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Integrated multi-omic analyses in *Biomphalaria-Schistosoma* dialogue reveal the immunobiological significance of FREP-SmPoMuc interaction

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Abstract

The fresh water snail *Biomphalaria glabrata* is one of the vectors of the trematode pathogen *Schistosoma mansoni*, which is one of the agents responsible of human schistosomiasis. In this host-parasite interaction, co-evolutionary dynamic results into an infectivity mosaic known as compatibility polymorphism. Integrative approaches including large scale molecular approaches have been conducted in recent years to improve our understanding of the mechanisms underlying compatibility.

This review presents the combination of integrated Multi-Omic approaches leading to the discovery of two repertoires of polymorphic and/or diversified interacting molecules: the parasite antigens *S. mansoni* polymorphic mucins (SmPoMucs) and the *B. glabrata* immune receptors fibrinogen-related proteins (FREPs). We argue that their interactions may be major components for defining the compatible/incompatible status of a specific snail/schistosome combination

Keywords

Compatibility polymorphism ; *Schistosoma mansoni* ; *Biomphalaria glabrata* ; FREPs ; SmPoMucs

Introduction

Host-parasite interactions are dynamic biological systems in which the host's defense mechanisms face the parasite's infectivity mechanisms (Combes, 2000; Howard and Jack, 2007). The coevolution of hosts and parasites involves huge reciprocal selective pressures on both partners. Therefore, in natural environment, all living organisms have developed defense systems capable of recognizing and activating efficient effectors to eliminate most of the pathogens encountered during their life span.

In invertebrate host/parasite interactions, invariable germ-line encoded or somatically diversified pathogen recognition receptors (PRRs) that recognize a set of pathogen associated molecular patterns (PAMPs) appear to be the ultimate outcome of this molecular arms race to limit pathogen invasion (Medzhitov and Janeway, 1997). These polymorphic and/or diversified receptors have been identified in echinoderms (SRCR or Sp185/333 of sea urchin (Ghosh et al., 2010; Pancer, 2000; Pancer et al., 1999), insects (Dscam of *Drosophila melanogaster* and Anopheles gambiae (Dong et al., 2006; Watson et al., 2005), crustaceans (Dscam of *Daphnia* (Brites et al., 2008) or *Litopenaeus vannamei* (Hung et al., 2013), and molluscs (FREPs of *Biomphalaria glabrata* (Zhang et al., 2004)). These studies indicated that these putative immune receptor variants vary considerably between individuals, yielding an enlarged repertoire of putative recognition molecules. We can speculate that large and individual repertoires of immune receptors might confer the immune recognition specificity of invertebrate immune systems and explain in part, the issue of host-pathogen compatibility.

The present chapter explores the molecular interactions between *Schistosoma mansoni* and the snail *Biomphalaria glabrata* that provides a model of choice in evolutionary biology for investigating host-parasite co-evolutionary dynamics and the mechanisms of invertebrate innate immune response (Lockyer et al., 2008; Mitta et al., 2012; Mone et al., 2011; Pinaud et al., 2016; Zhang et al., 2004).

The genus *Schistosoma* contains around twenty species, three of which, *S. haematobium*, *S. japonicum*, and *S. mansoni* are the principal agents of human schistosomiasis that represents the second most prevalent parasitic disease in the world affecting 200 million people and killing 200,000 persons every year (Chitsulo et al., 2004). Adult worms mate in a vertebrate host venous system.

They produce eggs that reach the aquatic environment by passing through the vertebrate intestinal or bladder wall. In water, eggs hatch and release a miracidium that specifically infects fresh water snail species. Schistosomes, like all trematode species, needs molluscs as intermediate hosts to carry out part of their life cycle before infecting humans. Inside snail tissues, the miracidium turns into primary sporocyst (Sp1) that gives sequentially a secondary sporocyst (Sp2) and then cercaria by asexual multiplication. After leaving the snails, cercariae actively look for a definitive vertebrate host to infect (Yoshino et al., 2016).

In South America, *B. glabrata* snails have important medical and epidemiological impacts due to their role as the main vector of *S. mansoni*, the agent of intestinal Schistosomiasis. Consequently, a better understanding of the molecular interactions and the compatibility (susceptibility/resistance status) between *B. glabrata* and *S. mansoni* would contribute to the discovery of new ways to prevent and/or control Schistosomiasis diseases in the field (Tennessen et al., 2015b).

A high level of specificity exists between the *Schistosoma* miracidia and the snail intermediate hosts. This specificity could be explained by the suitability/unsuitability between the physiological and biochemical states of the host and the parasite. However, the main factor supporting this specificity is the host immune system and the ability of the parasite to cope with this immunity and/or to circumvent it (Sapp and Loker,

2000; Van Der Knaap and Loker, 1990). Thus, during specific interactions, either the host wins and the parasite is eliminated or the parasite wins and infects the host. This aspect of host/parasite interactions is described under the spelling compatibility polymorphism (Mitta et al., 2012) (see Fig. 5). Interestingly, investigation of the fate of miracidium parasites after penetration in the same host snail revealed that the infected snail can contain both developing primary sporocysts and encapsulated sporocysts located side by side within its tissues (Theron et al., 1997, 2014). The phenotype (infective vs. uninjective) of a parasite is expressed as a function of the genotype of the particular host that it enters. Likewise, the phenotype (susceptible vs. unsusceptible) of a host is expressed as a function of the parasite genotype it harbours (Theron et al., 2014). Compatibility polymorphism is thus based on a matching-phenotype model in which the compatibility is tested independently for each entering miracidium and each exposed snail (Theron et al., 1997, 2014). Numerous studies using isolates or strains of *Biomphalaria* snails from diverse geographic localities that have been exposed to sympatric or allopatric strains of *S. mansoni* (see Fig. 5) have emphasized the high compatibility polymorphism that characterizes this host-parasite matching phenotype (Basch, 1976; Morand et al., 1996; Richards, 1975; Richards and Shade, 1987; Theron et al., 2014).

To go further in the understanding of this matching-phenotype, we must decipher the mechanisms through which snails and schistosomes interact. Integrative approaches are needed to combine a diversity of methodologies required to address the different levels of the molecular hierarchy potentially involved in host-pathogen compatibility and unravel the complexity of such biological systems (Fig. 1). The recent development of comparative genomic, transcriptomic, proteomic and epigenomic approaches, referred as multi-omic analyses, paved the way to a better understanding of the underlying molecular processes of snail/schistosome compatibility (Fig. 1). The molecular mechanisms underlying the high level of compatibility polymorphism observed in these interactions are based on the formation of immune complexes between *B. glabrata* fibrinogen related proteins (FREPs) and the *S. mansoni* polymorphic mucins (SmPoMucs), which were previously demonstrated as the most promising candidates involved in snail/schistosome compatibility (Dheilly et al., 2015; Mitta et al., 2012; Mone et al., 2010). On one hand, SmPoMucs are mucin-like molecules displaying a domain containing a variable number of tandem repeats (VNTR). Mucins are known to be involved in host-parasite interplay (Buscaglia et al., 2006; Hicks et al., 2000; Rathore et al., 2005; Theodoropoulos et al., 2001). SmPoMucs could act as pathogen associated molecular patterns (PAMPs) and would be the initiator of snail host immune recognition (Mone et al., 2010). On the other hand, fibrinogen-related proteins (FREPs) constitute a highly diversified family of soluble immune pathogen recognition receptors (PRRs) from *B. glabrata* (Adema et al., 1997; Mitta et al., 2012; Mone et al., 2010). FREPs combine one or two N-terminal immunoglobulin superfamily (IgSF) domains with a C-terminal fibrinogen-related (FBG) domain and participate in binding trematode parasites (Mone et al., 2010; Zhang and Loker, 2004; Zhang et al., 2008a).

