Identification of candidate virulence loci in *Striga hermonthica*, a devastating

parasite of African cereal crops

Suo Qiu[1,¶,#a], James M. Bradley[1,¶,#b], Peijun Zhang[1], Roy Chaudhuri[1], Mark Blaxter[2,#c],

Roger K Butlin[1,3]*, Julie D. Scholes[1]*

[1]School of Biosciences, University of Sheffield, Western Bank, Sheffield, S10 2TN, UK.

[2] Institute of Evolutionary Biology, School of Biological Sciences, The University of

Edinburgh, Ashworth Laboratories, Charlotte Auerbach Road, Edinburgh, EH9 3FL,UK.

[3]Department of Marine Sciences, University of Gothenburg, S-405 30 Gothenburg,

Sweden.

Current address:

[a,#]Institute of Evolutionary Biology, School of Biological Sciences, The University of

Edinburgh, Ashworth Laboratories, Charlotte Auerbach Road, Edinburgh, EH9 3FL,UK.

[b,#]Cell and Systems Biology, University of Toronto, 25 Willcocks St., Toronto, ON   M5S

3B2, Canada

[c,#]Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridge CB10

1SA, UK.

[¶]Joint first authors

*Corresponding authors: Professor Julie Scholes (E-mail: j.scholes@sheffield.ac.uk);

Professor Roger Butlin (r.k.butlin@sheffield.ac.uk).

Correspondence to:

Professor Julie Scholes

School of Biosciences

University of Sheffield

Sheffield, S10 2TN, UK

Abstract

Parasites have evolved suites of proteins, Virulence Factors (VFs), that are delivered into host plants to facilitate colonization. Whilst VFs mediating plant-microbe and plant-nematode interactions have been characterised extensively, less is known about VFs mediating parasitic plant interactions with their hosts. *Striga hermonthica* is an obligate, root-parasitic plant capable of parasitizing multiple cereal hosts in sub-Saharan Africa, causing devastating losses in yields. An understanding of the molecular nature and allelic variation of VFs in *S. hermonthica* is essential for breeding durable resistance and delaying the evolution of parasite virulence. To address this issue, we assembled a genome for *Striga hermonthica* and identified candidate VFs by combining *in silico* prediction of secreted proteins with pooled sequencing of parasites growing on a susceptible and a strongly resistant rice host. Consistent with predictions for parasites, like *S. hermonthica*, that can interact with multiple hosts, we identified multiple loci, potentially with a wide range of functions, implicated in overcoming host resistance. Extremely different allele frequencies were observed at 152 non-secreted and 38 putatively secreted VFs between *S. hermonthica* parasitising the resistant and susceptible rice varieties. Our candidate, secreted VFs encompassed functions such as host cell wall modification, protease inhibitors, oxidoreductase and kinase activities, as well as several with unknown functions. Consistent with maintenance of variation at virulence loci by balancing selection the candidate loci had significantly higher Tajima's D on average than the genomic background. Our results show that diverse strategies are used by *S. hermonthica* to overcome different layers of host resistance. Understanding the maintenance of variation at virulence loci by balancing selection will be critical to managing the evolution of virulence as a part of a sustainable control strategy.

Author statement

The parasitic weed *S. hermonthica* infects the staple cereal crops of sub Saharan Africa inflicting huge agricultural damage on poor subsistence farmers. Understanding coevolution between Striga and its hosts is essential for effective control strategies, yet little is known about the molecular or population genetic basis of virulence variation in this parasite. Here we combine the sequencing of the *S. hermonthica* genome with an experimental population genetic analysis to identify suites of genes that encode candidate virulence factors enabling some Striga individuals to overcome host resistance. This study revealed variation in multiple functional categories of genes that contribute towards parasite virulence. It provides strong evidence that any single mechanism of host resistance will be rapidly overcome by the allelic diversity in the parasite population, and thus highlights the pitfalls of relying on few resistant genotypes. Only by identifying this allelic diversity will it be possible to combine resistance alleles, in host varieties that are suitable for different agro-ecological zones, and in ways that achieve sustained control by delaying the evolution of virulence. Our data provide a key resource to aid researchers, plant breeders and plant pathologists in developing much-needed durable control against this parasitic weed.

Introduction

Plants are constantly challenged by parasites from across all kingdoms of life [1,2]. As a consequence, they have evolved sophisticated surveillance systems to detect and protect themselves against parasite invasion [3,4,5]. In turn, plant parasites have evolved suites of proteins, miRNAs, or other molecules which are delivered into host plants to facilitate colonisation (Virulence Factors (VFs)) [1,2,6,7] and they are pivotal in determining the outcome of a parasite-plant interaction. Despite substantial advances in understanding the

identity and mode of action of VFs in plant interactions with fungal, bacterial and nematode

parasites [1,6,7] much less is known about VFs mediating parasitic plant interactions with

their plant hosts [8-10]. Parasitic plants occur in almost all terrestrial habitats and have

evolved independently at least 12 times [8,11,12]. Regardless of evolutionary origin,

parasitic plants possess a multicellular organ called the 'haustorium', through which direct

structural and physiological connections are formed with their host plant [8,13]. This allows

them to abstract water, organic and inorganic nutrients. In addition, the haustorium is

increasingly recognised to play a role in host manipulation, through the movement of

parasite-derived proteins, miRNAs and other small molecules into the host plant [10,12-

16].

Striga is a genus of obligate, root parasitic plants within the Orobanchaceae [17,18]. One

species in particular, *Striga hermonthica* (Del.) Benth., is a notorious parasite of rain-fed

rice, maize, sorghum and millets, leading to devastating losses in crop yields for resource-

poor farmers in sub-Saharan Africa (SSA) [19,20]. Control of this parasite is extremely

difficult as *S. hermonthica* is an obligate outbreeder, with high fecundity, wide dispersal

and a persistent, long-lived seed bank [18] leading to a large effective population size [21].

Resistant crop varieties are a crucial component of successful control strategies [19] yet,

even for crop varieties considered highly resistant, genetic variation within parasite

populations is such that a few individuals can overcome host resistance responses and

form successful attachments [22,23]. In order to develop crop varieties that display durable

resistance against highly polymorphic populations of *S. hermonthica*, it is vital to gain a

comprehensive understanding of the repertoire, mode of action and genetic variability of

parasite VFs that suppress or circumvent host defence responses [10,24]

Here we have combined two complementary approaches to identify candidate genes

encoding VFs in *S. hermonthica.* First, we assembled and annotated the genome of *S.*

hermonthica, and developed a pipeline for computational prediction of putative secreted

proteins (the secretome) and candidate VFs. The assembled genome was then used as a

reference for an experimental, population genomics analysis, to compare DNA sequence

variants in bulked (pooled) samples of *S. hermonthica* grown on a susceptible (NERICA

7) or resistant (NERICA 17) rice host (Fig 1a i-ii). This allowed us to scan for loci in the *S.*

hermonthica genome where the selection imposed by the resistant host had elevated the

frequency of alleles contributing to successful colonisation (termed 'virulence' alleles) (Fig

1 b-d). A similar approach was used to identify candidate genomic regions associated with

resistance in *Solanum vernei* to the potato cyst nematode, *Globodera pallida* [25]. The

intersection between genes encoding predicted VFs and genes with highly significant

allele frequency differences in the genome scan of *S. hermonthica*, revealed a set of

candidate virulence loci encoding proteins with many functions, including cell wall

modification, protease, or protease inhibitor, oxidoreductase and putative receptor-like

protein kinase activities. Our results show that diverse strategies are used by *S.*

hermonthica to overcome different layers of host resistance and suggest a polygenic basis

of virulence in this parasite.

Results

The Striga hermonthica genome is very heterozygous

We obtained a single population of *S. hermonthica* seeds from farmer's fields in Kibos,

Kenya and infected a highly susceptible rice variety, NERICA 7 (Fig 1a). The genetic

diversity of the seed population is reflected in the subtle differences of flower colour and

morphology of attached parasites (Fig 2a). We sequenced, assembled and characterised

the genome of a single individual from this population, which to our knowledge, represents

the first genome assembly for *S. hermonthica*. The genome size was estimated by K-mer

analysis to be 1,475 Mbp, (Fig 2b). This agrees closely with a previous flow cytometry-

based estimate [26] and is more than twice the size of the recently sequenced genome of

S. asiatica [27]. The assembly consisted of 34,907 scaffolds > 1 kbp in length, with an N50

of 10.0 Mbp and 29 scaffolds making up half of the genome size (S1 Table). The *S.*

hermonthica genome was remarkably heterozygous (overall mean π = 0.011) (Fig 2 b,c)

when compared with other parasitic and non-parasitic plant genomes, likely reflecting the

fact that it is an obligate outbreeding species. In addition, the genome contained a large

proportion (69%) of repetitive DNA (Fig 2 b,c) which was dominated by long terminal repeat

(LTR) elements (S2 Table), a pattern also found for the leafless shoot-parasitic plants,

Cuscuta australis and *C. campestris* [28, 29] and the closely related parasitic plant *S.*

asiatica [27]. As expected, the density of repetitive elements along each scaffold negatively

correlated with the density of protein-coding genes (Fig 2c). In total, 29,518 protein-coding

genes were predicted from the *S. hermonthica* genome, which was comparable to *S.*

asiatica (34,577), the closely related non-parasitic plant *Mimulus guttatus* (28,140) and to

Arabidopsis thaliana (27,416) (S3 Table). Through our BUSCO analysis of gene set

completeness [30], we found 87.3% of 2,326 conserved single-copy orthologs in

eudiocotyledons were complete in the *S. hermonthica* genome, which is very similar to

that found in *S. asiatica* (88.7%) (Fig 3; S4 Table). Of the BUSCOs not found in the *S.*

hermonthica genome, over half were also absent from the *S. asiatica* genome (S4 Table).

