (e.g., GH16, GH17, GH18, GH26), suggesting that the fungus may deploy different CAZymes during spore germination and host colonization (22). Comparison to other wheat rust fungi with different alternate hosts suggests that they have derived varied CSEP complements to infect their hosts and, most likely, to specifically infect the Ga-host and Sp-host.

### 4.2. *Cronartium ribicola*

*Cronartium ribicola* is responsible for white pine blister rust disease, which is a serious concern for white pine plantations in North America. It has a heteroecious and macrocyclic life cycle, and its Ga-hosts are five-needle pines (*Pinus* spp.), whereas the Sp-hosts are species of *Ribes* (52). Basidiospores are specialized to penetrate needles of the Ga-host through their stomata, from which they progress over several months through the vascular tissue to reach the host stems and branches and then the bark tissue where swollen cankers form the following spring (52). No

**Table 1 Transcriptomic studies of life cycle stages of heteroecious rust fungi[a]**

| Rust species (disease) | Life cycle type, host plants | Reference genome | Life stage (spore type) | | | | |
|---|---|---|---|---|---|---|---|
| | | | Basidium, basidiospores (IV) | Spermogonium, spermatium (0) | Aecium, aeciospores (I) | Uredinium, urediniospores (II) | Telium, teliospores (III) |
| *Cronartium ribicola* (white pine blister rust) | Macrocyclic, white pine and *Ribes nigrum* | ND | Basidiospores infecting pine needle and pine bark (60) | ND | Aeciospores, not in planta (60) | Urediniospores, not in planta (60) | ND |
| *Gymnosporangium yamadae* (apple rust) | Demicyclic, *Juniperus chinensis* and *Malus domestica* | ND | ND | Spermogonia in planta (95) | Aecia in planta (95) | NA | Telia, teliospores (95, 96, 97) |
| *Melampsora larici-populina* (poplar leaf rust) | Macrocyclic, poplar cv. Beaupré and *Larix decidua* | 29 | Basidia, basidiospores (63) | Spermogonia in planta (63) | Aecia in planta, mixed with spermagonia (63) | Urediniospore and in planta infection (30, 63) | Telia, teliospores (40) |
| *Puccinia striiformis* f. sp. *tritici* (wheat stripe rust) | Macrocyclic, wheat cv. MX169 and *Berberis shensiana* | Several reference genomes (15, 16, 22, 82, 112) | Basidiospores infecting barberry leaf (111) | ND | ND | Urediniospores infecting wheat leaves (111) | ND |
| *Puccinia triticina* (wheat leaf rust) | Macrocyclic, *Triticum aestivum* L. and *Thalictrum speciosissimum* | 22 | ND | Spermogonia nectar droplets (109); spermogonia in planta (22) | Aeciospores (109); aecia in planta, mixed with spermagonia (22) | Dormant and germinating urediniospores, isolated haustoria (109); dormant and germinating urediniospores, urediniospores infecting wheat leaves (22) | Telia, teliospores (109) |

[a]The table does not include the extensive transcriptomic studies that focused only on the sporothallus host infection stage (i.e., urediniospores, host infection, or isolated haustoria) for *P. striiformis* f. sp. *tritici* and *M. larici-populina* or for other rust fungi not listed in this table (reviewed in 4, 28, 31, 62).

Abbreviations: NA, not applicable; ND, not determined.

reference genome is yet available for this fungus, but RNAseq data have been generated for the following life cycle stages: aeciospores, urediniospores, Western white pine *Pinus monticola* needles infected by basidiospores, and bark infected by the proliferating gametothallus (60). The *C. ribicola* transcriptome contains 13,591 unigenes, of which 80% are expressed in all the sampled stages and only approximately 6% were specifically expressed at a given stage. Stage-specific genes encoding CSEPs and CAZymes are reported at each life stage. Interestingly, expression patterns of several CAZymes were specific to infected bark tissues and may represent specific equipment for the colonization of woody material, whereas other GH family members were uniquely or predominantly expressed in *Ribes*-related spores, representing putative adaptation to the alternate hosts.