In this review, the roles played by FREPs and SmPoMucs in compatibility polymorphism has been compiled from integrated “Multi-Omic” approaches conducted on different snail and schistosome combinations displaying different compatibility phenotypes.

2. What have we learned from genomic approaches on FREPs and SmPoMucs?

Genetic studies conducted in the *S. mansoni*/*B. glabrata* model demonstrated that compatibility was heritable and could be selected in the laboratory either for susceptibility/resistance of the snail or for infectivity of the parasite (Davies et al., 2001; Richards, 1975; Richards et al., 1992; Richards and Shade, 1987; Webster and Davies, 2001), although the underlying genetic and molecular mechanisms were largely unknown. Compatibility may reside in a concordance of genetically determined phenotypes in snail and schistosome (Basch, 1975, 1976), a reduced set of parasite phenotypes in a population generally match with solely a fraction of the phenotypes present in the host strain (Theron and Coustau, 2005).

The first genome assembly and annotation of *S. mansoni* was published in 2009 (Berriman et al., 2009). It is composed of approximately 363 megabases in which 11,809 putative genes encoding 13,197 transcripts were identified. The functions of only a small percentage of these genes have been characterized so far. Moreover, the assembly was not perfect considering that 40% of the genome was repetitive and that the annotation mostly focused on possible therapeutic target genes against adult worms. This genomic resource has contributed to functional studies (Protasio et al., 2013), and was particularly helpful for investigating the mechanism of resistance to specific drugs (Valentim et al., 2013). Nevertheless, it has never been used to identify which genomic regions were under selection for the interplay with its intermediate host snail.

Additional efforts have been made in the characterization of gene loci in the *B. glabrata* genome associated with resistance or compatibility to *S. mansoni* infection. A first study using random amplified polymorphic DNA (RAPD) approach on several *B. glabrata* strains displaying a range of compatibility toward the same parasite isolate led to the identification of two heritable markers related to the resistance phenotype of adult snails (Knight et al., 1999). As these RAPD markers were located in repetitive genomic regions in the *B. glabrata* genome, further attempts to characterize the associated genes were not successful.

However, more recently a restriction site associated DNA sequencing (RADseq) study was conducted using two laboratory lines of snails that have been selected for *S. mansoni* resistance by experimental evolution (Tennessen et al., 2015b). RAD genotyping revealed in both lines a genomic region linked to resistance to the parasite. This region comprised 12 putative noncoding and 15 coding genes, among which 7 presented similarities for single-pass transmembrane proteins. Proteins from this family are implicated in different processes like cell migration, adhesion, growth, and typically act as receptors for extracellular signals (Tennessen et al., 2015b). The authors notably underlined their potential role in the immune system such as B- and T-cell receptors, Fc receptors, major histocompatibility complex (MHC) receptors and Toll-like receptors, which are all membrane-bound receptors recognizing foreign molecules (Tennessen et al., 2015b). Complementary RNAseq analysis revealed that the compatibility phenotype of the snail (susceptible or resistant) was not correlated to the level of gene expression but rather to differences in allele sequences (Tennessen et al., 2015a, 2015b).

Several molecules have been identified and implicated for immunity of *Biomphalaria glabrata* against *Schistosoma mansoni* (for review, see (Coustau et al., 2015)). However, few have been characterized at the genetic or genomic level, including one particular allele of the copper-zinc superoxide dismutase (SOD1) that is associated with snail resistance to *S. mansoni* infection (Blouin et al., 2013; Goodall et al., 2006) and of course the SmPoMuc and FREP molecules described thereafter.

2.1. *The Schistosoma mansoni polymorphic mucins (SmPoMucs)*

The only parasite molecules characterized at the genomic level and implicated in the interaction with the intermediate host snail are the *S. mansoni* Polymorphic Mucins (SmPoMucs) (for review, see (Gourbal et al., 2015)). These molecules were shown to be highly polymorphic at both population and individual level. In a population each miracidium expresses a unique combination of SmPoMucs derived from a limited set of genes. SmPoMucs are encoded by at least 6e9 multi-gene family members (Roger et al., 2008b). These genes are composed of 15 exons: the first two exons of the 50 gene region are considered as the variable region of SmPoMuc genes, while the 13 other exons are considered as the conserved 30 region. Indeed, southern blot experiments revealed that SmPoMucs contained a variable number of repeated exon 2 that never exceeded 20 copies. The different genes were classified in four paralogous sequence groups according to their sequence coding for the conserved 30-terminal part of the gene. All members of group 4 were considered as pseudogenes. Fluorescent in situ hybridization (FISH) allowed locating SmPoMucs genes in four distinct genomic regions of chromosomes 3 and 4. This genomic clustering associated with the presence of pseudogenes strongly supports that gene duplications would be at the basis of SmPoMuc gene diversification.

In SmPoMuc genes, Exon 2 (27 nucleotides) and its flanking introns are organized in 1 kb repeats, each separated by microsatellites. The high level of similarity of these genomic repeats between all members of SmPoMuc multigene family results in the frequent recombination and homogenization of the SmPoMucs genes. Diversification of SmPoMuc molecules is driven by complex genomic mechanisms. Calculation of synonymous to nonsynonymous substitutions ratios (K_S/K_N) and Tajima's neutrality test indicated that purifying selection acts on SmPoMuc genes (Roger et al., 2008b). In these genes, genomic repeats are subjected to concerted evolution and intron sequences are more homogenized than exons that evolve under purifying selection (Roger et al., 2008b). This results in combinatory events observed at the genomic level, probably linked to ectopic recombination and exon exchange between clusters.

The SmPoMucs genes were not present in the current *S. mansoni* genome annotation but can be found by similarity searches. However, the current assembly (v5) around these loci contains potential errors since similarities between genes of this family as well as the genomic repeats prevented their correct assembly into the genome.