Both *Striga* spp. share missing BUSCOs that are present in the genome of the closely

related non-parasitic plant, *Mimulus guttatus* (Fig 3b; S4 Table). Similarly, two shoot

holoparasites, *C. australis* and *C. campestris*, with a BUSCO completeness of 81.0 and

81.7% respectively, also shared many missing BUSCOs that were present in the genome

of the non-parasitic close relative, *Ipomea nil* (Fig 3c). This is consistent with previous

findings suggesting some missing BUSCOs are likely to be a result of the parasitic lifestyle

[27-29, 31].

The Striga hermonthica secretome

One way that parasite proteins can interact with host biology is through parasite-directed secretion. We identified 3,375 putatively secreted proteins in *S. hermonthica* (11.4 % of the proteome) (S1 Fig), many of which were homologous to *A. thaliana* secreted proteins (S5 Table), providing experimental evidence for secretion into the extracellular space. On average, the *S. hermonthica* secreted proteins were both significantly smaller and had a higher percentage of cysteine residues compared with the rest of the proteome (Fig 5 a,b). Genes encoding secreted proteins tended to be more clustered (within 15 kbp of their nearest neighbour) compared to all genes in the genome ($p < 10^{-5}$, 10^5 permutations) (S2 Fig) suggesting they are likely to be arrayed in tandem and belong to large gene families [35]. Functionally, the secretome was rich in protein domains involved in cell wall modification (e.g. endoglucanases, cellulases, pectinesterases, expansins, and pectate lyases), protease activity (e.g. papain-like cysteine proteases, aspartic proteases, and subtilase proteases) and oxidoreductase activity (peroxidases, copper oxidases, and cytochrome p450 proteins) (Fig 5c, S3 and S4 Figs). The cytochrome P450 domain, for example, was particularly frequent in the *S. hermonthica* secretome (3.13% of protein domains) compared with the rest of the proteome (0.25% of protein domains) (S3 Fig). Three other highly-abundant protein domains in the secretome were described as copper oxidases (S3 Fig) and are commonly found in laccases that are involved in the generation or breakdown of phenolic components, such as lignin [36]. Small cysteine-rich proteins are common characteristics of VFs from a range of phytoparasites [37, 38]. In *S. hermonthica*, 183 such proteins were identified (Fig 5a) and were similar to proteins annotated as carbohydrate binding X8 domain-containing proteins, protease inhibitor/lipid transfer proteins, PAR1-like proteins, pectinesterases, RALF-like proteins and thaumatin-like proteins (S4 Fig), many of which are likely to play a role in host-Striga interactions [27,39].

We identified several protein domains in the *S. hermonthica* secretome that were enriched

to a higher degree than observed in the secretome of the closely-related non-parasitic

plant, *M. guttatus* (Fig 5c, S3 Fig , S1 Data), suggesting these functions are relevant to the

parasitic lifestyle of *S. hermonthica*. The xyloglucan endotransglycosylase (PF06955)

domain, for example, was found in 17 *S. hermonthica* proteins (Fig 5c, S4 Fig). Xyloglucan

endotransglucosylases / hydrolases (XETs) have the potential to modify either the parasite

or host cell walls (or both) during parasitism [40]. XETs are secreted from the haustoria of

the parasitic plant *Cuscuta reflexa* during a susceptible interaction on its host *Pelargonium*

zonale, and contribute towards the pathogenicity of this parasitic plant [40]. Pectate lyase

superfamily (PF12708) and pectinesterase (PF01095) domains were enriched in the

secretome of *S. hermonthica* compared to *M. guttatus* and may act as VFs to modify host,

or parasite, pectin during penetration. We found a battery of different carbohydrate-active

glycosyl hydrolase (GH) domains that were enriched in the *S. hermonthica* secretome (Fig.

5c, S3 Fig). Eight *S. hermonthica* proteins were annotated as cellulases of the GH5 family

(containing domain PF00150) (S4 Fig) and were similar to secreted cellulases that function

as VFs in some phytoparasitic nematodes [41]. The degradation of cellulosic β-1,4-glucans

has been observed in susceptible sorghum roots infected by *S. hermonthica* [42] and may

be mediated by these secreted enzymes to facilitate the migration of *S. hermonthica*

intrusive cells between host root cortical cells. The identification of many putatively

secreted VFs in the *S. hermonthica* genome, that are likely to modify host plant cell walls,

raises an interesting question about how such proteins are targeted to avoid damaging the

parasite's own cell walls.

Population genomic analysis to identify candidate virulence loci

We next designed an experimental system that would allow us to identify a subset of VFs

with genetic variation relevant to the ability to infect some host genotypes and not others.

Hundreds of *S. hermonthica* individuals were harvested from either a very resistant

(NERICA 17) or susceptible (NERICA 7) rice cultivar, and pools of these individuals were

subjected to genome resequencing. After aligning the reads to our reference genome, we

detected 1.8 million SNPs in genic regions (defined as regions from 2 kbp upstream of the

start codon to the 1 kbp window containing the stop codon). These genic regions were split

into 150,741 1 kbp windows and of these, 194 (0.13%) had extreme and consistent allele

frequency differences between the bulked pools of *S. hermonthica* selected on the

resistant *versus* the susceptible hosts (S5 Fig; S2 Data). These highly differentiated

windows were located in 190 genes. These candidate loci potentially encode virulence

factors with allelic variants, influencing either structure or expression that contribute to the

ability of some individuals to parasitise NERICA 17. As expected for an outbred parasite

with a large population that encounters multiple host species and genotypes, many loci

were detected and they cover a range of predicted functions. Of these candidate VFs, 152

were not predicted to be secreted and were assigned to a wide range of functional

categories, including putative transcription factors, hormone signalling pathways,

transporters, repeat-containing proteins and a number of proteins of unknown function (Fig

6a; S2 Data). Some of these proteins may function to protect the parasite against host

defences and facilitate growth on the resistant rice variety. In addition, some may enter

the host by non-traditional pathways, for example, via the host-parasite xylem connections.

One sixth (24) of these non-secreted proteins had sequence similarity to proteins in the

Pathogen-Host Interaction (PHI) database [43]. These included *S. hermonthica* proteins

with sequence similarity to a putative leucine-rich repeat protein from *Ralstonia*

solanacearum, a mitogen-activated protein kinase from *Ustilago maydis*, a calreticulin-like

protein from *Magnaporthe oryza* and a cytochrome P450 from *Bursaphelenchus xylophilus*

(S2 Data).

The remaining 38 VFs were members of the *S. hermonthica* secretome and represent particularly strong candidates associated with the ability to parasitise NERICA 17 successfully (Fig 6a,b, S2 Data). These genes were categorised into six functional groups, the largest of which contained 12 genes associated with cell wall modification (Fig 6a,b), including genes encoding an expansin protein, a COBRA-like protein, a β-(1-2)-xylosyltransferase, two trichome birefringence-like (TBL) proteins, a pollen Ole e allergen and two exostosin family proteins, all of which can function to modify the extensibility or other mechanical properties of plant cell walls [2, 44-46] (Fig 6b). Groups of genes annotated as proteases (6 genes including subtilases, aspartyl proteases, and a cysteine proteinase), lipases (3 genes) and kinases (3 genes) were also found. The proteases were always associated with an inhibitor protein domain (Fig 6b). For example, the putative aspartyl proteases possessed one or more xylanase inhibitor domains (Fig 6b). There were also eight genes encoding proteins with a range of different putative functions, including a PAR1-like protein, a probable aquaporin, an α/β-hydrolase and two receptor-like protein kinases (Fig 6b). In addition, a further six genes were annotated as proteins of unknown function (Fig 6b).