### 4.3. *Gymnosporangium* Species

*Gymnosporangium* species are heteroecious and demicyclic, meaning they do not produce the uredinial stage (**Figure 1**). These fungi are known for the prominent gelatinous telia horns produced from galls formed on juniper trees (1, 97). In contrast to most other heteroecious rust fungi, the Sp-hosts of *Gymnosporangium* spp. are gymnosperms, whereas their Ga-hosts are dicots belonging to the Maloideae (e.g., apple and pear trees). They overwinter as sporothalli within host tissues and produce teliospores in the spring and colonize the Ga-host later in the season (3). No reference genome is available for a species of *Gymnosporangium*, possibly because of the very large estimated genome size (98). RNAseq studies were conducted at different life stages of different *Gymnosporangium* spp. Two studies report on transcript expression from telia of *Gymnosporangium asiaticum*, *Gymnosporangium japonicum*, and *Gymnosporangium yamadae* (96, 97). RNAseq transcriptomes reveal the expression of subsets of genes specific to each species (150–200 specific genes when compared to available rust genomes in databases). Interestingly, these three species all infect species of *Juniperus* but present some differences in the organ infected (leaves, branches, or trunk/bark). The transcriptome profiles are quite similar in the three species and most of the annotated cellular categories showed similar distributions (95). Approximately 700–800 secreted proteins are predicted in each species. Hemicellulose cleaving enzymes of the GH26 family and members of GH16, GH17, and GH18 families are among the most highly expressed genes. Similar GH families were also found among highly expressed genes of other rust fungi (e.g., *C. ribicola* or *P. triticina*) at other stages, suggesting that these CAZyme families can be recruited for different rust spores and their specific roles need to be clarified. RNAseq expression of *G. yamadae*–infecting leaves of the Ga-host was studied in spermatia and aecia 10 and 30 days post basidiospore inoculation, respectively (95). The overall gene profiles for these annotated cellular categories are rather similar to the teliospore stage of other rust infection transcriptome studies. This observation strengthens the idea of a core genetic program expressed by rust fungi during pathogenesis, whatever the host and the stage of the life cycle considered (**Figure 2**). Only a fraction of the transcripts showed a specific expression at the two stages on apple leaves, a significant portion of which encode secreted proteins. A higher proportion of CSEP genes of unknown function were expressed in telia and aecia than in spermogonia and may have a role in host alternation, although their precise role remains to be determined (95). Interestingly, conserved CSEP genes of the Rust Transferred Protein 1 (RTP1) family, previously reported to be expressed on the Sp-host of other rust fungi (33, 62, 75), are also expressed on the Ga-host of *G. yamadae* (95).

### 4.4. *Melampsora larici-populina*

*M. larici-populina*, the poplar rust fungus, is heteroecious and macrocyclic and alternates on poplar (Sp-host) and larch (Ga-host). This fungus causes important epidemics and economic damage