2.2. The fibrinogen-related protein (FREP) genes

Several studies have focused on *B. glabrata* fibrinogen-related proteins (FREPs) (for a review see (Gordy et al., 2015)) that have been shown in vitro to interact with the parasite molecules, SmPoMuc (Mone et al., 2010). FREPs are composed of a signal peptide (SP), target signal for extracellular secretion, immediately followed by an immunoglobulin superfamily domain (IgSF), which is finally connected by a length-variable interceding region (ICR) to a fibrinogen domain (FBG). Moreover two classes of FREPs are described, single-IgSF FREPs and tandem-IgSF FREPs, in which IgSF domain are arranged in tandem and separated by a short connecting region (Hanington and Zhang, 2011; Zhang et al., 2001). The two IgSF domains of the tandem-IgSF FREP are quite different in sequence and are not just a repeat of a unique domain. The two IgSF domains of tandem-IgSF FREP are more similar to each other than to the IgSF domain of a single-IgSF FREP (Dheilly et al., 2015).

Fourteen FREP families have been described up to date, mainly from *B. glabrata* M-line strain (Adema et al., 1997; Zhang and Loker, 2003; Zhang et al., 2008a). Families 2, 4 and 14 belong to single-IgSF FREP, while families 3, 7, 12 and 13 belong to tandem-IgSF FREP. The other FREP families couldn't be clearly assigned

to one of these groups, because solely one IgSF or FBG domain has been described for these genes. At the genomic level complete gene sequences are available for only 4 FREP families (2, 3, 4 and 7). The FREP 2 and 4 (single-IgSF) are encoded by 4 exons and 3 introns (Leonard et al., 2001; Zhang et al., 2008a), and FREP 3 and 7 (tandem-IgSF) are encoded by 6 exons and 5 introns. It has been shown that introns from single-IgSF FREPs are composed of repeats that varied in length and number, and that they presented low similarity between FREP2 and FREP4 (Leonard et al., 2001). The FBG region is the most conserved domain, while IgSF and ICR are the most variable domains (Dheilly et al., 2015; Leonard et al., 2001; Zhang et al., 2001; Zhang and Loker, 2004).

To characterize the diversity of FREPs, several approaches have been conducted. Southern blot analysis and PCR with degenerated primers on FBG domains revealed their abundance in *B. glabrata* genome and suggest the diversity of FREPs at this level (Zhang and Loker, 2004). Moreover, other studies have highlighted the capacity of somatic diversification by point mutation, gene conversion and ectopic recombination at the genomic level in all FREP families studied so far. Such diversification events have been reported for IgSF, ICR and FBG domains (Dheilly et al., 2015; Mone et al., 2010; Zhang et al., 2001, 2004). Altogether, these results suggest a high potential of diversification of FREP molecules. The assembly and annotation of the *B. glabrata* genome will provide a powerful expected tool to describe and understand the mechanisms responsible for diversity of FREPs at the genomic level.

Such diversity of FREP/SmPoMuc molecules provides support for the matching phenotype hypothesis and might partly explain the compatibility polymorphism observed between schistosomes and *Biomphalaria* strains.

3. What have we learned from transcriptomic approaches on FREPs and SmPoMucs?

Transcription embodies the first step of gene expression in cells. The absence or presence of specific transcripts at a precise time and/or specific localization in cells might give insights on activated or repressed genes in specific physiological or molecular pathways. With this objective, many techniques have emerged since the 1980s to massively and easily explore the transcriptomic compartment, from suppression subtractive hybridization (SSH) and microarray to massive RNA sequencing (RNAseq).

The history of the discovery and investigation of FREPs and SmPoMuc follows the improvement of next generation sequencing (NGS) methods and benefited from several methods until nowadays. In 2003, Marathon cDNA libraries brought the first information about sequence diversity of FREPs genes family. It also provided evidence for alternative splicing abilities of FREPs in *Biomphalaria* in response to parasite infection (Zhang and Loker, 2003).

Alternative splicing is described as a transcriptomic process to increase the production of host diversified recognition molecules and enhance host's ability to recognize pathogens. This provided the first insights toward the idea of a polymorphism and/or diversification in the FREPs family. In SmPoMuc, a similar pattern of alternative splicing, in addition to aberrant splicing or transsplicing, led to inclusion or exclusion of some parts of the genes that ultimately produce a high level of polymorphism from a restricted number of genes (Fig. 2), allowing to increase the diversity of proteins that are the potential targets recognized by the host immune system (Roger et al., 2008b). Concerning *Biomphalaria* factors, reverse transcription quantitative PCR (RT-qPCR) first revealed the differential expression of FREP family genes in

Biomphalaria following infection with two species of highly infective parasites (*S. mansoni* and *Echinostoma paraensei*) (Hertel et al., 2005). This result gave support for putative roles of FREP2 and FREP4 in *Biomphalaria* defense. Functional invalidations were rapidly developed in *Biomphalaria* system to explore the involvement of specific genes in compatibility phenotypes. The first RNAi (interference RNA) gene knockdown developed in *Biomphalaria* strain involved the injection of double-stranded RNA (dsRNA) into the snail hemolymph or their addition to co-cultures of *Biomphalaria* embryonic cell line (Bge cells) and parasites (Jiang et al., 2006). This technique opens the way to gene inactivation of FREPs. Rapidly, the related molecules in *Schistosoma* have also been explored. The first transcriptomic analysis of SmPoMuc revealed that mRNAs encoding mucin-like proteins were detected at three different developmental stages (i.e., egg, miracidium and primary sporocyst Sp1), particularly at a high-level at the miracidium stage of the parasite (Roger et al., 2008a). Mucins may be involved in the penetration of miracidia into the snail and help during the transition from the free-living miracidium stage to the first intramolluscal stage Sp1 (Theodoropoulos et al., 2001). Comparison between strains of parasites revealed that less-recognized compatible strain (SmBRE) expressed 3-fold less SmPoMuc than the best-recognized incompatible one (SmGH2) (Galinier et al., 2017; Roger et al., 2008a). These observations suggest that mucins can be the target of the recognition by the snail and that diversity of these putative antigenic proteins can increase parasite capacity to evade the host immune pathogen recognition receptors (PRRs). Proteins containing fibrinogen C-terminal domain have been shown to be triggered in response to compatible and incompatible *Schistosoma* infection but also following an infection with irradiated-miracidium (Ittiprasert et al., 2010). Based upon microarray experiments, the variation in FREP gene transcription was analyzed and revealed the over-expression of FREP2, 3, 4 and 6, the under-expression of FREP1, 5, 7, 10, 12 and divergent expression of FREP11 between 12 h and 32 days following *Schistosoma* infection (Hanington et al., 2010). This study provided the first evidence that FREPs constitute a complex immune-related family in which some members are over-expressed while other are underexpressed following the same infection response. The specificity of parasite detection appears to be more efficient when the host possesses a diversified repertoire of differentially-selected PRRs that can be used in response to different pathogens. After microarrays, massive RNA sequencing (RNAseq) with Illumina/Solexa technologies have generated a lot of new information associated with the deeper sequencing resolution. By investigating *B. glabrata* FREPs diversity from a de novo generated transcriptome, Dheilly and collaborators (Dheilly et al., 2015) revealed that within each FREP family member, a significant nucleotide variability was observed in transcripts resulting in polymorphism in the predicted amino-acid protein sequence. These authors showed that the FREP molecules did not show the highest level of transcript variants among lectin family members and also that, FREPs were not the more expressed in naïve snails. This means that putative other immune-related recognition receptor molecules within the large lectin family (including Ig-like fold, C-Type lectin, Leucine-rich repeat, Selectin, Galectin) may increase immune recognition capabilities in *Biomphalaria*. Several Variable Immunoglobulins (VIgGs), CREPs (C-Type lectin related protein) and GREPs (Galectin related protein) sharing IgSF domain with FREPs were also evidence. This indicates the existence of recombination or trans-slicing events between highly-variable molecules belonging to different lectin families. This demonstrates the strong evolutionary pressures playing on those closely-related molecules, which share similar putative immune-related features, which are able to generate additional polymorphism and diversity. Recent RNAseq approach confirmed the role of FREPs during a shift from a cellular to a humoral immune response in the innate immune memory of *Biomphalaria* (Pinaud et al., 2016). Despite an absence of up-