The 38 candidate VFs were investigated in more detail by quantifying changes in gene expression in haustoria at critical stages of parasite development on the susceptible rice variety NERICA 7, inspecting the distribution of SNPs throughout the promoter and genic regions, and testing for signatures of historical selection. Changes in gene expression were measured in an independent experiment by harvesting *S. hermonthica* haustoria generated *in vitro,* i.e. prior to attachment to the host (Fig 6c). Changes in gene expression of attached haustoria were measured relative to gene expression in haustoria generated *in vitro.* At 2 days after inoculation of the host root, parasite haustoria were attached and parasite intrusive cells had penetrated into the host root cortex. By day 4, the parasite

intrusive cells had penetrated between the endodermal cells and by day 7 had formed connections with the xylem vessels of the host, providing direct access to host resources (Fig 1a iii).

Prior to attachment to the host, some of the genes encoding candidate VFs were not expressed in haustoria (e.g. subtilase gene (SHERM_16883) and subtilisin-like protease (SHERM_08443) or were expressed at very low levels (e.g the peroxidase (SHERM_00887), glycosyl hydrolase (SHERM_(20042), both aspartyl proteases (SHERM_16482 and SHERM_26730) and an unknown protein (SHERM_03853) (S3 Data). However, all 38 genes were expressed in haustoria during the early stages of infection of the susceptible host, NERICA 7 (Fig 6c; S3 Data). There were two main patterns of gene expression. Firstly, 21 genes, including those mentioned above, had low levels of expression in haustoria 2 days post infection, followed by an increase in expression as infection progressed (Fig 6c; S3 Data). In contrast, 17 genes were highly expressed in haustoria 2 days post infection and expression then decreased progressively with time, e.g. genes encoding β-glucosidase, β-(1-2)-xylosyltransferase, and TBL protein SHERM_06484, all of which modify cell walls. The cysteine protease, PAR1, α/β-hydrolase and aquaporin genes also exhibited a similar expression profile (Fig 6c; S3 Data).

Most of the 38 genes had significantly differentiating SNPs in their promoter regions (from the start site to 2 kbp up-stream). Some of these SNPs may lead to a change in the regulation of gene expression (Fig 6b). Some genes, for example, the gene encoding the pollen Ole e allergen protein (SHERM_15460), one of the exostosin family proteins SHERM_12118), a probable aquaporin SIP2-1 (SHERM_13450) and one of the two protein TBL genes (SHERM_16484), also had non-synonymous SNPs in the coding region (Fig 6b), that may result in functional differences between the alleles of these genes in

individuals infecting NERICA 7 and NERICA 17. Finally, SNPs were also found within

predicted intron regions in many of the genes (Fig 6b).

The co-evolutionary interactions between hosts and parasites can generate balancing

selection [47]. We predicted that genes contributing to virulence would tend to have a

history of balancing selection because of the diverse range of hosts used by *S.*

hermonthica. To test this prediction, we compared Tajima's D between candidate loci and

the rest of the genome, expecting to see more positive values for loci with a history of

balancing selection [48]. We used the pools from the susceptible host for this comparison

because they represented the Striga population as a whole. As predicted, the 152

candidate loci in the *S. hermonthica* proteome (S6 Fig) and the 38 candidate loci in the

secretome (Fig. 6d) had significantly elevated Tajima's D, on average, compared to all the

genes in the genome (p < 0.0001 and p < 0.0003, respectively; 10^5 permutations). Some

loci had particularly high Tajima's D values, for example the two receptor-like protein

kinases (Fig. 6b). Interestingly, some loci showed large differences in Tajima's D between

the control and virulent *S. hermonthica* pools with the largest difference seen for the TBL

gene (SHERM_16484) with a negative ΔD (D_{Vir} - D_{Con}) of -0.9. This suggests strong

selection resulting in one common haplotype in the virulent pools in contrast to two or more

haplotypes at intermediate frequencies in the control pools. There were also large positive

ΔD values: 0.71, 0.16 and 0.20 for one of the putative receptor-like protein kinases

SHERM_01541, one of the aspartyl proteases, SHERM_16482, and the peroxidase

SHERM_00887, respectively. This suggests that a rare haplotype in the control pools is

present at intermediate frequency in the virulent pools. Overall, these changes indicate

that selection on the resistant host caused changes in frequency of multi-SNP haplotypes

at these loci, haplotypes that may have been created by areas of low recombination or by

recent invasion of new variants under positive selection [49] and which underlie the ability

of some *S. hermonthica* individuals to overcome resistance in NERICA 17.

Discussion

Plants secrete proteins involved in many biological functions, from nutrient acquisition, to

development and defence [3, 44]. However, unlike most plants, in parasitic plants such as

S. hermonthica a subset of secreted proteins is likely to function as VFs and contribute

towards parasite fitness by facilitating host colonization [10]. We used a combination of *in*

silico prediction of secreted proteins and pooled sequencing of parasites derived from

susceptible and resistant rice hosts, both facilitated by the first available genome

assembly, to identify a set of candidate VFs. These are secreted proteins encoded by

genes that had extremely different allele frequencies between replicated pools derived

from susceptible and resistant hosts, suggesting strong selection for particular variants

that facilitate successful colonisation despite host resistance. This experimental approach

has not been applied previously to investigate virulence of Striga, or any other parasitic

plant. Its success here paves the way to application of similar methods to other host-

parasite combinations, providing vital information on virulence mechanisms and their

genetic variability within and between parasitic plant populations from different regions of

Africa, and so underpinning the development of sustainable control strategies.

Our list of 38 candidate, secreted VFs points to key functions involved in pathogenicity,

including oxidoreductase, receptor-like protein kinase, protease and protease inhibitor,

and cell wall modification activities. The latter is consistent with growing evidence that cell-

wall modification is a critical step in plant invasions by many different parasites including

parasitic plants. Recently, the structural integrity of lignin was shown to be a crucial

component of resistance in roots of the rice variety Nipponbare to infection by *S.*

hermonthica [50]. In our study it is clear that the host cell wall is involved in resistance in

NERICA 17. Most *S. hermonthica* individuals from the Kibos population were unable to

penetrate the root endodermis or, if they breached the endodermis, they were unable to

establish functional connections to the host xylem vessels (Fig 1a iv-vi). Consistent with

this, the largest category of our candidate, secreted VFs included a putative peroxidase,

an expansin, pollen allergen-like proteins, a β-glucosidase, a β (1-2) xylosyltransferase,

and a TBL protein, all of which function to modify cell walls. The TBL protein,

SHERM_16484, had a strikingly different Tajima's D in the control pool compared to the

value in the virulent pool. This large difference suggests a change from intermediate to

extreme frequencies for multiple SNPs, consistent with selection favouring one haplotype

on the resistant NERICA 17, out of several haplotypes present in the population. In *A.*

thaliana and *O. sativa* TBL proteins belong to large gene families with functions related to

cell wall modifications. In *A. thaliana*, At-TBL44 has been implicated in pectin esterification

[51, 52], whilst in rice other members of this family appear to be involved in acetylation of

xylan moieties in cell walls [53]. In each case, alterations in enzyme activity altered

resistance in *A. thaliana* to powdery mildew and in rice to leaf blight [51, 53]. Recently an

11 kDa protein was isolated from the cell wall of the shoot parasite *C. reflexa* and identified

as a glycine rich protein (GRP) [54]. The protein and its minimal peptide epitope (Crip21)

bind to and activate a cell surface resistance gene in tomato (CuRe1), leading to resistance

to the parasite, illustrating the importance of cell wall modifications to host resistance.

In addition to cell wall modification, several candidate genes were annotated as having

protease activity, including two aspartyl proteases, three subtilisin or subtilisin-like genes

and a cysteine proteinase. Interestingly, all were predicted to possess a dual-domain

structure consisting of a propeptide inhibitor domain and a catalytic protease domain. In

other such protease enzymes the propeptide domain auto-inhibits the enzyme activity until

cleavage of this inhibitor domain activates the catalytic domain [55]. This provides a mechanism by which the parasite could initially secrete an inactive VF that only becomes active once in the host environment. A similar dual-domain structure was found for a highly expressed, haustorium-specific cysteine protease in the shoot parasitic plant, *C. reflexa*, which positively contributes towards pathogenicity [33]. Although the precise functions of other candidate VFs are unknown, for example the putative aquaporin, PAR1 protein, cytochrome P450 and the 5 proteins with no functional annotation, they provide exciting avenues for further investigation.

S. hermonthica has extremely high fecundity (>100,000 seeds per plant) [18], a persistent seed bank and is obligate out-crossing [56], leading to a very large effective population size [21]. Therefore, the high heterozygosity that we observed in the *S. hermonthica* genome was not unexpected. *S. hermonthica* parasitizes many different host species and varieties, often within the same geographical area. Populations therefore encounter many different forms of resistance, which they experience as a highly heterogeneous environment. This is expected to maintain genetic diversity at many loci contributing to virulence, which is consistent with observations from field studies that resistant varieties, of any particular crop species, are often parasitized by one or two *S. hermonthica* individuals [22,24]. A typical example is the host-parasite combination used here as a test system; the *S. hermonthica* Kibos population and the strongly resistant upland rice variety, NERICA 17 [23,57], one of 18 NERICA rice varieties grown widely by African farmers [57].

