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SHR4z, a novel decoy effector from the haustorium of the parasitic weed *Striga gesnerioides*, suppresses host plant immunity

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Summary

- Cowpea (*Vigna unguiculata*) cultivar B301 is resistant to races SG4 and SG3 of the root parasitic weed *Striga gesnerioides*, developing a hypersensitive response (HR) at the site of parasite attachment. In contrast, race SG4z overcome B301 resistance and successfully parasitizes the plant.
- Comparative transcriptomics and *in silico* analysis identified a small secreted effector protein dubbed Suppressor of Host Resistance 4z (SHR4z) in the SG4z haustorium that upon transfer to the host roots causes a loss of host immunity (i.e., decreased HR and increased parasite growth). SHR4z has significant homology to the short leucine-rich repeat (LRR) domain of SERK family proteins and functions by binding to VuPOB1, a host BTB-BACK domain containing ubiquitin E3 ligase homolog, leading to its rapid turnover.
- VuPOB1 is shown to be a positive regulator of HR since silencing of VuPOB1 expression in transgenic B301 roots lowers the frequency of HR and increases the levels of successful SG4 parasitism and overexpression decreases parasitism by SG4z.
- These findings provide new insights into how parasitic weeds overcome host defenses and could potentially contribute to the development of novel strategies for controlling *Striga* and other parasitic weeds thereby enhancing crop productivity and food security globally.

Key words: Effector, haustorium, parasitic weed, resistance, *Striga*, *Vigna unguiculata*

Introduction

Parasitism has evolved multiple times within the angiosperm kingdom and today parasitic weeds are found in a wide variety of ecological habitats around the world (Barkham et al., 2007; Westwood et al., 2010). Parasitic weeds derive all (holoparasitic) or part (hemiparasitic) of their nutrition from their host plants by attaching to either the roots or aerial parts with a unique organ, the haustorium, which forms a physical and physiological conduit between host and parasite (Kuijt, 1969). While most parasitic weeds remain innocuous members of plant communities, some have established themselves as noxious and persistent pests in agricultural fields and pose a serious constraint to crop productivity. Among the latter are the witchweeds (*Striga* spps.), a genus of root hemiparasites which currently infest about two-thirds of the farmland under cultivation in sub-Saharan Africa (Parker, 2009). It has been estimated that witchweed infestations cause losses of yield in excess of 10 B USD annually (Scholes and Press, 2008), directly affecting the lives and livelihoods of more than 300 million small share-holder, low-input farmers in this region.

Striga species form two main groupings based on host preference (Mohamed et al., 2001). One group, that includes *S. hermonthica, S. asiatica*, and *S. aspera*, parasitizes members of the Poaceae (grass family) including the agronomically important food and forage grains: maize (*Zea mays*), sorghum (*Sorghum bicolor*), rice (*Oryza sativa*), and pearl and finger millets (*Pennisetum glaucum, Eleusine coracana)*. The second group includes *S. gesnerioides*, the most morphologically variable and widely distributed of the witchweeds, which preferentially attacks dicots, including wild and cultivated legumes, and members of the Convolvulaceae, Euphorbiaceae and Solanaceae (Mohamed et al., 2001). Among the primary hosts for *S. gesnerioides* is cowpea (*Vigna unguiculata* L. Walp), the most important food and forage legume in the African Sahel (Timko and Singh, 2008; Singh, 2014).

While most cowpea cultivars are susceptible to parasitism by *S. gesnerioides*, several resistant landraces and local accessions have been identified (Timko and Singh, 2008; Lane, et al., 1993). Resistance in these cowpea genotypes takes on one of two different phenotypic manifestations: a hypersensitive response (HR) at the site of parasite attachment to the host root with a subsequent discoloration and death of the parasite within 3-4 days, or little to no host root response at the

attachment site but subsequent arrested growth of the parasite seedling early in the tubercle swelling stage of development (Lane, et al., 1993; Botanga and Timko, 2005). In the case of tubercle arrest, the parasite fails to make vascular connections with the host and shows no evidence of cotyledon expansion. Lane et al (1993) were the first to document that some cowpea genotypes (cultivars and local accessions) showed a differential ability to be parasitized by *S. gesnerioides* isolates collected from different geographical locations, leading to the suggestion that distinct races of the parasite exist in West Africa (Lane, et al., 1993; 1994). Subsequent studies of host differential resistance responses and molecular phylogenetic analysis of parasite isolates led Botanga and Timko (2005) to propose that at least 7 distinct races of *S. gesnerioides* parasitic on cowpea are present West Africa. These were designated: SG1 (Burkina Faso), SG2 (Mali), SG3 (Nigeria and Niger), SG4 (Benin), SG4z (localized to the Zakpota region of Benin), SG5 (Cameroon), and SG6 (Sénégal). SG4 and SG4z are virtually indistinguishable based on their molecular genetic profiles (Botanga and Timko, 2006) and SG4z appears to be a recently evolved variant of SG4 that originated in farmer's fields after prolonged cultivation of B301, a cowpea cultivar widely grown because of its prior resistance to all known races of *S. gesnerioides* in West Africa (Supplemental Figure S1). The breakdown of resistance under host-driven selective pressure is anticipated since in all reported studies thus far, race-specific resistance is conferred by single dominant genes distributed in two gene clusters in the cowpea genome (Singh and Emechebe, 1990; Timko and Singh, 2008). Using a molecular marker-assisted positional cloning strategy, Li and Timko (2009) subsequently isolated the *RSG3-301* gene from cowpea that confers resistance to *S. gesnerioides* race SG3 and showed that it encodes a typical nucleotide-binding domain and leucine-rich repeat containing (NLR) protein with a N-terminal coiled-coil domain (CC), followed by a central nucleotide binding site (NBS) and a C-terminal leucine-rich repeat (LRR) domain. The characterization of *RSG3-301* led to the suggestion that race-specific *Striga* resistance in cowpea is an example of effector-triggered immunity (ETI) in which intracellular NLR proteins (such as RSG3-301) are activated either directly or indirectly upon recognition of pathogen/parasite effectors (Oren et al., 2016, Jubic, et al., 2019) .