regulation of FREP genes following sympatric infection, a global over-representation of FREPs was observed following immune homologous challenge. Knockdown of FREP2, 3 and 4 genes revealed that the 100% acquired resistant snails became partially susceptible (15%) after a reinfection with the same parasite and confirmed the key role of FREPs among lectin families, while highlighting that additional factors might complement FREP function in *Biomphalaria* innate immune memory.

4 What have we learned from epigenomic approaches on FREPs and SmPoMucs?

Heritable phenotypic diversity has traditionally been attributed to genetic diversity. We know today that this view of heritability is incomplete and must include other diversity-generating mechanisms that can be heritable such as those regulated by epigenetic elements. The epigenetic changes refer to a set of molecular processes that can affect gene expression by non-coding RNA, through methylation of nucleic acids in DNA, chemical modification of histones and re-localization of a locus inside different nucleus territories (Keung et al., 2015). These epigenetic factors have been shown to be under the influence of the environment and their modifications can have consequences on the chromatin compaction and therefore may affect gene expression. In this sense, epigenetic modifications can result in new phenotypes that might be adaptive. The epigenotype is mitotically and to some degree meiotically heritable, but unlike in the genotype, changes in the epigenotype are generally reversible (Keung et al., 2015). Therefore, the epigenetic information system (EIS, epigenetics) can provide heritable, novel phenotypes that do not rely on variation in DNA sequence (Geoghegan and Spencer, 2012). If this EIS influences the capacity to generate different phenotypes, both the better adapted phenotype and the capacity to generate this phenotype will be selected and conserved in the next generation (Gomez-Diaz et al., 2012). This hypothesis has been investigated in the context of the arm race hypothesis that occurs during the interaction between *S. mansoni* and its intermediate host *B. glabrata*. The availability of the genome for both partners (Berriman et al., 2009) (*Bg* genome submitted: see Vector Base website (<https://www.vectorbase.org/>)) and the recent advances in genome-wide analysis have allowed the first description of different bearers of epigenetic information in different contexts. Histones are highly conserved proteins and commercial drugs and antibodies are usable to target these proteins and study their modifications in diverse species. Chromatin immunoprecipitation (ChIP) analysis targeting these modified histones has been applied to *S. mansoni* and allowed the visualization of the position of modified histones in vivo in different environmental contexts and at different developmental stages of this parasite (Picard et al., 2016; Roquis et al., 2015). Several studies have demonstrated how histone modifications in *S. mansoni* are engaged to generate phenotypic plasticity during the interaction with its intermediate host. SmPoMuc are highly conserved genes in their coding region and upstream putative promoter regions. Despite this high degree of conservation observed between compatible and incompatible strains of *S. mansoni*, their patterns of expression strongly differ between these two strains (Roger et al., 2008a). ChIP analysis revealed that different modified histones are present in the upstream regulatory regions of the SmPoMucs genes which could be responsible for an epigenetic-based regulation of their expression (Perrin et al., 2013). Other studies further showed that treatment of the parasite with drugs that target histone acetylation led to an increased compatibility with the snail (Fneich et al., 2016). The snail environment has also been demonstrated to be an epigenome modifier itself as infection of allopatric and sympatric snails with genetically identical *S. mansoni* resulted in the generation of different epigenotypes in each genetically identical parasite (Roquis et al., 2016). Furthermore, *S. mansoni* is a typical organism with a tRNA methyltransferase DNMT2 for which DNA methylation is really low and has been controversial (Raddatz et

al., 2013). DNA methylation can be studied by a large panel of techniques such as bisulfite conversion (BS) assay that allows studying cytosine methylation at the single base resolution (Grunau et al., 2001), methylation-sensitive amplification polymorphism (MSAP) (Reyna-Lopez et al., 1997) and immunological and cytochemical approaches based on the use of conserved antibodies. By combining some of these different methodologies, Geyer et al. (2011) studied methyl-5-cytosine (m5C) in *S. mansoni* and linked the presence of the cytosine methylation machinery to platyhelminth oviposition processes (Geyer et al., 2011). Finally, different works reported stage and sex-specific expression of several non-coding RNAs, which indicate that they might be involved in parasite development. On the side of the intermediate host, *B. glabrata* epigenome has also been shown to be under the influence of the environment (Knight et al., 2016). DNA methylation and locus topography have been shown to be modified in response to *S. mansoni* infection (Arican-Goktas et al., 2014; Knight et al., 2016). A methylome of *B. glabrata* has been generated by BS-Seq analysis (Cosseau et al. unpublished data and Fig. 3). This snail displays a typical invertebrate mosaic-like DNA methylation pattern, with highly methylated regions, predominantly in the CpG context, interspaced with poorly methylated regions. Unlike plants and mammals, the role of DNA methylation for regulation of gene expression is controversial in invertebrates and other bearers of epigenetic information such as small RNA, histone modifications and locus position in chromosome territories certainly deserve further attention to elucidate the involvement of epigenetic regulation events on the observed patterns of gene expression. FREP genes are typical phenotypic variants for which we anticipate an epigenetic-based gene regulation. Such a regulation is expected in the case of the generation of phenotypic diversity followed by natural selection upon environmental changes (Cortes et al., 2012). FREPs genes in *Biomphalaria glabrata* are mainly localized in low methylated regions (Fig. 3). The absence of DNA methylation in these regions has been hypothesized to contribute to stochastic transcriptional opportunities (Gavery and Roberts, 2014), which is typically what is expected to explain part of the transcriptional diversification observed in highly diversified FREP gene families. These FREP genes are therefore candidates for which valuable tools such as ChIP-assays against modified histones will be worth pursuing. This will certainly open new perspectives and improve the knowledge of the sophisticated molecular pathways involved in the host parasite interplay.