This type of parasite interaction with multiple hosts leads to two predictions that are supported by our data. First, multiple loci, potentially with a wide range of functions, are likely to be implicated in overcoming host resistance. We detected 190 strong candidates for contribution to virulence, with extreme allele frequency differences between our control and virulent pools, including many gene families. It is likely that many additional candidate

VFs would be revealed, by repeating this comparison on other resistant hosts. An

important question for the future will be to determine how individual VFs are implicated in

overcoming resistance for specific hosts or across a range of hosts. Second, maintenance

of variation at virulence loci by balancing selection will lead to elevated Tajima's D relative

to the background, reflecting persistence of multiple alleles at these loci. We found the

overall Tajima's D in *S. hermonthica* to be negative, perhaps reflecting population

expansion following the spread of agriculture, but our candidate loci had significantly

higher Tajima's D on average, consistent with balancing selection on these loci.

Understanding the maintenance of variation at virulence loci by balancing selection will be

critical to managing the evolution of virulence as a part of a sustainable control strategy

[58].

Effective control of *S. hermonthica* is essential for food security and poverty alleviation for

small-holder subsistence farmers, but it remains elusive. The use of resistance crop

varieties is recognised as sustainable and cost effective [19], but the durability of resistant

varieties is compromised by the potential for rapid evolution of parasite virulence. Thus,

the long-term success of host resistance, as a control strategy for S*. hermonthica* and

other parasitic weeds, requires knowledge of the virulence factors involved, their allelic

variation within and between Striga populations and their interaction with different host

resistance alleles. Only then will it be possible to combine resistance alleles, in host

varieties that are suitable for different agro-ecological zones and in ways that achieve

sustained control by delaying the evolution of virulence. Our experimental approach and

identification of candidate VFs and allelic variation within a *S. hermonthica* population, is

a critical first step in this direction.

Materials and Methods

Collection and extraction of S. hermonthica DNA for genome and pooled sequencing

An accession (population sample) of *S. hermonthica* seeds was collected from individuals' parasitising maize in farmers' fields in the Kibos region of Kenya (0° 5' 30.1272" S; 34° 46' 4.6416" E). To obtain *S. hermonthica* for genome sequencing and the bulked sample analysis (BSA), rice seedlings of the varieties, NERICA 7 and NERICA 17, were grown in rhizotrons and infected with germinated *S. hermonthica* seeds (to ensure synchronous attachment) as described in [22]. Plants were grown in a controlled environment room with a 12 h photoperiod, a photon-flux density of 500 µmol.quanta.m^{-2}.s^{-1} at plant height, a day / night temperature of 28 / 24 °C and 60 % relative humidity. For the construction of a reference genome, one *S. hermonthica* individual was randomly harvested from NERICA 7. For the pooled sequencing, 300 *S. hermonthica* individuals were harvested from NERICA 7 and from NERICA 17. Individual parasites were harvested when > 30 mg in weight, divided into 20 mg aliquots and immediately frozen in liquid nitrogen. The 300 individuals collected from NERICA 7 or NERICA 17 were divided into three pools of 100 individuals (three biological replicates). Each pool was randomly divided into 25 groups of four individuals (20 mg of tissue per individual) for DNA extraction. To remove high levels of secondary metabolites and polysaccharides in *S. hermonthica*, lysed plant tissues were first washed in a buffer containing 2 % polyvinylpyrrolidone, 0.25 M NaCl, 0.2 M Tris-HCl and 50 mM EDTA. DNA was then extracted following the CTAB protocol [59]. The quality of DNA was checked by gel electrophoresis and quantified by Nanodrop. An equal quantity of DNA from each of the 25 samples was then combined to form one biological replicate. The six DNA samples (three from *S. hermonthica* individuals from NERICA 7 and three from NERICA 17) were then subjected to paired-end sequencing using an Illumina HiSeq

machine at the Beijing Genomics Institute (BGI), China. The libraries, insert sizes and

sequencing depth are shown in S6 Table. DNA from the individual harvested from NERICA

7 for the production of a reference genome was sequenced on an Illumina HiSeq2500

sequencer at Edinburgh Genomics, UK. Six paired-end DNA libraries were constructed

with different insert sizes as described in S6 Table.

De novo assembly of the *S. hermonthica* genome

For *de novo* genome assembly, adapters and low quality reads (Q < 20) were removed

using CutAdapt [60]. Only reads longer than 50 bp for the paired-end libraries and 30 bp

for the mate-pair libraries were retained for subsequent analyses. Duplicated reads

generated by PCR amplification in the library construction process were removed using

FASTUNIQ [61]. Sequencing errors, which can create difficulties for the short-read

assembly, were corrected using the software BLESS, a *k*-mer spectrum-based method

designed to remove *k*-mers with a small number of occurrences [62]. After filtering, ~2.7

billion reads were generated from the short insert libraries and 0.76 billion reads from

mate-pair libraries. This corresponded to ~230 X and ~54 X coverage of the *S.*

hermonthica genome, respectively.

The cleaned and filtered reads were used to assess the *S. hermonthica* genome size,

repetitiveness and heterozygosity, compared with 12 other plant species (S6 Table), in the

module preQC, implemented in the software sga (https://github.com/jts/sga). The Illumina

sequencing reads of these 12 species were downloaded from the NCBI Sequence Read

Archive (SRA; https://www.ncbi.nlm.nih.gov/sra/) with the source project numbers given in

S6 Table. This analysis showed *S. hermonthica* was highly heterozygous and therefore

the software Platanus, which is specifically designed for highly heterozygous genomes,

was chosen to assemble the *S. hermonthica* genome [63] (S1 Table).

To further improve the *S. hermonthica* genome assembly, Chicago and Dovetail Hi-C libraries were prepared and sequenced at Dovetail Genomics, California, USA (https://dovetailgenomics.com/plant-animal/) (S1 Table). For construction of Chicago libraries, DNA from the same *S. hermonthica* individual (as used for initial sequencing) was sequenced on a standard Illumina HiSeq 2500 platform. For the Hi-C libraries, plant tissues from an F1 individual from a cross between the sequenced individual and another *S. hermonthica* individual (Kibos accession) were used for the library construction and sequencing. Sequences from both the Chicago and Hi-C libraries were then used to improve the contiguity of the initial genome assembly using the Dovetail HiRise Assembler software. RepeatModeler was used to generate a *S. hermonthica*-specific repeat library and RepeatMasker was then used to classify repeat elements in the genome. A repeat-masked version of the genome was used for annotation [64, 65].

Annotation of the S. hermonthica genome

The genome was annotated using three methods. Firstly, gene structures were inferred using a *S. hermonthica* transcriptome dataset generated by the Parasitic Plant Genome Project (PPGP) [9]. This dataset included Illumina sequencing of cDNAs collected from *S. hermonthica* individuals at eight developmental stages [39]. The reads were mapped onto the *S. hermonthica* genome assembly using TopHat to identify exon regions and splice positions [66]. Transcriptome-based gene structures were predicted using Cufflinks [67] and candidate coding regions were then constructed in Transdecoder (https://github.com/TransDecoder/). Secondly, the protein sequences from five species: *Arabidopsis thaliana* (TAIR10), *Mimulus guttatus* (v2.0), *Solanum lycopersicum* (ITAG2.4), *Oryza sativa* (IRGSP1.0) and *Sorghum bicolor* (79), were used to determine consensus gene models in the genome. The protein sequences from rice and sorghum were included because they are natural hosts of *S. hermonthica* and previous studies showed that

horizontal gene transfer is common between the host species and *Striga* [68, 69]. The

protein sequences were mapped onto the *S. hermonthica* genome using TBLASTN and

pairwise alignments were then input into Genewise [70] to predict gene models in *S.*

hermonthica. Thirdly, an *ab initio* method was used for *de novo* prediction of genes in the

S. hermonthica genome by the software, Braker [71]. Braker combines the advantages of

the two gene prediction software packages: GeneMark-ET and AUGUSTUS, and

integrates RNA-Seq read information into final gene predictions [72, 73]. Finally, Evidence

Gene Modeler was used to integrate various gene models from the transcript data,

mapped proteins, and the predicted gene models from the *ab initio* method [74]. The

completeness of the gene set was assessed using BUSCO v5 using the 2,326 core

orthologs from eudicots_odb10, with default settings.