The nature of the gene expression changes occurring during compatible and incompatible *Striga*-cowpea associations was examined by Huang et al (2012) who documented dramatically different

expression profiles in the roots of B301 plants being attacked by *S. gesnerioides* races SG3 and SG4z. These investigators specifically noted that many of the genes and pathways up-regulated in the RSG3-301-mediated HR to SG3 attack were unaltered or repressed in the susceptible interactions to SG4z, suggesting that the parasite is targeting specific components of host defense mechanism (Huang, et al., *2012*).

In this study, comparative transcriptomics and *in silico* computational analysis was used to identify a small soluble effector protein that is highly expressed in the SG4z haustoria transferred to the host root, and capable of suppressing the host innate immunity by binding to a host BTB-BACK domain containing ubiquitin E3 ligase homolog (VuPOB1). We show that silencing of VuPOB1 expression in transgenic B301 lowers the frequency of HR and increases the levels of successful parasitism by SG4, whereas overexpression of VuPOB1 resulted in decreased parasitism by SG4z suggesting VuPOB1 functions as a positive regulator of the HR response. These studies provide novel insight into the role of secreted effectors as part of the strategy used by parasitic weeds overcome host immunity to complete their life-cycles.

Materials and Methods

Plant Materials. All experiments involving viable *S. gesnerioides* seeds, developing parasites, and host-parasite interactions, were performed in an APHIS-approved quarantine facility in the Department of Biology at the University of Virginia (Facility Number 669; APHIS Plant Protection and Quarantine Permit No. P526P-14-03013). *S. gesnerioides* seeds were collected from plants growing on cowpea cultivars in the following locations: SG3 (Maiduguri, Nigeria - 2008), SG4 (Cana, Benin - 2009) and SG4z (Zakpota, Benin - 2006)) (Supplemental Figure S1). Seeds of the multi-race resistant cowpea cultivar B301 were provided by Dr. Lucky Omoigui (University of Agriculture Mukurdi, Nigeria) and California Blackeye no. 5 (BE) seeds were purchased commercially (W. Atlee Burpee & Co., Warminster, PA).

***Striga*-host root interactions assays and statistical analysis.** Seeds of the *S. gesnerioides* race SG4 and SG4z were surface sterilized with 10% bleach for 10 mins and pre-conditioned at 30˚C for 9 days.

Seeds were pre-germinated by incubation with cowpea root exudates for 2 days (Mellor et al., 2012) and then gently transferred to transgenic and non-transgenic cowpea roots using a paintbrush. At 10, 20, and 30 days post-inoculation (dpi) the occurrence number of all parasite-host root interaction events (i.e., attachment, tubercle swelling, cotyledon expansion and hypersensitive response) were scored and the percentage for each interaction type was determined. Ten (10) or more individual plants were used per treatment and the occurrence percentage for each interaction event were subject to paired t-test for statistical significance using R version 3.2.2.

Transcriptome profiling using RNA HiSeq analysis. For RNA-seq library preparation, parasite seedlings were removed from the host roots directly into RNAlater (Ambion, Inc., Austin, TX), quickly frozen in liquid Nitrogen, and stored at –80˚C until used for RNA extraction. Experiments were conducted in triplicate for all parasite races at 2 host genotypes and 3 time-point conditions. Host tissue was harvested as previously described (Huang et al., 2012). Library preparation for subsequent Illumina single-end sequencing was carried out as previously described (Yang et al., 2015). Complete details on RNA-seq library preparation from host and parasite, transcriptome assembly, and gene annotation are provided in Supplemental Data, Methods S1.

Identification of differentially expressed race-specific effector candidates. High-quality Illumina reads from each stage-specific library were mapped to the reference transcriptome via Bowtie2 (Langmead and Salzberg, 2012). Transcript abundance was evaluated independently in each library with eXpress v1.5.1 (Roberts et al., 2011) using the default settings (except --r-stranded) and Transcripts Per Million (TPM) were calculated to represent transcript abundance and used for expression visualization. Raw read counts were normalized and processed for differentially gene expression analysis using DESeq2 v1.6.3 (R version 3.2.2) (Love et al., 2014). The two factor full generalized linear model (GLM) was used to exam race differential expression at each developmental stage. Race-specific expressed genes were defined as genes differentially expressed during the interactions with both resistant and susceptible hosts.

To identify potential candidate effectors, genes differentially expressed across different developmental stages were first subjected to intuitive classification based on stage pairwise

comparison (Supplemental Table S1). We expect effectors to be highly expressed during the initial interaction of parasites with hosts. We anticipate three potential gene expression groupings: (i) genes with continuous high expression during the two attachment stages; (ii) genes with expression peaks at the early attachment stage; and (iii) genes with expression peaks at the late attachment stage. Following expression analysis, all contigs with complete protein coding regions were examined for the presence of signal peptides and transmembrane domains using SignalP (Petersen et al., 2011) and TMHMM (Krogh et al., 2001). Contigs predicted to contain signal peptides and no transmembrane domain were further examined for the presence of ER localization sequences by scanning using the PROSITE database (Hofmann et al., 1999). Contigs containing signal peptides, lacking transmembrane domains, and lacking ER localization domains (i.e., predicted to be transport to extracellular region) were included in the "secretome" data set. Contigs in the secretome data set with expression patterns thought to match those of candidate effectors as described above were placed in the "effector candidates" group Supplemental Table S2). A large number of contigs did not have complete coding regions and in particular could not be analyzed for signal peptides at their 5' ends. Those contigs that met all of the other criteria (i.e., lacking transmembrane domains, ER retention signals, and haustorial specific expression peaks in post-attachment stages of the host-parasite interaction) were also included in "effector candidate" group. A detailed description of the methodology used in subsequent 3' and 5' RACE cloning of effector candidates from haustorial RNAs, and for determining effector gene structure in genomic DNA can be found in the Methods S1.