5 What have we learned from proteomic approaches on FREPs and SmPoMucs ?

Proteomic approaches allow investigating and characterizing features of immune-related actors by focusing on the last form of the gene expression process, i.e. protein that can explain the phenotype. Although genomic, transcriptomic and epigenetic approaches can inform on the sequence diversity at the gene and transcript level and on gene expression regulation, only proteomic approaches can provide information about the molecular interaction between proteins from hosts and pathogens and give insights into their function. This can come from characterization by gel-based approaches (SDS-PAGE, 2D-PAGE) or gel-free approaches (Label-Free protein sequencing) of entire proteomes coupled with tandem mass spectrometry, or by analyzing post-translational modifications of proteins, protein localization in cells, organs or tissues and/or by analyzing protein/protein interactions. All these approaches have been used in the *S. mansoni*/*B. glabrata* interaction to identify parasite antigens and the potential corresponding pathogen recognition receptors in the host's hemolymph.

Following snail infection by *S. mansoni*, incompatible miracidia are recognized, encapsulated and killed in few hours, while compatible miracidia remain unaffected. This observation suggests constitutive antigenic

differences between those strains of parasite. Thus, a global comparative proteomic approach has been conducted to characterize these antigenic differences (Roger et al., 2008a, 2008c). The main difference identified corresponded to SmPoMucs that exhibited a pronounced qualitative and quantitative polymorphism between compatible and incompatible parasite strains (Roger et al., 2008c). SmPoMuc were further investigated (Roger et al., 2008a, 2008c) and the data obtained showed that these proteins are: (i) only expressed at larval stages interacting with the mollusc, (ii) located in the apical gland of miracidia and sporocysts, (iii) secreted and released in excretory-secretory products and finally (iv) displayed a high degree of polymorphism at the protein primary structure level and (v) are highly glycosylated. The analysis of the global glycosylation status of SmPoMucs, for both strains through chemical deglycosylation experiments, highlighted higher glycosylation levels for variants of the incompatible strain as compared to compatible strain (Roger et al., 2008a). All of these characteristics make SmPoMucs key candidates of the compatibility polymorphism and matching phenotype process, being potential pathogen antigens recognized by the host recognition receptors.

Mucins have indeed already been identified as important actors of the host-parasite interaction (Theodoropoulos et al., 2001), FREPs were originally discovered as circulating glycoproteins present in the plasma of *B. glabrata* and able to agglutinate rabbit erythrocytes (Boswell and Bayne, 1984). This agglutination was shown to be inhibited when carbohydrates were pre-incubated with plasma before the addition of rabbit red blood cells, suggesting the key role of sugar in this interaction. Further studies demonstrated that an agglutination reaction also occurred when *Biomphalaria glabrata* plasma was exposed to sporocyst of trematode parasites species (*S. mansoni* and *Echinostoma paraensei*) (Couch et al., 1990; Loker et al., 1984, 1994). Furthermore, the intensity of the reaction independent of the snail strain used regarding the infection (naïve or infected) and its resistance (susceptible or resistant) toward the parasite considered (Bayne et al., 1986; Couch et al., 1990; Loker and Hertel, 1987). These agglutination factors were finally demonstrated to precipitate *E. paraensei* secretory/excretory products, which later turned out to contain FREPs (Adema et al., 1997; Zhang et al., 2001).

To shed light on the molecules from the host that specifically recognize parasite proteins and therefore trigger the appropriate immune response, an interactome approach has been performed on the *Biomphalaria-Schistosoma* host-pathogen system. In addition to several enzyme inhibitors, hormones and metalloenzymes, two families of lectins from *B. glabrata* were identified as able to interact with *Schistosoma* proteins: several galactose binding lectin-like proteins and FREPs (Mone et al., 2010). Moreover, many different proteins from the parasite interacting with host proteins were also identified including three glycoprotein families that are integral membrane protein Sm23, secretory glycoprotein k5 and SmPoMuc (Mone et al., 2010). To go further, co-immunoprecipitations were mandatory to demonstrate which snail protein(s) bind to SmPoMucs. CO-IP assays were thus conducted using proteins from *Biomphalaria* hemolymph, parasite extract containing SmPoMuc and antibody raised against C-terminal part of SmPoMucs. This revealed three proteins directly interacting with SmPoMuc include an alpha amylase, a thioester-containing protein (TEP) and a specific protein from the FREP family, FREP2 (Mone et al., 2010). It was hypothesized that TEP would interact with the complex primarily formed by FREP2 and SmPoMuc but the mechanisms and the binding sites involved in such an immune complex are currently unknown (Gordy et al., 2015; Mone et al., 2010).

Finally, in *B. glabrata*, it has been hypothesized that these PRR molecules could serve as complementary or collaborative recognition factors that interact with each other to form complexes (Adema and Loker, 2015).

Among them, FREPs have been shown by western blot analysis to occur in *Biomphalaria* hemolymph as multimers of high molecular weight although the mechanism responsible for this multimerization and its importance in the role of FREP-based pathogen recognition has yet to be investigated (Adema et al., 1997; Gordy et al., 2015; Zhang et al., 2008b).

Nevertheless, such multimerization could be an additional process for increasing the mollusc pathogen recognition repertoire, by generating a wide range of multimers each with a different level of parasite antigen specificity. As such, it could increase the potential for specific recognition of parasite molecules, allowing a high capacity of immune system to recognize non-self and to trigger specific anti-pathogen innate immune responses (Adema et al., 1997; Schulenburg et al., 2007).

FREPs diversification would have been the evolutionary response of host to maintain its recognition capacity and could be engaged with hemocytes to mediate phagocytosis after parasite recognition (Hanington and Zhang, 2011; Peterson et al., 2009). These results highlight how, even at the protein level, the interaction between FREPs and SmPoMuc could be at the basis of the process of compatibility between different strains of *Schistosoma* and *Biomphalaria* (Coustau et al., 2015; Mitta et al., 2012).

6. A multi-omic integrative view of FREPs/SmPoMucs compatibility polymorphism

The molecular mechanisms underlying the high level of immune specificity observed in these interactions are based on the formation of complexes between a specific set of highly polymorphic or diversified immune recognition molecules named the fibrinogen-related proteins (FREPs) from *B. glabrata* and polymorphic parasite's glycosylated mucin molecules, named *Schistosoma mansoni* polymorphic mucins (SmPoMucs) (Dheilly et al., 2015; Mone et al., 2010). Results obtained from “Omic” approaches on these two candidates were summarized in the Fig. 4 for different sympatric snail and Schistosome combinations displaying different levels of compatibility (see legend of Fig. 4 for details on *S. mansoni* and *B. glabrata* strain origins and phenotypes).