483 **Functional annotation of the S. hermonthica proteome**

Putative protein functions were assigned to *S. hermonthica* proteins using BLASTp

analyses against the SwissProt and TrEMBL databases, and against the proteomes of

Arabidopsis thaliana (version 30 downloaded from TAIR) and *Oryza sativa* (version 7

downloaded from MSU); which were chosen for their extensive functional annotations. A

BLASTp analysis was also conducted against the pathogen-host interaction database

(PHI-base, version 4.2), which contains curated biological information on host and parasite

genes shown to be involved in pathogen-host interactions (maintained by Rothamsted

Research, UK: http://www.phi-base.org/index.jsp). BLASTp analyses were run locally

using the NCBI BLAST package (version: ncbi-2.3.0+) and a hit was taken to be significant

if e-value < 10^{-5}, bit score > 30 and percentage identity > 30. Protein motifs and domains

were determined by searching databases including Pfam, PATHER, GENE3D, CDD,

PRINTS, PROSITE, ProDom and SMART with InterProScan (Zdobnov and Apweiler

2001). Gene Ontology (GO) terms for individual proteins were retrieved from the

corresponding InterPro descriptions.

Inference of Orthogroups (OG)

Orthologous gene groups (Orthogroups or OGs) among 13 plant species (*Amborella*

trichopoda, *Musa acuminata*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, *Aquilegia*

coerulea, *Solanum lycopersicum*, *Mimulus guttatus*, *Striga hermonthica*, *Arabidopsis*

thaliana, *Medicago truncatula*, *Populus trichocarpa* and *Vitis vinifera*) were inferred using

the software OrthoFinder v2 [75]. Except for *S. hermonthica*, the proteomes of these

species were downloaded from Phytozome V12.1 (https://phytozome.jgi.doe.gov/

pz/portal.html). The number of genes per species for each OG was transformed into a

matrix of *Z*-scores to quantify gene family expansion / contraction in comparison with other

plant genomes. The significance of expansion or contraction was determined using CAFE

v4.2 [76]. Specifically, multi-species alignments of 42 single-copy genes from the

OrthoFinder results were first constructed using MAFFT v7.407 [77]. A maximum likelihood

(ML) tree using the alignments of the 42 single-copy genes was constructed in MEGA X

78], then converted into an ultrametric tree and calibrated based on a divergence time of

82.8 Ma–127.2 Ma between *A. thaliana* and *P. trichocarpa* [79]. The ultrametric tree and

the gene numbers in each orthogroup from the OrthoFinder results were then used for the

CAFE analysis. The functional annotation of each OG was predicted based on sequence

similarity to the InterPro protein family database where the majority of the proteins in the

OG shared the same Pfam annotation.

Prediction and analysis of the S. hermonthica secretome

518 Secreted *S. hermonthica* proteins were predicted according to the default thresholds using

519 SignalP3.0 and SignalP4.1 [80, 81] (S1 Fig). SignalP3.0 predicts a secretion signal peptide

according to both neural network (SignalP3.0-NN) and hidden Markov model (SignalP3.0-HMM) algorithms, whilst SignalP4.1 uses a neural network algorithm. Putative secreted proteins were searched for transmembrane spanning regions in the remaining portion of the protein sequence (without secretion signal) using TMHMM2.0 [82]. Proteins with a secretion signal but without a predicted transmembrane helix were retained as the 'secretome'. Putative secreted proteins were ranked according to the agreement between the different SignalP algorithms: '3' = predicted by all three SignalP algorithms (SignalP3.0-NN, SignalP3.0-HMM and SignalP4.1); '2' = predicted by at least two algorithms and '1' = predicted by one algorithm. TargetP1.1 was used to provide additional predictions on the cellular localization of the putative secreted proteins ('S' = extracellular; 'C' = chloroplast; 'M' = mitochondrial) [83]. The closest matching *Arabidopsis thaliana* protein for each *Striga hermonthica* putative secreted protein was queried against the SUBA4 online database, which combines bioinformatic and experimental evidence to predict subcellular localisation of *A. thaliana* proteins (https://suba.live) [84]. The consensus location of each queried protein was recorded and the number of each subcellular location was expressed as a percentage of the total. Using this approach, the most common subcellular location predicted in the secretome was 'extracellular', supporting the validity of our secretome prediction pipeline for *S. hermonthica* (S5 Table).

To determine whether a putative secreted protein was expressed during early stages of parasitism a BLASTn search using the protein's coding sequence was conducted against the stage 3 (~ 48 hours post infection) and stage 4 (~ 72 hours post infection) transcriptome dataset downloaded from the PPGP [38]. BLASTn hits were taken to be significant if they returned an e-value $< 10^{-10}$, percentage identity of > 95 % and a bit score of > 60. To identify enriched Pfam domains in the *S. hermonthica* secretome compared with the rest of the proteome (non-secretome), the frequency of each Pfam domain was first calculated

as the number of occurrences of each domain over the total number of domains in the secretome or non-secretome. Enrichment fold for each domain was then calculated as the frequency of hits in the secretome over the frequency in the non-secretome. The significance of enrichment was assessed using a Chi-squared test with a false discovery rate (FDR) correction for multiple testing [85] and was taken to be significant when the corrected p value was < 0.1.

To test whether genes in the secretome clustered together in the genome, a permutation test was performed. For each gene, 'MinDis', defined as the distance between the start positions of the focal gene and its nearest neighbouring gene, was calculated. Genes without a neighbouring gene on the same scaffold were excluded. MinDis was also calculated for 10,000 randomly sampled gene sets with the same size as the secretome. The numbers of clustered genes with MinDis falling into a series of distance intervals were counted and were then compared with the numbers in the same distance interval in the 10,000 random gene sets.

Refining the secretome to select subsets of potential virulence factors

The initial secretome was refined into subsets of VFs based on a series of structural and functional characteristics (S1 Fig). First, those putative secreted proteins that met the following criteria were retained: SignalP ranking = 3; TargetP location = S; and have a BLASTn hit against either stage 3 or 4 *S. hermonthica* transcriptome libraries (suggesting gene expression during early stages of parasitism). From these, four subsets of proteins were identified. Subset 1 comprised small, secreted cysteine-rich proteins (SSCRPs) < 500 amino acids in length and containing ≥ 4 % cysteine residues. Subset 2 consisted of proteins containing Pfam domains that were significantly enriched in the *S. hermonthica* secretome. Only those Pfam domains that were enriched to a greater extent in the *S.*

hermonthica secretome compared with their enrichment in the *M. guttatus* secretome

(determined in exactly the same way as for *S. hermonthica*) were used to obtain this

subset. Subset 3 comprised proteins with no known Pfam domains or orthogroup

assignment. Subset 4 contained proteins with similarity to proteins involved in host-

pathogen interactions based on having a match against the PHI-base (S1 Fig).

Identification of candidate virulence loci using pooled sequencing data

The raw sequence reads from the six pools were trimmed to remove low quality bases and

adapter sequences using CutAdapt [60]. The cleaned reads were mapped to the *S.*

hermonthica reference genome using both BWA [86] mem v 0.7.15 and NOVOALIGN

(http://www.novocraft.com). Mapped reads were sorted using Samtools [87] and PCR

duplicates were removed using Picard (http://broadinstitute.github.io/picard/). Reads

around indels were realigned using the modules RealignerTargetCreator and

IndelRealigner implemented in gatk [88]. SNPs were called using bcftools

(https://samtools. github.io/bcftools/). An R script described in [89] was used to infer the

read coverage distribution for each SNP for each pool of sequenced reads, based on

three-component mixture models. An upper boundary of the coverage for the intermediate

component (single and low copy number regions) was obtained from the fitted distribution

for each pool. The high-coverage component is expected to be enriched in repetitive

sequences while the low coverage component is enriched in erroneous reads or

inadequately sampled genome regions. In order to provide robust data for subsequent

analyses, the inference of an allele and its frequency was considered to be reliable only if

the following criteria were met. (a) Coverage from each pool was ≥ 4 and $\leq$ the upper

boundary inferred from the mixture model distribution. (b) The sum of the coverage across

the six DNA pools was ≥ 36. (c) The count of the minor allele frequency, summed over

pools, was > 2. (d) The ratio of the coverage of the second highest allele to the total

coverage was ≥ 0.1. (e) The ratio of the combined coverage of the third and fourth highest alleles to the total coverage was < 0.02. Only the highest and second highest frequency alleles were considered for the subsequent analyses. Although three or four alleles were present at some positions, the additional alleles were always rare. Cut-offs were determined following exploration of a range of alternative values. They removed only variants that are very unlikely to differ significantly among pools and so they did not influence the ranking of candidate loci.

The likelihood of the observed read counts for the two most common alleles, across the six pools was calculated according to equation 3 from Gompert and Buerkle [90] to allow for the two levels of sampling associated with pooled sequencing data (sampling of reads and of individuals). We compared three allele-frequency models for each SNP using the Akaike information criterion (AIC): a null model with a single allele frequency for all pools, a control-virulent model with one frequency for the control pools (from the NERICA 7 host) and one for the virulent pools (from the NERICA 17 host) and a replicate model with a different allele frequency for each of the three pairs of pools (one control and one virulent) that were sequenced together. The control-virulent model was the model of interest while the replicate model was intended to check for consistency across pairs of pools. Therefore, two ΔAIC values were obtained: $\Delta AIC_{cv} = AIC_{null} - AIC_{control-virulent}$ and $\Delta AIC_{rep} = AIC_{control-virulent} - AIC_{replicate}$. High positive values of ΔAIC_{cv} represent better fits than the null model and indicate significant differences between control and virulent pool types. SNPs with positive ΔAIC_{rep} values were likely to be affected by artefacts caused by sequencing methods and were excluded from the following analyses. All analysis steps were repeated independently for SNPs based on BWA and NOVOALIGN mapping as recommended by Kofler et al. [91].