Construction of transgene expression plasmids and generation of *ex vitro* composite plants. The full length SHR4z protein was predicted based upon the first ATG at 5' end and longest open reading frame (ORF) to an in-frame stop codon and this coding region was cloned and mobilized into Gateway® pDONR™221 Vector (Invitrogen, Carlsbad, CA) using Gateway BP Clonase Enzyme mix (Invitrogen, Carlsbad, CA). A truncated version of the SHR4z full length protein in which the predicted signal peptide was removed (SHR4z**Δ**SP) was also cloned into the Gateway® pDONR™221 Vector. In SHR4z**Δ**SP an ATG codon was inserted at 5' end of truncated sequence to allow for translation initiation. Both pDONR-SHR4z and pDONR-SHR4z**Δ**SP were further recombined with pK7WG2D (Karimi et al., 2002) using the LR recombination reaction in the

Gateway LR Clonase Enzyme mix (Invitrogen, Carlsbad, CA). pK7WG2D expressing small FLAG-tag were used as control. All pK7WG2D plasmids (pK7WG2D-SHR4z, pK7WG2D-SHR4z**Δ**SP and pK7WG2D-FLAG) were transformed into *Agrobacterium rhizogenes* strain R1000 for heterogeneously expression in cowpea cultivar B301.

B301 seeds were sterilized with 10% (v/v) bleach for 10 min and pre-germinated on sterile rockwool. *Ex vitro* composite plants were then generated as previously described (Mellor et al., 2012) using *A. rhizogenes* strains carrying the pK7WG2D-SHR4z, pK7WG2D- SHR4z**Δ**SP and pK7WG2D-FLAG plasmids. Seedlings with regenerated roots were moved to rhizotrons 20 d after transformation and grown at 30˚C for 14 d before *Striga* inoculation. At 10, 20, and 30 dpi parasite-host root interaction events were scored and the percentage for each interaction type was determined for non-transgenic (no visible GFP) and transgenic (GFP expressing) roots, respectively (see Methods S1).

Yeast two-hybrid (Y2H) screening. Total RNA from *Striga*-infected host roots was isolated and used to construct a normalized cDNA 'prey' library in a modified pDEST22 vector (Bio S&T Inc., St. Laurent, Quebec, Canada). Y2H screening was performed using ProQuest system (Invitrogen). PDONR-SHR4z**Δ**SP was recombined with pDEST32 to generate DNA binding domain 'bait' fusion (pDEST32-SHR4z**Δ**SP). PDEST32-SHR4z**Δ**SP was transformed to yeast strain MaV203 and positive transformants selected by planting on Leu deficient YNB plates (Leu-YNB). A single verified transformant was selected for the preparation of competent cells and the competent cells were then transformed with the cowpea host root cDNA 'prey' library. The resulting transformants were plated on triple amino acid deficient YNB plates (Leu-/Trp-/Ura- plates) to select interaction-positive clones. The putative host interacting proteins candidates were further characterized and sequenced and one such candidate subject to further analysis (see Methods S1). A transcript (UP12_14781) encoding the full length cowpea VuPOB1 protein was retrieved from Cowpea EST database (Version 1.42 of "HarvEST:Cowpea"). Coding frame of VuPOB1 was determined by selecting the longest CDS with 100% match to Y2H in-frame insertion and cloned from a B301 root cDNA library using gene specific primers (Table S3, BTB_ORF_F and BTB_ORFnoT_R).

Co-immunoprecipitation and bimolecular fluorescence complementation (BiFC) analysis. Epitope tags myc- and HA- were added to C-terminals of protein SHR4z**Δ**SP and VuPOB1 by PCR. The purified PCR products were ligated into YFP vectors SPYCE and SPYNE to generate expression constructs myc- SHR4z**Δ**SP-YN and HA-VuPOB1-YC. Agroinfiltration was performed on three of the largest leaves of a 6 week-old *N. benthamiana* plant as previously described (Wydro et al., 2006). Assays were conducted in the presence or absence of MG132 (100 μM) infiltrated into leaves 6 h before harvesting of tissue for co-immunoprecipitation and immunoblotting (see Methods S1). BiFC and confocal microscopy was performed at 3 d post initial infiltration of leaf tissues with Agrobacterium harboring the above constructs. Leaves were treated with MG132 (100 μM) for 6 h before harvesting and examined under Leica sp8 scanning confocal microscope. Split-YFP was excited with 488 nm from laser with emissions collected between 497 – 544 nm.

Ectopic overexpression and RNAi silencing of VuPOB1 in B301 roots. An ~ 250 bp fragment within the coding region of VuPOB1 was amplified and mobilized into the Gateway® pDONR™221 Vector (Invitrogen, Carlsbad, CA) and then recombined into pK7GWIWG2D(II) (Karimi et al., 2002) to generate inverted repeat construct pK7GWIWG2D-VuPOB1-RNAi. The full length PCR amplicon of VuPOB1 was mobilized into a Gateway® pDONR™221 Vector (Invitrogen, Carlsbad, CA) generating pDONR-VuPOB1 (with C-terminal stop codon) and subsequently recombined with pK7WG2D (Karimi et al., 2002) to generate overexpression construct pK7WG2D-VuPOB1. The sequence verified vectors were then transformed into *A. rhizogenes* R1000 and used to generate *ex vitro* composite B301 plants. Transgenic and non-transgenic roots were analyzed by qRT-PCR to confirm silencing of VuPOB1 expression (see Methods S1) in B301 roots.
Cowpea composite plants expressing the pK7WG2D-VuPOB1overexpression construct or RNAi silencing construct pK7GWIWG2D-VuPOB1-RNAi were moved to rhizotrons and grown for 14 d at 30˚C, inoculated with *Striga* seedlings, and the occurrence percentage for each interaction event score (see above and Methods S1).