We found that the polymorphism and expression levels of FREPs and SmPoMucs would be linked to the compatibility level between *S. mansoni* and *B. glabrata* (Galinier et al., 2017). In BgBAR/SmLE interaction, snail hosts presented a low diversity but a high level of expression of FREPs while the parasite expressed low levels of highly diversified SmPoMucs (Fig. 4). In this context, the increased diversification combined with the decrease expression levels of the SmPoMucs glycoproteins could allow the parasite to escape from being recognized by FREP immune receptors. The same strategy could also be described for the BgVEN/SmVEN interaction, but as the host has a low expression of FREPs, the recognition selective pressures on the parasite were reduced and thus a moderate expression of SmPoMucs has been maintained. In this case, the parasite invests more in SmPoMuc diversification than in reduction of expression to circumvent recognition. A totally different strategy could be observed in BgBRE/SmBRE interaction. In this case, the snail host presents a high diversity and low expression of FREPs and the parasite a low diversity and low expression of SmPoMucs. We could hypothesis that it could be difficult for the parasite to diversify the SmPoMucs more than the host FREP molecules (BgBRE snails exhibited the highest FREP diversity) and thus parasite would reduce SmPoMuc expression to limit recognition. Finally, in BgGUA/ SmGH2 interaction, intermediate patterns can be observed, with moderate diversity/moderate expression of host FREPs and moderate diversity/high expression of parasite SmPoMucs. In this case, SmPoMucs were highly expressed and could be easily recognized by FREPs, which could explain the low compatibility observed in this host-parasite couple. On one hand, these results seemed to

demonstrate that a high diversity and a low expression of SmPoMucs are required to enhance parasite infectivity. On the other hand, the diversity of FREPs seems not to be correlated to the compatibility level as it could be expected from an ongoing arms race between FREPs and SmPoMucs. Indeed, BgBRE and BgGUA (highly compatible snail strains) displayed the higher numbers of variants by comparison with BgVEN and BgBAR (low compatible snail strains). The explaining factor seems to be the high level of expression of FREPs that is required to enhance snail resistance toward *S. mansoni*. Allopatric interactions would also confirm these hypotheses; for example, SmVEN parasite succeed in infecting BgBAR but solely at a prevalence of 55% because SmPoMuc diversification is high but expression remains quite high and BgBAR FREPs would manage to form complexes with SmPoMucs. In SmGH2, the moderate diversity and high expression of SmPoMucs appeared to be a deleterious pattern that could perhaps explain the high incompatibility phenotype of this strain (prevalence never exceeds 60%).

Based on these hypotheses, the expected compatibilities between all potential combination of the four strains of hosts and parasites were estimated and compared to the experimentally observed compatibility phenotypes (see table in Fig. 4, experimental prevalence and intensity values are indicated). Interestingly, most of the expected and observed phenotypes were in agreement. Only two cases for which expected phenotypes did not fit with observed phenotypes were identified: (i) SmBRE in BgGUA expected to have a moderate compatibility because of the moderate expression of FREPs from BgGUA that are expected to recognize a large part of SmBRE parasites but is actually compatible (see Table Fig. 4); (ii) SmGH2 in BgBRE expected to have a moderate compatibility because of the low expression of FREPs in BgBRE and the high expression of SmPoMucs in SmGH2, and is actually incompatible (see Table Fig. 4).

This discrepancy between expected and observed phenotypes would be expected. Indeed, if recognition by FREPs seemed to explain most of the compatibility, we know, based on knockdown experiments (Hanington et al., 2012; Pinaud et al., 2016), that FREPs were not the unique determinant of compatibility and that additional factors were involved in *Biomphalaria* immune response. The activation of signalling pathways following recognition leading to hemocytes-dependent phagocytosis or encapsulation and to the release of humoral factors have to be considered.

Finally, the results described above on *B. glabrata*/*S. mansoni* interactions, challenged our view of the role or function of FREP diversification. These results lead us to a totally new unexpected hypothesis: we argue that quantity rather than diversity of FREPs would benefit the overall recognition and elimination of a specific *S. mansoni* pathogen. Local adaptation or co-evolution would indeed favour the selection of a specific subset of FREPs that would be expressed at a high level to recognize a specific subset of SmPoMucs expressed by the parasite.

Nevertheless, it remains unclear to what extent FREP diversity benefits immune function of *B. glabrata* (Adema, 2015). Polymorphism and/or diversity of FREP immune receptor variants yield an enlarged repertoire of putative recognition molecules between individuals. This diversification provides a mechanism to increase host's recognition ability, and as such, the potential for specific recognition of pathogens. Indeed, *Biomphalaria glabrata* snails are confronted with an environment filled with complex changing communities of microorganisms and potential pathogens (bacteria, fungus, yeasts, trematodes, nematodes, etc ...). Therefore, it can be expected that snails have to co-evolve with these pathogen communities and should possess sophisticated recognition systems for dealing with them. FREP diversification would be part of this enhanced immune recognition capabilities used to recognize and eliminate most of these pathogens.

7. And now, what next?

To further understand the interaction between *S. mansoni* and *B. glabrata* different lines of research can be considered.

- 6.1.) The interaction between FREPs and SmPoMucs is part of an immune complex involving different partners, like the thio-ester containing protein (BgTEP) and perhaps other molecules that remain to be characterized (Gourbal et al., 2015; Mone et al., 2010). Identification of such molecules will necessitate coimmunoprecipitation or pull-down experiments with recombinant FREP or TEP incubated with parasite extracts and snail plasma. Another question to solve is to identify the nature of the interacting domains and how molecules interact with one another. To answer this question, approaches such as those described above could be conducted using targeted mutagenesis or truncated recombinant proteins.
- 6.2.) To explore the function and role of highly-variable molecules like FREPs and SmPoMucs, transcriptomic approaches from RT-qPCR to NGS have suffered from technical limitations, notably the lack of genomic resources in non-model species (Dheilly et al., 2014). However, emerging technologies such as single-cell RNA sequencing will provide technical breakthroughs for this field of research. We can expect that individual diversity and expression of both FREPs and SmPoMuc will be unraveled using this technology. For example, sequencing the hemocyte transcriptome for the same individual before and after pathogen infection might give insights on pathogen recognition receptor diversification in an invertebrate model.
- 6.3.) Analysis of transcriptomic data from different strains of *B. glabrata* (Dheilly et al., 2015) strongly suggests the existence of other FREP subfamilies and consequently additional genes that have to be clarified with the use of the future *B. glabrata* genome annotation (Bg genome available at Vector Base website (<https://www.vectorbase.org/>)).
- 6.4.) Whole-genome re-sequencing of parasite populations using PacBio and/or illumina genomic sequencing would be a powerful tool to circumvent the limits of actual genome assemblies for multi-gene families containing genomic repeats. The highlycontiguous de novo assemblies using PacBio sequencing can close gaps in current reference assemblies and longer reads would be useful to sequence through extended repetitive regions (Rhoads and Au, 2015). Whole-genome re-sequencing might permit to explore how patterns of genetic variation change across the genome and detect, based on population genetic tests, the genes under directional and balancing selection (Crellen and Iantorno, 2015; Rhoads and Au, 2015). Genetic approach by quantitative trait locus (QTL) mapping or extreme QTL (X-QTL) mapping using *S. mansoni* genome data would also be useful to identify genomic regions that were under selection for the interplay with its intermediate snail host.
- 6.5.) Methods for the functional characterization of genes of interest (i.e., invalidate and/or restore gene functions to analyze the corresponding phenotype modifications: functional genomics) are still lacking in schistosomes and snails. Except gene knockdown by RNA interference (RNAi) that is successfully used in both animal models, knockout or stable transgenic systems have to be developed now. Development of tools to introduce DNA constructs into adult and larval schistosome stages to express reporter genes have been tentatively developed using particle bombardment, electroporation or virus-based infection strategies. Unfortunately, until now non stable transformations were obtained (Beckmann and Grevelding, 2012; Crellen and Iantorno, 2015; Kines et al., 2008, 2010; Mann et al., 2008; Rhoads and Au, 2015). Such developments in *Biomphalaria* still lag behind. Finally, gene knockout using the CRISPR-Cas genome-editing technique (Komor et al., 2016) provides the ability to edit genomes within living cells or organisms representing a major advance for both host and parasite models.