The effective population size in *Striga* is likely to be large [18] and this is consistent with high diversity in our samples (overall mean π = 0.011). Therefore, we also expected that linkage disequilibrium would break down quickly. To define a suitable window size to search for regions potentially implicated in virulence, the extent of linkage disequilibrium in *S. hermonthica* was investigated. Firstly, the distribution of the gene lengths, i.e. from the start codon to the stop codon of a gene, for all the genes in the *S. hermonthica* genome was obtained and 4 kbp, which is the 75th percentile of the gene length distribution, was used as the upper limit for the length of region to be considered. The SNP with maximum $\Delta AICcv$ within a gene was identified and the region up to 4 kbp from the SNP was divided into eight 500 bp intervals. The mean difference of $\Delta AICcv$, relative to the maximum $\Delta AICcv$, for SNPs in each 500 bp interval was then calculated. The mean difference was typically only elevated in the 500 bp interval containing the SNP with maximum $\Delta AICcv$, so it was clear that linkage disequilibrium above background levels did not normally extend beyond 1 kbp (see S7 Fig). Therefore, 1 kbp windows were used to detect genomic regions potentially associated with virulence on the basis of allele frequency differences between pools from the susceptible and resistant hosts.

Regions starting from 5 kbp upstream of the start codon and ending no further than 2 kbp downstream of the stop codon of a gene were divided into 1 kbp-windows and the mean $\Delta AICcv$ across all the SNPs in each window was calculated. A permutation test was performed to obtain the probability of observing the mean $\Delta AICcv$ value, or higher, for each window based on the distribution of $\Delta AICcv$ across the regions as a whole. Specifically, a mean $\Delta AICcv$ was calculated for a window containing n SNPs. One hundred thousand samples of n SNPs from the pool of all SNPs within the analysed regions genome-wide, were drawn randomly. For each of these samples, a mean $\Delta AICcv$ value was calculated and an expected distribution for the mean in the target window was

obtained. The probability that the observed mean ΔAICcv of a window could be matched

or exceeded by chance was obtained by counting the number of permuted mean ΔAICcv

with values lower than the observed mean ΔAICcv value for that window and then divided

by 100,000. Finally, we retained genic regions for which this probability was less than or

equal to 2×10^{-5} for both the BWA and NOVOALIGN analyses in any window. This cut-off

was chosen to provide experiment-wide significance given the number of protein-coding

genes in the analysis (29,518). In the secretome, a more relaxed cut-off of 1×10^{-4} was

used to reflect the prior expectation that the secretome would be enriched with

pathogenicity-related genes and the smaller number of genes in this set (3,375). Thirty two

genes met this criterion for both Novoalign and BWA (Fig 6; S2 Data). In addition, six

genes encoding putative secreted proteins that passed the 1×10^{-4} cut-off for either

Novoalign or BWA were included in the candidate set because they either contained large

numbers of non-synonymous SNPs or contained high impact SNPs that can alter protein

structure (e.g. due to protein truncation) (Fig 6; S2 Data).

Two population statistics were calculated for each genic region in the control pool using

the software Popoolation [92]. These were nucleotide diversity (π) and Tajima's D, a

statistic describing the allele frequency spectrum used for testing whether a DNA

sequence is evolving under a process that departs from the standard neutral model, such

as selection or demographic change [93].

Analyses of candidate virulence genes

The candidate virulence genes, defined as all genes containing windows that meet the cut-

offs for allele-frequency differentiation between control and virulent pool types, were

categorised into functional groups based on the annotations of the closest matching

homologs from the *A. thaliana* and *O. sativa* proteomes, as well as the Pfam domain

annotations. For each gene, the numbers of SNPs were counted for the promoter region

(within 2 kbp upstream of the start codon), the intronic region and coding region, and the

numbers of non-synonymous SNPs were determined. To quantify the allele frequency

differences between control and virulent pools for these candidate virulence genes, the

proportion of SNPs with high fixation index (F_{ST}) values in the significant window was

calculated. The F_{ST} values between the control and virulent pools for each SNP were

calculated using Popoolation2 [94]. The 95th percentile was obtained from the F_{ST} values

for all SNPs across the genic regions as a whole. For each genic region, we counted the

number of SNPs in its significant window(s) with F_{ST} value higher than the 95th percentile

and then divided by the total number of SNPs in the significant window. This provided a

measure of differentiation for each candidate virulence gene between the *S. hermonthica*

grown on the two host varieties.

680   Expression profiling of candidate virulence genes

To determine the expression profiles for candidate virulence genes, an RNA-seq analysis

was conducted for *S. hermonthica* (Kibos accession) collected at 2, 4, or 7 days post

infection on NERICA 7. Rice plants were grown and infected in the rhizotron system as

described previously [22]. *S. hermonthica* were collected from the roots of NERICA 7 by

cutting a 1 mm section of rice root around each *S. hermonthica* attachment. In addition,

unattached *S. hermonthica* haustoria were induced *in vitro* by the addition of 10 µM DMBQ

[95]. For each treatment, four biological replicates were generated. RNA was extracted

using the RNeasy plant Minikit (Qiagen) and 2 µg of RNA was treated with DNase to

remove DNA contamination (dsDNase, Thermoscientific). The resulting RNA was purified

and concentrated using the RNeasy MinElute cleanup kit (Qiagen) and then sent to

NovoGene Co. Ltd. (China) for sequencing (https://en.novogene.com/). Cleaned reads

were mapped to the *S. hermonthica* genome using Tophat2, version v2.0.12 (default

settings except 'mismatch' = 2). Transcript abundance was determined as read count and

measured by HTSeq (version 0.6.1) with default settings, except that the '-m' option was

set to 'union'. The FPKM values for each gene at each time point were used to calculate

a fold change in expression relative to the haustorial sample. To determine whether gene

expression changed significantly during infection, a one-way ANOVA was carried out for

each gene. If the assumptions of ANOVA were not met, data were log transformed. For

each gene, $\log_2$ fold expression values, across the time points, were centred around 0 and

scaled by the standard deviation for plotting as a heatmap. Heatmap plotting was

conducted using the pheatmap function in R and clustering of genes by expression profiles

was conducted using the 'complete' method with the 'Euclidean' distance measure.

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transverse sections through rice roots infected with *S. hermonthica*.

Data Availability

Raw reads for the pooled *S. hermonthica* sequences and for the *S. hermonthica* genome

sequence, the assembled genome sequence and annotations have been submitted to the

European Nucleotide Archive (ENA) browser at (http://www.ebi.ac.uk/ena/data/view/)

under the following accession numbers: Genome Assembly GCA_902706635; Project ID

PRJEB35606; Sample ID ERS4058863 and Contig accession CACSLK010000001-

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Supporting information

Supplementary Figures S1 – S7.pdf

S1 Fig. Three step pipeline to predict the *Striga hermonthica* secretome and subsets of candidate pathogenicity-related genes. Step 1: Three SignalP algorithms were used to identify *S. hermonthica* proteins with a predicted secretion signal at their N-terminus. These proteins were then checked for a transmembrane helix in the remaining part of the protein and only retained if no transmembrane domains were identified. Step 2: A range of structural and functional information was gathered for putative secreted proteins. Step 3: the secretome was refined into sets of candidate pathogenicity-related genes using the information gleaned in step 2. In addition to the three secretome subsets, a stand-alone BLASTp analysis was carried out against the pathogen-host interaction database.

S2 Fig.Testing gene clustering in the secretome. The red dot represents the number of genes in the secretome with MinDis falling into each 5Kb distance interval. The upper and lower limits of the boxes are the first and third quartiles in the 10,000 permutations in each distance interval; medians of the data are shown as bands in the boxes; the whiskers

represent the smallest and biggest datum that are still within 1.5 times interquartile range

of the lower and upper quartile, and the outliers are shown as black dots.

S3 Fig. Relative abundance of Pfam domains in the *Striga hermonthica* secretome

or in the rest of the proteome (non-secretome). The relative abundance of each Pfam

domain is plotted as a percentage of all Pfam domains found in the secretome or the non-

secretome. The 45 Pfam domains with the highest difference in relative abundance

between the two data sets are shown.