Results

Differential response of cowpea cultivar B301 to different races of *S. gesneriodies.*

Seeds of *S. gesnerioides* germinate in response to the presence of chemical signals (strigolactones) in the rhizosphere released from the roots of potential host plants (Bouwmeester et al., 2003). Two day-old germinated seedlings of the various *S. gesnerioides* races parasitic on cowpea in West Africa (e.g., SG4, SG4z, SG3) are phenotypically indistinguishable prior to host contact (Figure 1A, Supplemental Figure S1). However, following attachment to the host root, differences in host and parasite phenotype become evident depending on the partners of the interaction (Botanga and Timko, 2005; Lane et al., 1996). California Blackeye no. 5 (BE) is susceptible to all races of *S. gesnerioides* identified thus far (Botanga and Timko, 2005) and by 3 days post-inoculation (dpi) BE roots show significant numbers of attached *Striga* seedlings from all three races. No obvious discoloration is found on the BE roots at the site of parasite attachment at this early stage. By 10 dpi, all three races of the parasite (SG4, SG4z and SG3) have successfully penetrated the BE root cortex and established connections with the host vascular system. There is the formation of an enlarged tubercle and evidence of cotyledon expansion of the parasite, an indication of successful vascular connection between host and parasite (Figure 1B).

In contrast, a very different phenotypic response is observed in the interactions between the *Striga* races and B301, a cultivar from Botswana resistant to all *Striga* races except SG4z (Botanga and Timko, 2006). At 3 dpi, B301 roots show only very minor distinguishable phenotypic differences when challenged by *Striga* races SG4z, SG4 and SG3 with the only notable effect being the appearance of a slight discoloration or browning of the host root at the attachment interface of SG3 and SG4 (Figure 1A). However, at 10 dpi, dramatically different phenotypic responses are observed among the races (ANOVA p value < 0.01 for AT, CE, HR and TS). Both SG4 and SG3 have elicited an easily visible HR with associated browning and necrosis of the B301 root at the site of parasite attachment. There is also arrested growth of the parasite and a browning of the parasite. The HR reaction of B301 to SG3 challenge is more pronounced than that observed with SG4 (Turkey's HSD adjusted p value = 0.02), with just slightly more arrested growth at the tubercle stage of parasite development than observed in SG4 **(**Figure 1B). In stark contrast, SG4z seedlings fail to elicit a strong

HR response on B301 roots. Similar to what is observed on the susceptible BE, SG4z-B301 interactions have significantly more tubercle swelling events and more frequent observed seedlings with evidence of cotyledon expansion (a characteristic associated with the formation of successful vascular connections with the host) by comparison to SG3 and SG4 –B301 interactions (Turkey's HSD adjusted p value < 0.001).

Transcriptomic profiling of *Striga* races during compatible and incompatible cowpea interactions.

To uncover alterations in gene expression in SG4z that could potentially underlie its ability to overcome or bypass the innate immunity / defense pathways present in B301 activated by the closely related SG4, we generated transcriptome profiles of SG4 and SG4z, and a more distally related race, SG3, during compatible and incompatible interactions with BE and B301 roots. Three developmental stages were sampled: 2 day-old germinated seedlings prior to host contact; parasite seedlings at an early host attachment stage (3 dpi); and parasite seedlings at a late host attachment stage (10 dpi). A total of 45 different libraries were generated and sequenced using an Illumina RNA HiSeq platform yielding more than 300 million 1 X 150 bp single-end reads.

To functionalize this data, we built a hybrid transcriptome assembly (SGall) that combines reads from all races and developmental stages and annotated it to coding proteins using ESTscan (Iseli, et al., 1999).The SGall assembly contains 145,407 protein-coding contigs with a N50 contig length of 1218 bp and captures 89.55% to 98.88% of the conserved single copy genes (Table 1). The quality of the SGall assembly and gene capture frequency are very similar to the haustorial transcriptome assemblies reported from three other root parasitic plants including the closely related *S. hermonthica* (Zhang, et al., 2015). Therefore, we believe that SGall represents the large majority of the expressed genes in the *S. gesnerioides* haustorial transcriptome. When the SGall assembly was compared to comparable developmental-stage specific transcriptome assemblies from *S. hermonthica* (i.e., Stages 1, 2 and 4) by reciprocal blastn (evalue < 1e-10), ~ 63.6% of *S. hermonthica* unigenes found homologous counterparts in the SGall assembly.

To determine which factor contributed most to expression profile differences among the parasite races, we mapped the reads from all 45 RNAseq libraries to the SGall hybrid transcriptome assembly and then performed expression correlation clustering across the 15 conditions (parasite race X developmental time X host genotype). This analysis showed that host genotype and parasite developmental stage contribute significantly to differences among the various parasite race expression profiles (Supplemental Figure S2). At the germinated seedling stage, all three parasite races clustered together indicating that while differences exist among the races, such differences are not pronounced prior to host interaction. As might be expected, given the recent evolutionary derivation of SG4z from SG4 (Lane, et al., 1994; Botanga and Timko, 2006) , SG4 and SG4z samples clustered closer to each other than either did to SG3. Similarly, samples from the same post-attachment developmental stage clustered together, with the exception of those from the late attachment stages where compatible (susceptible) and incompatible (resistant) host-parasite pairing are being analyzed and clear distinctions are evident.