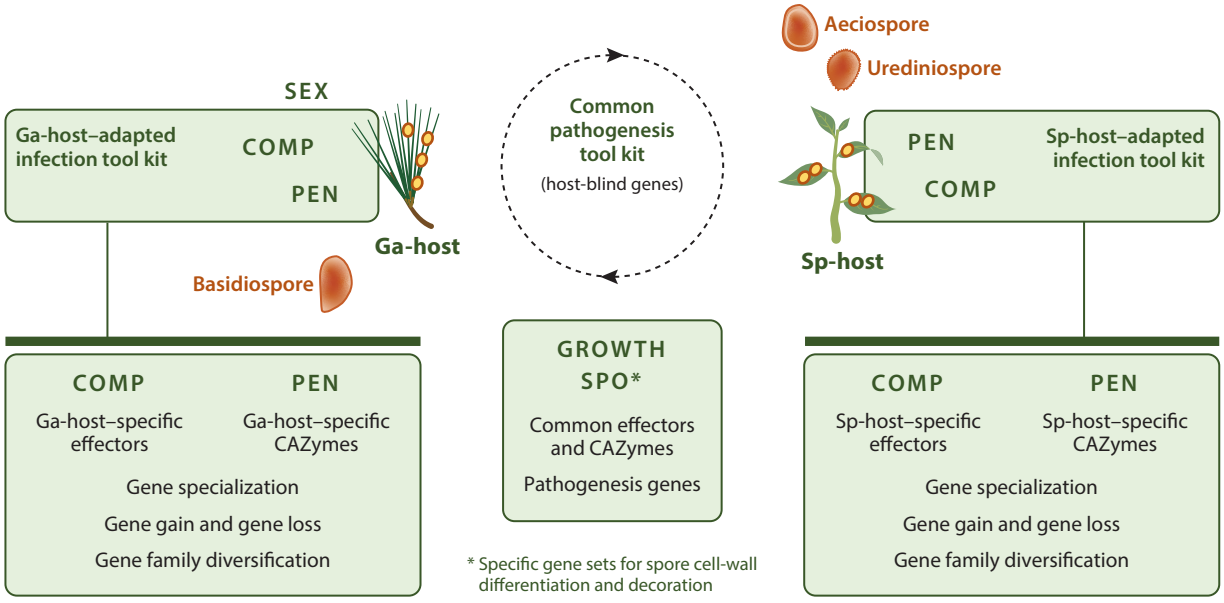



**Figure 2**

Infection-related genetic programs of a heteroecious rust fungus life cycle. Schematic representation of the heteroecious life cycle of rust fungi highlighting major genetic programs related to host adaptation and virulence expressed during infection of the gametothallus host (Ga-host) and the sporothallus host (Sp-host). The adapted infection tool kits and the common pathogenesis tool kit required for realizing infection on the host plants are detailed in corresponding boxes. Abbreviations: CAZymes, carbohydrate-active enzymes; COMP, compatibility; GROWTH, biotrophic growth; PEN, penetration; SEX, mating and fertilization; SPO, sporulation.

in poplar plantations (43). The sequencing of the poplar genome and *M. larici-populina* enabled early transcriptomics, including time-course infection experiments of the Sp-host and microdissection of uredinia, resting and germinating urediniospores, and telia on decaying poplar leaves (**Table 1**; 30, 40, 41). *M. larici-populina* basidia produced from teliospores on dead poplar leaves in spring after overwintering and spermogonia and aecia on the alternate Ga-host were studied by RNAseq (63) (**Table 1**). At each surveyed life stage, specific groups of CSEPs can be identified. The dissection of the infection process on poplar leaves reveals expression of CSEP genes in successive waves in a coordinated temporal scheme (30). Laser microdissection of uredinia confirmed that different genetic programs are expressed in the spore-forming area and haustoria-enriched area (41). Some CSEP genes show late expression in uredinia when new spores are produced in the host plant. These results highlight the complexity of the processes at play during infection from early colonization to late sporulation (**Figure 1*b***). Stage-specific CSEPs are also expressed in early differentiating telia (40). A recent study has shown that fungal pathogen CSEPs can have a role in interacting with surrounding microorganisms (84). It is tempting to speculate that CSEPs expressed in the telia of rust fungi are similarly involved in interactions with microbes present in decaying plant tissues (**Figure 1*b***). Transcriptome profiles of spermatia and aecia collected on larch needles are similar to each other, but they differ from basidia (63). This suggests that an adapted genetic program may be expressed for early colonization of the Ga-host. An enrichment in CSEP genes is noted among specifically highly expressed genes on either the Ga-host or Sp-host, representing adapted gene sets for the host-specific compatibility tool kits (**Figure 2**). CAZyme-encoding genes are also expressed at penetration-related stages

(63). These genes are limited in number and the vast majority are expressed similarly during infection of both host plants, illustrating once more the expression of a common pathogenesis gene set during biotrophic growth, independent of the host (63, 72). A hallmark of the genome of *M. larici-populina* is the expansion of multigene families, which represent almost two-thirds of the secretome (42). Host-specific CSEP genes are equally found among singletons and in gene families, and when large gene families are scrutinized, a majority of the gene members are usually expressed on just one of the two hosts. This is particularly striking for SP family 1, with 111 members all preferentially expressed on the Ga-host. A few families present a dual profile with some members expressed on the Ga-host and others on the Sp-host (63). Such a result raises interesting questions about the mechanisms that drive diversification and specialization in the course of evolution to evade the host immune system or adapt to host susceptibility genes targeted by CSEPs.