S4 Fig. Functional categorization of 4 subsets of proteins selected from the *Striga*

***hermonthica* secretome.** *S. hermonthica* proteins were assigned into functional groups

based on the closest BLASTp match with *Arabidopsis thaliana*. If no clear annotation was

available, the closest match with *Oryza sativa* was used. If no clear matches were obtained

for either *A. thaliana* or *O. sativa,* the Pfam domains were used to infer function. Subset 1:

small, cysteine rich proteins. Subset 2: proteins with Pfam domains enriched in the

secretome of *S. hermonthica* to a greater degree than in the secretome of *Mimulus*

guttatus. Subset 3: proteins with no known PFAM domain or orthogroup assignment.

Subset 4: proteins with a BLASTp match against a protein in the pathogen-host interaction

database. For subsets 1, 2 and 4, all *S. hermonthica* protein functions which were only

represented once were categorized as 'other'. Subset 3 only contained 25 *S. hermonthica*

proteins and therefore all protein functions were shown, even if they only occurred once.

Venn diagram shows the overlap between the four subsets of secreted *S. hermonthica*

proteins.

S5 Fig. | Mean ΔAICcv in relation to the numbers of SNPs in 1kb windows in genic regions.

ΔAICcv is a measure of the magnitude, and consistency across pools, of the allele

frequency difference between *S. hermonthica* growing on resistant NERICA 17 (virulent)

and those growing on susceptible NERICA 7 (control). Significant windows in genic regions

contributing to the secretome (red) and non-secretome (green) were identified by permutation.

S6 Fig. Comparison of Tajima's D for the 152 putative VFs (green) and all the genes in the genome (grey) for the control pools. The 152 candidate loci in the proteome had significantly elevated D on average ($p < 0.0001$ 10^5 permutations) compared to the rest of the genome.

S7 Fig. Distribution of mean ΔAICcv difference to the maximum ΔAICcv ratios in each distance interval. The upper and lower limits of the boxes are the first and third quartiles of the ratios in each distance interval; medians of the data are shown as bands; means are shown in red dots; the whiskers represent the smallest and biggest datum that are still within 1.5 times interquartile range of the lower and upper quartile, and the outliers are shown as black dots.

Supplementary Tables 1 – 7.xlsx

S1 Table. Summary statistics for the Striga hermonthica genome assembly. Only contigs / scaffolds with length ≥ 1000 bp are considered). Final assembly was generated using Chicago and HiC scaffolding, which allowed substantial improvement in the N50.

S2 Table. Repeat elements identified in the *Striga hermonthica* genome.

S3 Table. Comparison of the *Striga hermonthica* genome annotation with other plant species.

S4 Table. BUSCO completeness analysis using 2,326 core orthologous genes for eudicots (version: eudicots_odb10). The parasitic plant genomes have lower BUSCO completeness compared with their non-parasitic close relatives. *Striga hermonthica* BUSCO completeness is very similar to that obtained for the recently assembled *S. asiatica* genome.

S5 Table. (a) The *Striga hermonthica* putative secreted, or non-secreted proteins with a

hit against an *Arabidopsis thaliana* protein were first identified and then used as the input

into the SUBA4 online database (https://suba.live) to identify the consensus subcellular

location based on bioinformatic and experimental evidence. (b) The number of *A.*

thaliana proteins for each subcellular location was recorded and presented as a

percentage of the total.

S6 Table. Sequencing information for the *Striga hermonthica* reference genome and

pooled samples for BSA analysis.

S7 Table. Plant species included in the analysis of genome size, heterozygosity and

repetitiveness.

Supplementary Data

S1 Data.xlsx is associated with Fig 4 and S3 Fig. The file contains all data associated

with the Pfam enrichment analysis of the secretome of *Striga hermonthica* and *Mimulus*

guttatus.

S2 Data.xlsx is associated with Fig 6. The file contains the list of 152 non secreted and

38 secreted virulence factors with Gene IDs, protein lengths, cysteine % content, details

of SNPs in different regions of the genes, analysis of Tajima's D and Pfam and functional

annotation.

S3 Data.xlsx is associated with Fig 6. The file contains the FPKM values used for

calculation of gene expression changes in *S. hermonthica* haustoria following infection of

the rice variety NERICA 7 for the 152 candidate, non-secreted and 38 secreted virulence

factors, together with gene IDs and annotation.

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Fig. 1. | Experimental strategy for the identification of *Striga hermonthica* virulence loci. *Striga hermonthica* (Kibos accession) were grown on susceptible (NERICA 7) and resistant (NERICA 17) rice hosts (**a**). The whole rice root systems show many *S. hermonthica* individuals parasitising the roots of NERICA 7 (i) whilst only two individuals (red circles) were able to overcome the resistance response of NERICA 17 (ii) Scale = 1 cm. Transverse sections show *S. hermonthica* invading rice roots for a representative susceptible (iii) and resistant (iv–vi) interaction seven days post inoculation. In the successful host-parasite interaction parasite intrusive cells (PIC) have breached the endodermis and have made connections with the host's xylem (iii). In the resistant rice variety several phenotypes are observed; The parasite invades the host root cortex but is unable to penetrate the suberized endodermis (iv, v); the parasite penetrates the endodermis but is unable to form connections with the host xylem (v). H = host root. P = parasite. Scale = 5 μm. Our experimental strategy was based on the prediction that many *S. hermonthica* genotypes would grow on NERICA 7 but only highly virulent genotypes would grow on NERICA 17 (**b**). Samples of 100 *S. hermonthica* plants were bulked to generate three sequencing pools from each host variety (**c**). We expected that background loci would not differ in allele frequency between pools, but virulence alleles (and neutral alleles in linkage disequilibrium) would have increased frequency in all pools from the resistant host, allowing us to identify candidate loci (**d**).

Fig. 2. | *Striga hermonthica* **is an obligate outbreeding parasitic plant with a highly heterozygous and repetitive genome. a,** Flowering *S. hermonthica* growing on the rice host, NERICA 7, derived from a seed batch collected from the Kibos region of Kenya. Scale = 5 cm. **b,** Comparison of genome size, heterozygosity and repetitiveness between *S. hermonthica* and 12 other plants. The estimate of the genome size (Mbp) was based on k-mer count statistics. The estimate of heterozygosity was based on variant branches in the k-de Bruijn graph. The repetitiveness of the genomes was based on frequency of repeat branches in the k-de Bruijn graph. K: k-mer length. **c,** Genomic features calculated in 1 Mbp windows with a slide of 250 kbp for the largest 40 scaffolds in the *S. hermonthica* genome assembly. Outer bar plot (red): gene density (percentage of the window comprised of genic regions). Mid bar plot (blue): repeat density (percentage of window comprised of repetitive sequence). Inner line plot (green): nucleotide diversity (mean Pi for genic regions). Axes tick marks around plot circumference denote 4 Mbp. Vertical axis tick marks are defined in the centre.

Fig. 3 | a, BUSCO completeness analysis for *Striga hermonthica* genome, compared with 16 other published plant genomes. The number of missing BUSCOs for two Striga **b** and two Cuscuta species **c**. The overlap shows genes that are missing from both Striga or Cuscuta species respectively.

Fig. 4. | Orthogroup analyses. a A time tree for *S. hermonthica* and 12 other species generated in MEGA, based on 42 single-copy genes inferred from OrthoFinder. The number of significantly expanded (red) and contracted (blue) orthogroups based on CAFE analysis are shown above the branches. **b** Significantly expanded orthogroups in *S. hermonthica*, after removing proteins encoded as transposable elements, compared to 12 other plant species. Orthogroups only found in *S. hermonthica*, have family names in red. Higher Z-scores indicate the orthogroups are more expanded in a species while lower Z-scores indicate the orthogroups are more contracted in a species.

a, **b**, **c** panels figure.

Panel b table:

		Secretome	Non-secretome
Number of proteins		3,375	26,143
Protein length (aa)	Mean	377 [a]	400 [a]
	Standard error	4.24	1.81
	Minimum	49	49
	Maximum	3,890	5,081
Cysteine content (%)	Mean	2.28 [b]	1.91 [b]
	Standard error	0.03	0.01
	Minimum	0	0
	Maximum	12.87	20.48

[a] Mean protein length was significantly shorter for secreted proteins compared with non-secreted proteins (Welch two sample t-test: -5.03, df = 4700, p-value = 5.052e-7).