The existence of polymorphic and/or diversified putative host immune receptors and parasite antigens that vary considerably in pathogen and snail populations no longer has to be demonstrated. However, understanding the function of such polymorphic or diversified molecules in invertebrates will be now a new challenge to solve and will deserve further investigations.

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Figure 1

Integrative multi-Omics approaches of host-parasite interaction.

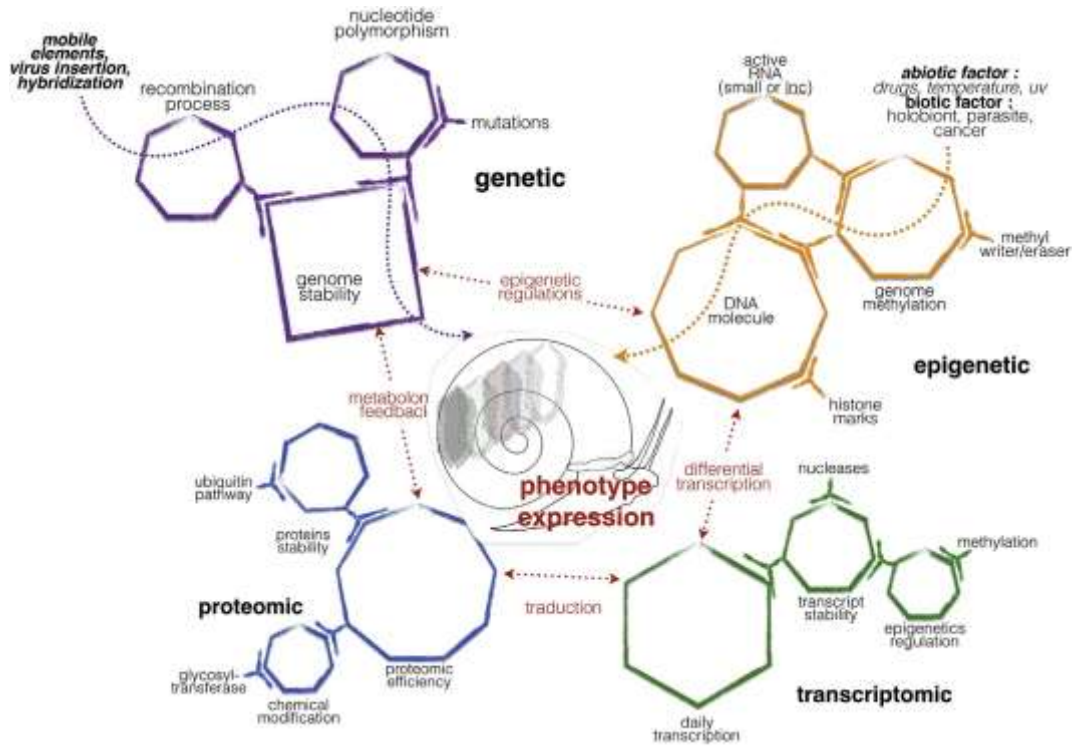


Figure 2

Transcriptional SmPoMuc polymorphism defines a specific SmPoMuc pattern for each individual in a population of *S. mansoni* parasites. Hypothetical exon/intron structures of SmPoMucs were used here to describe the transcriptional processes involved in the genesis of SmPoMuc diversity or polymorphism.

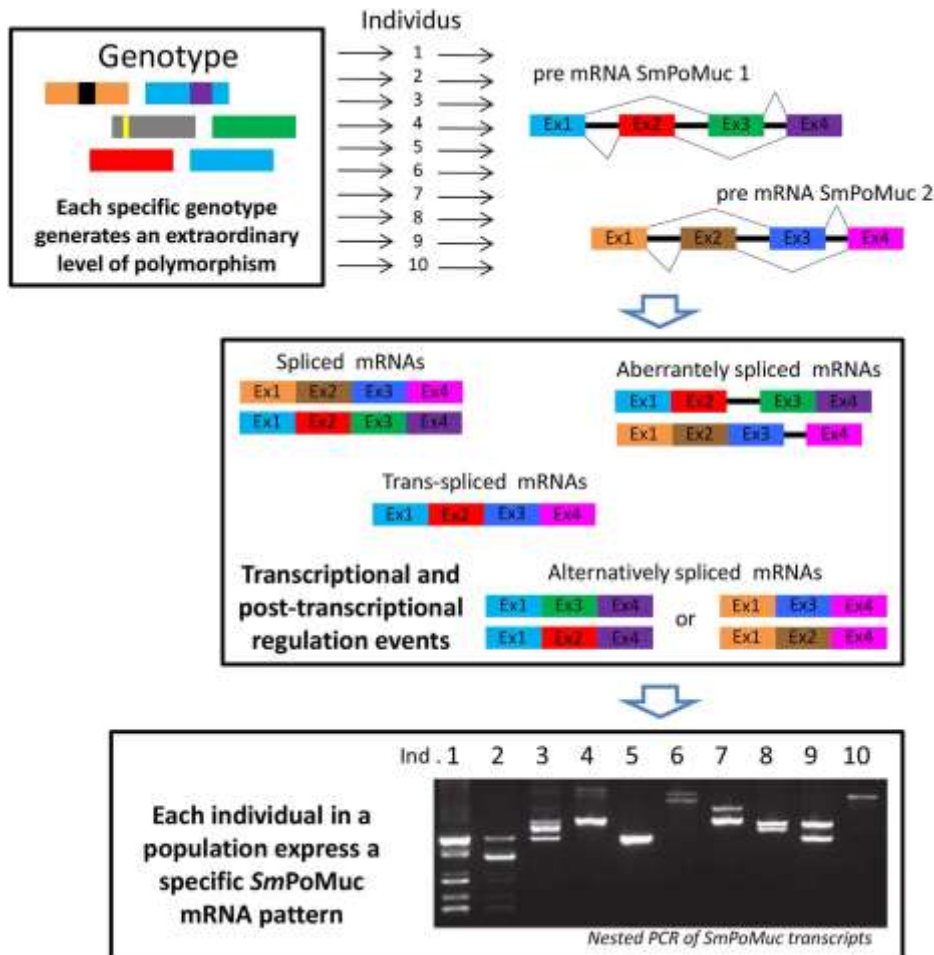


Figure 3

Genome extract of the *B. glabrata* genome assembly: LGUN_random_Scaffold990:3335-119,948. Methylated cytosines are represented by blue bars. The height of the bar represents the percentage of methylation obtained for the methylated cytosines. The highest percentage of methylation observed in this window is 96%. CDS are represented according to a previously published transcriptome (Dheilly et al., 2015). Two FREPs have been annotated in this window (from position 44,955 to 47,325 and from position 99,422 to 114,087) (Richard Galinier, personal communication). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