**Biogenic radiation:** a series of host jumps to ecologically codistributed but not necessarily related host plants

### 4.5. *Puccinia striiformis* f. sp. *tritici*

*P. striiformis* f. sp. *tritici* is a heteroecious and macrocyclic rust fungus infecting wheat as its Sp-host and barberry as its Ga-host. It causes the stripe rust disease, which leads to severe economic damage to wheat cultivation (81). This fungus has recently attracted attention from different groups focusing on either genomics and transcriptomics (16, 24, 48) or the functional analysis of CSEP genes (reviewed in 62, 93, 94). Several genome references of different isolates are available and have paved the way for transcriptomic studies (15, 16, 22, 82, 112) (**Table 1**). As with the poplar rust fungus, time-course series of wheat leaf infection have demonstrated the expression of waves of CSEP genes during biotrophic growth, indicating that temporal regulation of the infection genetic program may be conserved in rust fungi (24). A recent study addressed for the first time the transcriptome profiling by RNAseq during early infection stages of *P. striiformis* f. sp. *tritici* on its two host plants (111) (**Table 1**). Gene expression profiles were collected at one and two days post urediniospore inoculation of wheat leaves and at three and four days post basidiospore inoculation of barberry leaves. More CSEP genes are expressed on the Ga-host barberry or on both hosts than on the Sp-host alone. However, the CSEP genes account for a larger proportion of the genes expressed on the Sp-host wheat. As with *M. larici-populina*, the proportion of CSEPs among genes expressed on a single host is greater than in genes expressed commonly on the two hosts. A few CSEPs selected from the Ga-host–specific set were assayed in a heterologous plant system and showed the capacity to interfere with plant immune functions. This result suggests that such CSEP genes may be involved in host specificity (111). Different subsets of CAZymes are also specifically expressed at early stages of infection on the two host plants reflecting possible adaptation to the host tissue composition. Pectinase-encoding genes are particularly highly expressed on the Ga-host, whereas they are not on the Sp-host. Interestingly, cutinase genes are strongly expressed on wheat leaves, although penetration is achieved through stomata. Zhao et al. (111) propose that this expression pattern underlies the adhesion process on the host surface, as reported in previous studies (23, 104). Moreover, these authors discuss the higher number of Ga-host– versus Sp-host–specific expressed CSEP genes, speculating on a more ancient association with the Berberidaceae Ga-host for ancestors of this rust species, from which *P. striiformis* f. sp. *tritici* evolved following a more recent switch to wheat as an Sp-host (111), fitting the model of rust fungal evolution wherein biological specialization drives rust fungal coevolution with the Ga-host and biogenic radiation with the Sp-host (2, 3).

Altogether these studies illustrate that infection-related transcriptomes of heteroecious rust fungi are composed of commonly expressed genes in the two host plants, allowing expression of a conserved rust pathogenesis program along with limited subsets of host-specific genes on each

host (**Figure 2**). The global set of commonly expressed genes—retrieved independently of the rust species or spore stage—suggests that these genes participate in basic cellular functions that support biotrophic growth, nutrition, and sporulation and pertain to host-blind, infection-related functions (72). Indeed, some CSEP gene families are conserved in rust genomes and are similarly expressed on different hosts in different heteroecious rust species (e.g., the RTP1 family) (33, 75, 95). The host-specific gene subsets are enriched in CSEP, which may compose the initial tool kit to establish compatibility specifically on each host. The specific expression profiles unraveled for a few CAZyme genes tend to indicate that a penetration tool kit is likely required to ensure adhesion and/or entry inside the host tissue and specific GH genes may be associated with different modes of penetration, e.g., through stomata or the epidermis. In all the studies reported here, only a few time points were covered on the Ga-hosts. A more detailed dissection of the infection process in the Ga-host is still needed to better define the differences and commonalities between the compatibility tool kits required for infection of the two hosts as well as how coevolution with their divergent host plants drives rust evolution. Similarly, transcriptomics at later stages of interaction when new spores are formed or in early versus late teliospores could help identify genes commonly involved in spore differentiation and those specific to each spore type.