Differences in the SG4 and SG4z transcriptome profiles identify effector candidates.
Differences in gene expression have been suggested to underlie variations in virulence (Whitehead and Crawford, 2006) and therefore we hypothesized that among *S. gesneriodies* races differential expression would account for the differential resistance elicitation of SG4z compared to SG4 in its interaction with B301. Among the possible differences we anticipated that SG4z might have lost or significantly diminished levels of expression of one or more gene product functioning as an avirulence factor that is recognized by B301.This would preclude its detection by the host immunity system allowing SG4z to establish a compatible interaction with B301. Alternatively, SG4z may have altered the expression of an existing gene or gained new gene expression function (e.g., a new effector) that allows it to subvert the B301 defense pathways thereby yielding the host susceptible to parasitism.

To address these possibilities, we first examined the degree to which transcripts are differentially expressed between SG4z and SG4 at the various stages of interaction with BE and B301. Not surprisingly given their recent evolutionary divergence, relatively few contigs (135 in total) were

found to be significantly differentially expressed (FDR < 0.01 log2FoldChange > 1) between the SG4 and SG4z transcriptomes of germinated seedlings (0 dpi) and of these 135 contigs 50 were upregulated in SG4z and 85 were upregulated in SG4 (Supplemental Table S1). A total of 259 contigs were differentially expressed between SG4 and SG4z at the early attachment stage (3 dpi) on B301, whereas a slightly larger number (300 contigs) are differentially expressed at 3 dpi on BE (Supplemental Table S1, Supplemental Figures S3B, S3C). In addition, the magnitude of the expression differences between SG4z and SG4 were significantly increased at 3 dpi compared to 0 dpi (Supplemental Figures S3A; wilcoxon rank sum test p-value < 2.2e-16). At the late attachment stage (10 dpi), 17,242 contigs (~11.9%) were differentially expressed between SG4z and SG4 parasitizing B301 but only 191 contigs (0.13%) were differential expressed in the interaction with BE. This large difference in the number of differentially expressed genes at 10 dpi reflects the fact that one race (SG4z) is participating in a compatible interaction with B301 while the other (SG4) is in an incompatible interaction in which host penetration is arrested and *Striga* is dying (Supplemental Table S1, Supplemental Figures S3B, S3C). A total of 205 and 150 contigs are shared between both interactions (B301 and BE) and are significantly differentially expressed between SG4z and SG4 at 3 dpi and 10 dpi, respectively (Supplemental Figure S3C). Combined, 267 contigs have race-specific differential expression at either post-attachment stages (Supplemental Figure S3C). Hierarchical clustering of these 267 contigs showed that 122 are upregulated in SG4z at either 3 dpi or 10 dpi while 131 have higher expression in SG4 (Supplemental Figure S3D).

Since effectors and avirulence factors would be expected to be released from the haustorium upon interaction of the parasite with its potential host (serving to elicit host defense responses or functioning to suppress them) we hypothesized that there might be significant alterations in their expression relative to germinated parasite seedlings. We also expect that proteins moving from parasite to host would likely contain secretion signals and other structural features to facilitate this movement (Goritschnig, et al., 2012; Hacquard, et al., 2012; Rafiqi , et al., 2013). Therefore, we developed a candidate effector identification pipeline to identify contigs differentially expressed in post-attachment SG4z and SG4 haustoria that encode proteins with the hallmarks of secreted protein effectors (Supplemental Figure S4). Thirty-four (34) of the 267 differentially expressed contigs met

all or most of the criteria for effector candidates under our scheme (Figure 2A; Supplemental Figure 5A).

To verify that the differential expression across developmental stages and interactions based on RNASeq analysis accurately changes in transcript abundance we carried out qRT-PCR on a subset of the candidates (Supplemental Figure S5B). All of those tested confirmed their differential expression and one such validated candidate, contig SGall_040908.1, that reproducibly showed no detectable expression in SG4 and significantly higher (100-fold or greater) expression in SG4z compared to SG3 at both 3 dpi and 10 dpi (Figure 2B). We selected this candidate for further detailed analysis.

Characterization SHR4z, a small soluble protein effector that suppressed host immunity. Contig SGall_040908.1 contains only a partial coding sequence and therefore we performed 5'- and 3'-RACE using total RNA isolated from SG4z haustoria growing on B301 roots to obtain the full length of 942 nt transcript encoding a 195 amino acid protein (Supplemental Figure S6A). The encoded full length protein has a 25 amino acid predicted extracellular (apoplastic) targeting signal peptide at the N-terminus. There are four LRRs in the protein with one located adjacent to the signal sequence at the N-terminus and three arranged in tandem near the C- terminus of the protein (Supplemental Figure S6A). The predicted 3-D structure of the protein indicates that three tandem LRRs forms a stack of beta-bend ribbons which serve as potential protein interaction domain (Fig. S6B). Phylogenetically, the short leucine-rich repeat (LRR) domain of the protein has significant homology to members of the SOMATIC-EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) gene family (Supplemental Figure S11). Based on its unique high level of expression in SG4z haustorium and its demonstrated role in mediating the loss of the HR in SG4z-B301 interactions (described below), we designated the gene as *Suppressor of Host Resistance 4z* (*SHR4z*).