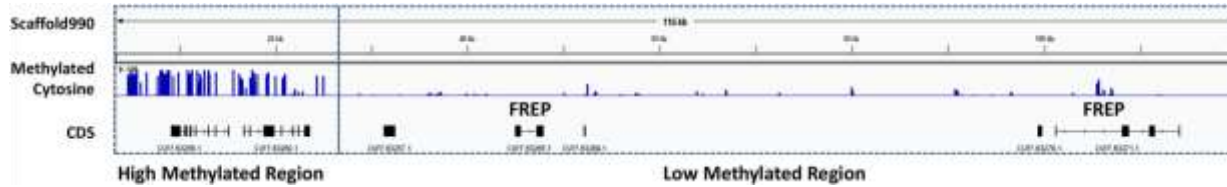
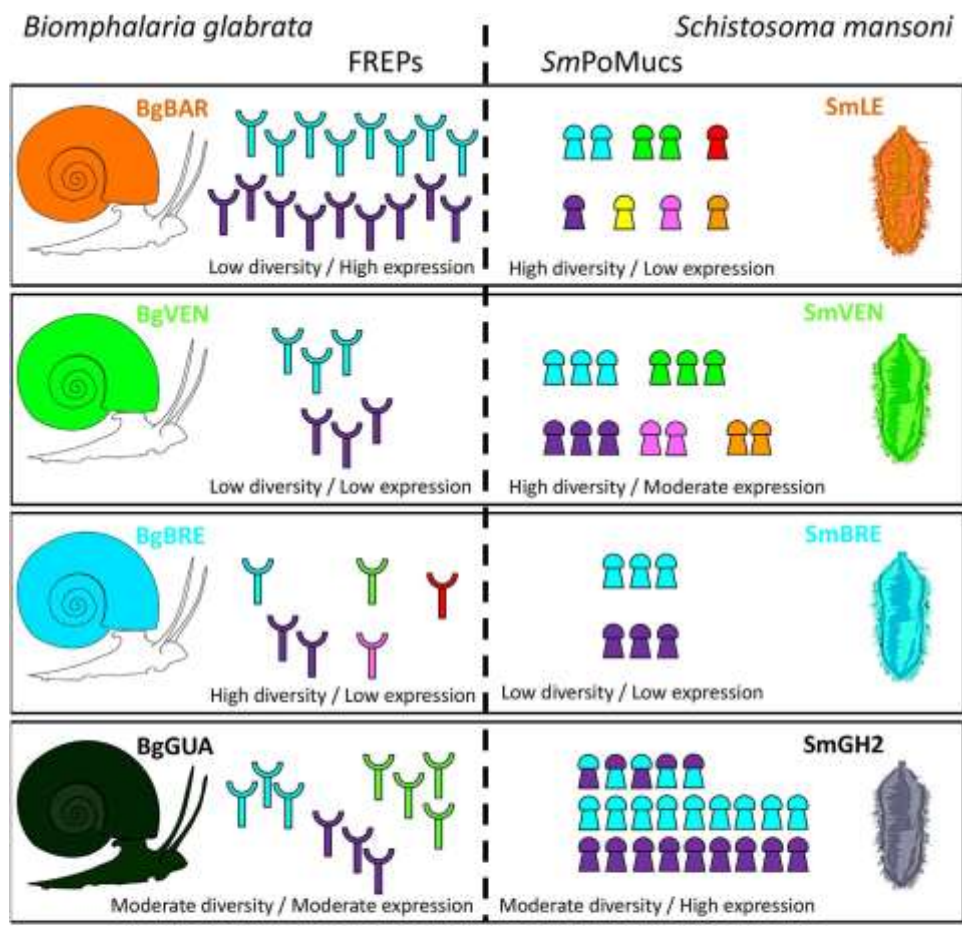


Figure 4

Diversity and expression levels of FREPs and SmPoMucs in four sympatric host/parasite combinations. Host and parasite were designated BgBAR/SmLE (for Belo Horizonte, Brazil), BgVEN/SmVEN (for Guaraca, Venezuela), BgBRE/SmBRE (for Recife, Brazil), and BgGUA/SmGH2 (for le Lamentin, Guadeloupe) (Theron et al., 2014). The number of FREPs and SmPoMucs symbols represents the level of expression. The color of FREPs and SmPoMucs symbols represents the level of diversity. Mixed colors in SmGH2 represent the SmPoMucs with intermingled repeats which are a specificity of this Guadeloupian parasite strain.

In each table cell was indicated in the left corner the expected/observed compatibility phenotype (C: compatibility; M: moderate compatibility; IC: incompatibility) and in the right corner the prevalence (P, percentage of snail infected) and intensity values (I, number of parasites per infected snails) for all sympatric and allopatric combinations following experimental infection with 20 miracidia (Theron et al., 2014). Expected compatibilities were estimated based on the hypothesis proposed from the observations of sympatric compatibility phenotypes (see part 5. for details). Highlighted table cells correspond to host/parasite interactions in which expected and observed phenotype data are not in concordance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



Expected / Observed compatibility phenotypes

	SmLE	SmVEN	SmBRE	SmGH2
BgBAR	C / C: P= 96% I = 3.7	M / M: P= 55% I = 3.9	IC / IC: P= 12% I = 2.3	IC / IC: P= 0% I = 0.0
BgVEN	C / C: P= 100% I = 7.4	C / C: P= 100% I = 7.1	C / C: P= 80% I = 5.5	M / M: P= 44% I = 4.7
BgBRE	C / C: P= 100% I = 8.4	C / C: P= 100% I = 8.2	C / C: P= 100% I = 7.1	M / IC: P= 4% I = 1.0
BgGUA	C / C: P= 100% I = 7.4	C / C: P= 100% I = 6.5	M / C: P= 83% I = 3.0	M / M: P= 60% I = 2.6

Figure 5

Key definitions related to snail-schistosome interactions

- **Compatibility:** a pair of specific host and parasite genotypes/phenotypes in which an individual parasite infects an individual host and completes the part of its life cycle appropriate to the host.
- **Incompatibility:** a pair of specific host and parasite genotypes/phenotypes in which an individual parasite did not infects an individual host and fails to complete the part of its life cycle appropriate to the host.
- **Sympatric interaction:** host and parasite species coexist in the same geographic area. They experiment frequent encounters one another.
- **Allopatric interaction:** host and parasite species originate from different geographic area.

They never experiment encounters one another.