## 5. CONCLUSION AND FUTURE DIRECTIONS

The complex life cycle of heteroecious rust fungi is marked by a succession of events that is constrained by the host plants and environmental conditions. Genomics has provided evidence of gene diversification and the presence of a large repertoire of CSEPs that are likely key to establishing a successful interaction with both host plants. Extensive transcriptomic studies illustrate that such genes are fine-tuned at the genetic level and that reduced gene subsets are specifically required for infection of either the Ga-host or Sp-host. These subsets have been selected during the course of evolution, and they reflect adaptation to the different host plants. Considering the specific events occurring on each host—i.e., entering the host tissue, interplay with the host immune system, and interaction with susceptibility genes—the evolutionary pressure from each host may differ and the specialization and diversification of the penetration and compatibility gene tool kits on the Ga-host and the Sp-host are illustrations of this evolution in Pucciniales. The fertilization stage, which occurs on the Ga-host, is probably an additional source of disequilibrium between the evolution of rust fungi and their hosts, which may explain why a stronger coevolutionary signal was observed for rust fungi and their Ga-hosts than for Sp-hosts (2). Also, adaptation of the host plant to its own environment as well as host extinction events can shape the evolution of the Pucciniales. When all the events depicted in **Figure 1*b*** are taken together, the transcriptomics surveys conducted thus far are still limited. Only the development of asexual urediniospores on the Sp-host has been extensively surveyed with appropriate time courses to dissect successive genetic programs expressed during infection. Waves of expression of distinct gene subsets have been observed, and it remains to be determined whether a similar expression regulation is achieved on the Ga-host and whether it is orchestrated by conserved genetic determinants such as master regulatory genes in different rust families.

Nevertheless, the breakthrough studies reported in this review are clearly paving the way to dissect the genetic tool kits underlying host adaptation in rust fungi. Although transcriptomic surveys are merely descriptive, they are also a unique source of information of utmost importance for studying the unique biology of obligate biotrophs like rust fungi. RNA-seq is indeed an approach of choice to address long-lasting questions about host adaptation in the Pucciniales.

## FUTURE ISSUES

1. Genome sequencing and transcriptome analyses of stages on the Ga-host and Sp-host are needed in more heteroecious species, including tropical rust fungi, to better define the gene subsets composing the penetration and compatibility genetic tool kits of rust fungi and their host specificity and identify temporal signals from core tool kits.
2. Genome sequencing and transcriptome analysis of heteroecious rust species that possess the exact same Ga-host or Sp-host should reveal whether similar penetration and compatibility genetic tool kits were selected during their evolution.
3. Genome sequencing and transcriptome analysis of autoecious and macrocyclic rust fungi during infection of their unique host plant should help to determine whether they possess reduced genetic tool kits for penetration and compatibility and whether these tool kits are expressed similarly on a unique host at stages that correspond to infection of Ga-hosts and Sp-hosts in heteroecious rust fungi.
4. Genome and transcriptome analysis of infection in rust fungi that possess reduced life cycles for comparison with macrocyclic autoecious and heteroecious rust fungi, especially correlated species pairs (80), may reveal whether members of correlated pairs possess diversified tool kits for infection, reflecting the capacity to infect an alternate host or loss of the capacity to perform such an infection (thus reflecting possible extinction of an alternate host).
5. In-depth transcriptome studies of time-course infections of both the Ga-host and Sp-host should be performed to capture all developmental transitions, using microdissection capture or micromanipulation techniques and single-cell expression approaches to overcome the limitations of the small number of cells from which RNA can be isolated.
6. Once such genetic tool kits have been established in several species spanning various families, more precise phylogenetic analyses of host-specific genes and host-blind genes should reveal evolutionary processes that shaped diversification in the Pucciniales.

## DISCLOSURE STATEMENT

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