To confirm the presence of the *SHR4z* gene in the parasite genome and define its structure, 5 pairs of primers were designed across the full length coding region and used in genomic PCR amplifications. The *SHR4z* gene was found to consist of 6 exons and 5 introns (Supplemental Figure S6C). Since *SHR4z* transcripts were only detected in SG4z seedlings, we checked to see whether the SHR4z gene

was present in other races of *S. gesnerioides* found in West Africa (Supplemental Figures S6D and S6E). Our data show that while *SHR4z* is present in the genomes all of the *S. gesnerioides* races tested it is not expressed. Sequence analysis of the SHR4z genes amplified from SG4z and the other *S. gesnerioides* races did not uncover any variation in the protein coding regions of the genes and only a small number of sequence variations in the introns. Intriguingly, the ability to amplify *SHR4z* coding sequences from genomic DNA of all individuals within a given race varied, with SG4z, SG3 and SG5 having a significantly higher proportion of individuals (ANOVA p-value < 0.001) (Supplemental Figures S6E) giving amplicons with the primer combinations tested. The reason for this is not known, but may simply reflect that minor variations exist in the gene coding regions that influence the ability of the primers to bind to their target efficiently.

Ectopic overexpression of SHR4z in B301 roots leads to suppression of parasite-elicited HR.
To test the hypothesis that SHR4z is a secreted effector involved in manipulating host root innate immunity, we ectopically overexpressed the full length SHR4z protein and a truncated version of SHR4z lacking the apoplastic targeting signal peptide (SHR4z**Δ**SP) in B301 roots (Supplemental Figures S7A and S7B) using an *ex vitro* composite plant transformation system for cowpea (Mellor et al., 2012). We rationalized that the full length SHR4z is needed to place the protein on the surface of the haustorium where is can be transferred to the host. Thus, we might expect that the full length and truncated SHR4z proteins (minus the targeting signal) function differently depending on whether they were secreted to the cell apoplast or retained in the cytoplasm. As shown in Figure 3A, transgenic B301 roots expressing the C-terminal mCherry fusion of full length SHR4z protein exhibit mCherry expression on the outer surface of the cowpea root cell consistent with the secretion signal targeting the protein to the apoplast. In contrast, transgenic roots expressing SHR4z**Δ**SP, the truncated secretion signal-less version of SHR4z, did not exhibit a similarly strong mCherry signal. However, in the presence of protease inhibitor MG132, the expression of SHR4z**Δ**SP became visible and was mainly concentrated in cytoplasm, suggesting that the truncated protein is turned over rapidly in the cytoplasm.

Transgenic B301 roots expressing the SHR4z and SHR4z**Δ**SP proteins and non-transgenic roots were then challenged with *Striga* race SG4 known to elicit a strong HR in B301 roots (Figures 3B and 3C). Transgenic B301 roots expressing SHR4z**Δ**SP showed a significantly lower frequency of HR events than control non-transgenic roots with SG4 starting at 10 dpi (paired t-test 10 dpi: p-value = 0.03; 20 dpi: p-value < 0.01; 30 dpi: p-value = 0.01) and significantly more tubercle swelling events at 30 dpi (paired t-test p value = 0.01) indicating that the overexpression of SHR4z**Δ**SP in host root was suppressing the host resistance response. We did not observe any cotyledon expansion events on the transgenic B301 roots expressing SHR4z**Δ**SP challenged with SG4. Cotyledon expansion is a well characterized phenotypic indicator of successful vascular connection between *Striga* and it host. The absence of cotyledon expansion on SHR4z**Δ**SP expressing transgenic B301 roots suggests that despite its ability to suppress HR, SHR4z alone may not be sufficient to completely overcome host innate immunity to the extent that it fully allows successful parasite growth.

In contrast, B301 roots expressing the full length SHR4z did not show a corresponding decrease in HR frequency (Supplemental Figure S8). We infer that targeting the SHR4z to the apoplast of the host cell makes it unavailable to influence host defense pathways. Collectively, these findings support a role for SHR4z as a suppressor of the host resistance response in the host cells as we previously speculated.

To test whether SHR4z overexpression increases the susceptibility of B301 to SG4z parasitism, we compared the frequency of successful parasite growth on transgenic B301 roots expressing SHR4z**Δ**SP to that of non-transgenic control roots (Figure 3D). A significant reduction in frequency of HR events was found (paired t-test 10 dpi: p-value=0.01; 20 dpi: p-value < 0.01; 30 dpi: p-value < 0.01), but in general, no significant differences were found in the frequency of cotyledon expansion events between transgenic and non-transgenic roots challenged with SG4z (Figure 3D, paired t-test 20 dpi: p value=0.49; 30 dpi: p value =0.45). Therefore, we infer from this finding that overexpressing SHR4z**Δ**SP in B301 enhances host susceptibility to the already hypervirulent SG4z by suppressing HR but not by promoting parasite growth.

Since resistance to *Striga* in B301 is conferred in a race-specific manner involving a number of different race-specific R genes, we also tested to see whether the resistance response of B301 to other races of *S. gesnerioides* is altered by SHR4z**Δ**SP overexpression. To this end, we inoculated non-transgenic control and transgenic B301 roots expressing SHR4z**Δ**SP with SG3 seedlings (Figure 3E). Transgenic B301 roots overexpressing SHR4z**Δ**SP had a significantly lower frequency of HR events than non-transgenic at 10 dpi (paired t-test p-value < 0.001), but no significant reduction in HR frequency at 20 dpi and 30 dpi (paired t-test 20 dpi: p value=0.33; 30 dpi: p value=0.059), suggesting that subtle differences in avirulence factors could also influence a parasite race's ability to differentially activate resistance mechanisms. The response difference between 10 dpi and later time points could also reflect the switch from basal resistance responses typified by pattern-triggered immunity (PTI) to a more amplified and faster effector triggered responses (i.e., the nexus between PTI and ETI).

SHR4z binds a cowpea homolog of the ubiquitin E3 ligase POB1.