[b] Secreted proteins had a significantly greater % cysteine content compared with non-secreted proteins (Welch two sample t-test: t = 11.77, df = 3844, p-value < 2.2e-16)

Panel c table:

Key	Pfam accession	Pfam domain description
1	PF02469:	Fasciclin domain
2	PF14368:	Probable lipid transfer
3	PF00759:	Glycosyl hydrolase family 9 (cellulase)
4	PF00722:	Glycosyl hydrolases family 16
5	PF05938:	Plant self-incompatibility protein S1
6	PF04043:	Plant invertase/pectin methylesterase inhibitor
7	PF00190:	Cupin
8	PF06955:	Xyloglucan endo-transglycosylase (XET) C-terminus
9	PF01650:	Peptidase C13 family
10	PF05686:	Glycosyl transferase family 90
11	PF03662:	Glycosyl hydrolase family 79-- N-terminal domain
12	PF00150:	Glycosyl hydrolase family 5 (cellulase)
13	PF00188:	Cysteine rich secretory protein family
14	PF00112:	Papain family cysteine protease
15	PF00704:	Glycosyl hydrolases family 18
16	PF01095:	Pectinesterase
17	PF14416:	PMR5 N terminal Domain
18	PF13839:	GDSL/SGNH-like Acyl-Esterase family in Pmr5 & Cas1p
19	PF01501:	Glycosyl transferase family 8
20	PF08263:	Leucine rich repeat-terminal domain
21	PF12708:	Pectate lyase superfamily protein
22	PF10502:	Signal peptidase-- peptidase S26

Fig. 5. | *Striga hermonthica* **secretome. a,** Relationship between protein length (log scale) and cysteine content (as a % of total amino acid number) for putatively-secreted (blue) and non-secreted (red) proteins in the *S. hermonthica* proteome. Secreted proteins < 500 amino acids in length and with a cysteine % > 1 standard deviation above the mean, were selected as a subset of small, cysteine rich proteins. **b,** Descriptive statistics for length and cysteine content for secreted and non-secreted proteins. **c,** Pfam domains enrichment (log fold-change) in the *S. hermonthica* secretome, relative to the proteome as a whole, compared to the corresponding enrichment in the *Mimulus guttatus* secretome. INF denotes infinite enrichment (Pfam domain only found in the secretome). Points above the 1:1 diagonal were enriched more in the *S. hermonthica* secretome relative to *M. guttatus* and have been coloured accordingly. Red symbol: domains only enriched in the *S. hermonthica* secretome. Yellow symbol: domains enriched more in the *S. hermonthica* secretome than in the *M. guttatus* secretome. Green symbol: domains enriched more in the *M. guttatus* secretome than in the *S. hermonthica* secretome. Blue symbol: domains present only in the secretome in both species. Sizes of the points were weighted according to the frequency of occurrence of each Pfam domain in the *S. hermonthica* secretome. Annotations for the most significantly enriched of the Pfam domains (p < 0.01) that were also enriched more in the *S. hermonthica* secretome relative to the *M. guttatus* secretome, are given in the accompanying table with their functional descriptions.

Gene ID	Proportion of differentiating SNPs	SNP presence P	SNP presence NS	SNP presence I	Tajima's D Con	Tajima's D Vir	Annotation of closest matching *Arabidopsis thaliana* protein (BLASTp)	Pfam domain(s) descriptions
SHERM_12802	0.3	x		x	-0.286	-0.030	β-galactosidase	Galactose binding lectin domain; Glycosyl hydrolase family 35
SHERM_14338	0.12	x	x	x	-0.412	-0.359	β-(1-2)-xylosyltransferase	Protein of unknown function (DUF563)
SHERM_01732	0.18			x	-0.371	-0.131	β-glucosidase	Glycosyl hydrolase family 1
SHERM_23996	0.41	x			-0.755	-0.639	COBRA-like protein	COBRA-like protein
SHERM_12118	0.21		x		-0.936	-0.664	Exostosin	Exostosin family
SHERM_11713	0.24	x			-0.069	-0.153	Exostosin	Exostosin family
SHERM_24347	0.36	x	x	x	-0.326	-0.239	Expansin	Pollen allergen; Lytic transglycolase
SHERM_20042	0.38	x			-0.623	-0.600	Glycosyl hydrolase	Glycosyl hydrolase family 3 C-terminal; Glycosyl hydrolase family 3 N-terminal
SHERM_15460	0.28	x	x		-0.577	-0.433	Pollen allergen & extensin	Pollen proteins Ole e I like
SHERM_00887	0.33	x		x	-0.426	-0.223	Peroxidase	Peroxidase
SHERM_19992	0.39	x			-0.556	-0.473	Protein trichome birefringence-like	PMR5 N-terminal; GDSL/SGNH-like Acyl-Esterase found in Pmr5 & Cas1p
SHERM_16484	0.12	x	x		0.107	-0.793	Protein trichome birefringence-like	PMR5 N-terminal; GDSL/SGNH-like Acyl-Esterase found in Pmr5 & Cas1p
SHERM_00245	0.23	x		x	-0.396	-0.351	α/β-hydrolase	α/β-hydrolase family
SHERM_20624	0.23		x	x	-0.162	-0.061	Cytochrome c1,2-heme protein	Cytochrome C1 family
SHERM_02175	0.29	x			-0.705	-0.645	PAR1 protein	PAR1 protein
SHERM_13450	0.5	x	x		0.108	0.286	Aquaporin SIP2-1	None
SHERM_13105	0.37	x		x	0.096	-0.004	Plastid-lipid-associated protein	PAP_fibrillin
SHERM_12715	0.16	x		x	0.152	0.237	Protein OSB1	None
SHERM_24308	0.36	x		x	-0.305	-0.231	RING/U-box protein	Ring finger
SHERM_19811	0.4	x			0.714	0.752	RNA pseudouridine synthase	RNA pseudouridylate synthase
SHERM_19595	0.21	x		x	0.302	0.408	Cysteine proteinase	Papain family cysteine protease; Cathepsin propeptide inhibitor domain (I29)
SHERM_16482	0.34	x	x	x	-0.482	-0.319	Aspartyl protease	Xylanase inhibitor N-terminal
SHERM_26730	0.67	x	x		-0.450	-0.527	Aspartyl protease	Xylanase inhibitor N-terminal; Xylanase inhibitor C-terminal
SHERM_20834	0.14	x			-0.450	-0.527	Subtilase	Subtilase family; Peptidase inhibitor I9
SHERM_16883	0.17	x			0.398	0.369	Subtilase	Subtilase family; Peptidase inhibitor I9; PA domain
SHERM_08443	0.75		x		-1.407	-1.374	Subtilisin-like protease	Subtilase family; Peptidase inhibitor I9; PA domain
SHERM_24050	0.22	x	x	x	0.659	0.699	Receptor-like protein kinase	Protein kinase; Protein tyrosine kinase; Carbohydrate-binding protein of the ER
SHERM_01541	0.33	x	x		1.003	1.713	Receptor-like protein kinase	Protein kinase; Protein tyrosine kinase; Carbohydrate-binding protein of the ER
SHERM_02417	1	x		x	-0.509	-0.583	UMP-CMP kinase	Adenylate kinase
SHERM_28852	0.44	x			-0.262	0.208	GDSL esterase/lipase	GDSL-like Lipase/Acylhydrolase
SHERM_20637	0.17	x		x	-0.668	-0.669	Triacylglycerol lipase 2	Partial α/β-hydrolase lipase region; Putative esterase
SHERM_02635	0.5	x			-0.240	-0.306	Pentatricopeptide repeat protein	PPR repeat; PPR repeat family
SHERM_03263	0.36	x		x	-0.921	-0.931	Unknown protein	None
SHERM_13437	0.39	x			-0.525	-0.725	Unknown protein	None
SHERM_03853	0.19	x			-0.154	-0.251	Unknown protein	None
SHERM_25707	0.38	x			-0.934	-0.946	Unknown protein (DUF593)	Zein-binding
SHERM_00726	0.16			x	-0.424	-0.626	Unknown protein (DUF604)	Protein of unknown function (DUF604)
SHERM_01710	0.24	x	x	x	-0.582	-0.671	Unknown protein	None

Fig. 6. | Identification of *Striga hermonthica* genes that display significant allele frequency differences between pools of individuals parasitising the susceptible rice variety (NERICA 7) and those that successfully parasitise the resistant rice variety (NERICA 17). a Functional categorisation of non-secreted proteins and secreted, candidate virulence factors (VFs). **b** The 38 genes encoding putative secreted *S. hermonthica* proteins with their associated measure of differentiation (proportion of differentiating SNPs within the significant window) between the control and virulent sets of pools. The presence of SNPs in the promoter region (P), non-synonymous SNPs in the coding region (NS) and those in the intronic regions (I) are indicated with an X. The annotation of the closest matching *Arabidopsis thaliana* protein is shown along with coloured boxes that correspond to the functional category assigned in the pie chart in **a**. Tajima's D was calculated for individuals grown on NERICA 7 (Con) or NERICA 17 (Vir). **c**. Clustered gene expression profiles of the 38 candidate VFs in *S. hermonthica* haustoria parasitising NERICA 7 at 2, 4 and 7 days post-inoculation (dpi). Log$_2$ fold change in expression is shown relative to expression levels in haustoria induced *in vitro*. The gene IDs and putative functions based on best BLASTp hit against the *A. thaliana* proteome correspond with part **b**. Significant changes in gene expression in haustoria during the infection time course are shown *** ($p < 0.001$); ** ($p < 0.01$); * ($p < 0.05$); ns non-significant (ANOVA). **d.** Comparison of Tajima's D for the 38 putative VFs (red) and all the genes in the genome (grey) for the control pools.