To identify host targets of SHR4z, we carried out a yeast two-hybrid (Y2H) screen using SHR4z**Δ**SP as bait and a cDNA prey library constructed from RNA isolated from B301 roots parasitized by SG4 and SG4z. From a screen of ~2 $X10^6$ colonies, we identified 5 clones that gave a reproducibly strong positive signal (Figure 4A). The inserts from these clones were recovered, sequenced, and annotated and all five were found to contain the same insert encoding a BTB (Bric-a-Brac, Tramtrack, and Broad Complex)-BACK (BTB and C-terminal Kelch) domain-containing protein (Gingerich et al., 2007) with a high level of similarity to the Arabidopsis AtPOB1 and *Nicotiana benthemiana* NbPOB1 proteins (76% and 74% amino acid sequence identity, respectively) (Fig. S9A). Both AtPOB1 and NbPOB1 have been previously identified as essential ubiquitin-protein ligases (E3s) involved in plant immune responses and are thought to ubiquitinate PUB17, a downstream component in the pathway, for subsequent degradation (Orosa et al., 2017).

Using HMM domain searching against the recently published cowpea reference genome assembly (Muñoz-Amatriaín et al., 2017), we identified six BTB-BACK proteins in cowpea. Phylogenetic comparisons (Supplemental Figure S9B) indicate that the cowpea BTB-BACK protein identified in

our screen clusters with the other cowpea BTB-BACK proteins and 3 of six fall in a clade along with the Arabidopsis AtPOB1/AtLRB2, AtLRB1, and tobacco NtPOB1 proteins. Based on its high level of sequence similarity and phylogenetic relatedness, we confidently designated the cowpea interacting protein as VuPOB1 (Vigun10g140300.1). The two additional cowpea BTB-BACK domains proteins that also fall within this cluster we have designated as VuPOB2 (Vigun10g140200.1) and VuPOB3 (Vigun06g120500.1).

The predicted macromolecular structures of SHR4z and VuPOB1 indicate a strong likelihood for interaction between the two proteins (Supplemental Figure S9C). To confirm a physical interaction between VuPOB1 and SHR4z**Δ**SP *in vivo*, bimolecular fluorescence complementation (BiFC) assays were performed using a split YFP system in *N. benthamina* leaves. Single and paired plasmids expressing the SHR4z**Δ**SP-YN and VuPOB1-YC constructs were Agro-infiltrated into *N. benthamiana* leaves in the presence and absence of MG132, a cell-permeable proteasome inhibitor that reduces the degradation of ubiquitin-conjugated proteins. At 3 d post infiltration confocal microscopy was performed. Reconstituted YFP fluorescence could only be seen in the cytoplasm of the transgenic cells expressing both constructs (VuPOB1 and SHR4z**Δ**SP) in the presence of MG132 indicating that these two proteins interact with the host cell cytoplasm (Figure 4B) but that the interaction leads to their rapid turnover.

To further confirm the nature of this interaction co-immunoprecipitation (Co-IP) studies were performed by co-expressing epitope-tagged proteins VuPOB1 and SHR4z**Δ**SP proteins (HA-POB1 and myc-SHR4z**Δ**SP, respectively) in *N. benthamina* leaves. Consistent with our previous observations (see Figure 3A) suggesting that SHR4z**Δ**SP is under rapid proteasome-mediated degradation in plant cells, expression of myc-SHR4z**Δ**SP was barely detectable by immunoblot analysis (Figure 4C: input). However, co-infiltration with the proteasome inhibitor MG132 allows for the detection of the SHR4z**Δ**SP protein alone and as a co-immunoprecipitate with VuPOB1 (Figure 4C: IP) further confirming the interaction of the two proteins *in planta.*

VuPOB1 is a positive regulator of cowpea HR upon *Striga* parasitism.

To uncover how VuPOB1 may be functioning in cowpea innate immunity, we first measured the levels of VuPOB1 transcripts in the various compatible and incompatible cowpea-*Striga* interactions. qRT-PCR analysis of VuPOB1 transcript levels in B301 roots parasitized by SG4z and SG4 showed a substantial but transient increase in VuPOB1 transcript levels at 3 dpi compared to 0 dpi and 10 dpi. In contrast, no significant expression change was observed in BE roots subjected to similar parasitism (Figure 5A). Interestingly, in their analysis of differential gene expression during compatible and incompatible *Striga*-cowpea interactions, Huang et al. (2012) observed that among the genes most significantly up-regulated (5.57- and 6.04-fold induced at 6 dpi and 13 dpi, respectively) in B301 roots parasitized by *S. gesnerioides* race SG3 was VuPOB2, the paralog of VuPOB1 (Huang et al., 2012) (Supplemental Figure S9B). Cumulatively, these findings suggest a role for VuPOB1 (and perhaps the other paralogs) the cowpea resistance response to *Striga*.

To test whether VuPOB1 is necessary for the resistance response of B301, we used RNAi to silence VuPOB1 expression in B301 roots. As shown in Figure 5B, VuPOB1-silenced B301 roots challenged with SG4 showed a significantly lower frequency of HR events (paired t test 10 dpi: p-value < 0.001; 20 dpi: p-value < 0.001; 30 dpi: p-value < 0.001) and a greater frequency of tubercle swellings events (paired t test 20 dpi: p-value=0.04; 30 dpi: p-value=0.01) than non-transformed control B301 roots indicating that VuPOB1 is a necessary and significant component of innate immunity. VuPOB1-silenced B301 roots challenged with SG4z showed significantly fewer HR events (paired t test 10 dpi: p-value < 0.01; 20 dpi: p-value < 0.001; 30 dpi: p-value = 0.01), more tubercle swelling events (t test 20 dpi: p-value < 0.01), but comparable frequency of cotyledon expansion events (Supplemental Figure S10). These data suggest that silencing of VuPOB1 may only marginally enhance susceptibility of B301 to SG4z by suppressing HR but not promoting parasite growth.

When we examined the effects of ectopically overexpressing VuPOB1 on *Striga*-host interactions, a quite strikingly different outcome was observed in the normally successful parasitism of B301 by SG4z parasitism. Rather than the normal compatible interaction, B301 overexpressing VuPOB1 roots

displayed a higher frequency of HR events (paired t test 10 dpi: p-value < 0.01; 20 dpi: p-value < 0.001; 30 dpi: p-value < 0.001) and lower frequency of tubercle swelling (paired t-test 10 dpi: p value=0.049; 20 dpi: p value < 0.01; p value=0.03). More significantly, there were no clear evidence of cotyledon expansion events among attached SH4z parasites (Figure 5C). These data clearly indicate that overexpression of VuPOB1 results in decreased parasitism by SG4z suggesting that VuPOB1 functions as a positive regulator of HR and host immunity in cowpea.

Discussion

In this study we used high-throughput RNAseq based transcriptomic profiling and transgenic expression analysis to identify a novel secreted effector protein, SHR4z, from a root parasitic angiosperm that is capable of modifying host plant immunity. The presence of secreted haustorial effectors was speculated on in our previous studies (Li and Timko, 2009) in which we showed that resistance in cowpea to *S. gesnerioides* is conferred by specific resistance genes, such as *RSG3-301,* hallmark of effector triggered immunity (ETI). The identification of SHR4z, whose unique high level expression in SG4z allows suppression of the HR triggered by SG4, also underscore the fact that the evolution of new components in the arms race between cowpea host and their parasite *Striga* is a continually ongoing process. In this case, host-driven selection of variants capable of overcoming host immunity within the SG4 population was likely facilitated by the repeated cropping of the multirace resistant cultivar B301 in farmer's fields in the Zakpota region of Benin over the several decades since its introduction (Lane et al., 1994). The occurrence of a mutation that resulted in high level expression of SHR4z and its associated suppression of the host plant's ability to mount a HR upon parasite attack, would have allowed these individuals to complete their lifecycle. Because of the autogamous reproduction present in *S. gesnerioides*, we expect that this trait would be rapidly fixed and build up within the region it occurred, presumably in and around Zakpota. The sensitivity of high-throughput comparative transcriptomics provided the possibility for identifying rare transcripts differentially expressed between SG4z and is evolutionary precursor SG4 and thus potential candidate effectors involved in overcoming the race-specific resistances present in B301.

The use of secreted effectors to modulate host resistance is widespread in nature and has been described in interactions between plants and a wide variety of pathogenic microbes, fungi, and nematodes. This work extends the use of secreted effectors for host immunity manipulation to parasitic weeds thereby opening a new understanding of the mechanism of the intriguing plant-plant interactions. Our findings indicate that the SHR4z effector is formed in the parasite haustorium prior to host attachment and deployed in the early post-attachment stages of the host-*Striga* interaction. The 25 amino acid secretion signal targets the SHR4z to the apoplast of the haustorial cell where it is available for entry into the host cell to carry out its suppressive function. Our data show that when the full length SHR4z protein is expressed in host roots (following *Agrobacterium*-mediated transient transformation), the apoplastic targeting signal at N-terminal of SHR4z moves the protein to the host cell apoplast where it is not functional in suppression of immunity. Thus, presence in the host cell cytoplasm is required for function. How the effector moves from parasite apoplast to host cytoplasm and what molecules are involved remains to be determined.

In oomycetes and fungus, it has been long known that the typical virulence effectors are among pathogenic secretome and have clear N-terminal signal peptide to allow translocating from pathogen cells to apoplastic region. They can function in apoplast or engage cell entry by overrepresented host-targeting motifs following N terminal signal peptide. The known host-targeting motifs, like RXLR, LFLAK, and CHXC amino acid sequences, were first discovered by examining sequence alignment of known AVR proteins (Whisson et al., 2007) and further tested by molecularly manipulating motifs for translocating detection (Albrecht et al., 2005). However, apart from its apoplast targeting signal, no obvious host-targeting motifs are observed in SHR4z.

Since parasitic weeds and their hosts share most cellular features in common, unlike pathogenic microbes and fungi that require specialized structures for the transfer of effectors into their plant prey, the movement of small proteins between *Striga* and its host likely occurs without the development of specialized structures or protein transfer routes via plasmadesmatal connections established after parasite attachment. When, where and how the apoplast targeting signal is removed from the SHR4z

protein and mechanism of effector trafficking between parasitic plant and host is clearly an area in need of additional future study.

Regardless, once delivered to the host cell, SHR4z is capable of suppressing HR by interfering with the normal transduction pathway activated during plant immune responses (Figure 6). The exact mechanism remains to be determined. SHR4z shares significant sequence and structural similarity with in the N-terminal region to the SERK (SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE) subfamily of LRR-RLKs (Supplemental Figure S11) and is predicted to interact with the BTB/POZ interface of VuPOB1. via the three LRRs at C-terminal of the protein (Supplemental Figure S9) SERK proteins are known to function as co-receptors, physically interacting with numerous cell-surface receptors to regulate various developmental and immune responses in plants including BRASSINOSTEROID signaling (Nam and Li, 2002; Li et al., 2002), male sporogenesis (Albrecht et al., 2005; Colcombet et al., 2005) and plant defense (Kemmerling et al., 2007; Chinchilla et al., 2007; He et al., 2007; Heese et al., 2007). For example, AtSERK3/BAK1 interacts with FLS2 immediately after perception of bacterial flagellum to trigger plant innate immunity. It also works redundantly with AtSERK4/BKK1 to control cell death. Similarly, the rice homolog of SERK1 (OsSERK1) is known to be involved in defense response and significantly induced at the RNA level by rice blast fungus (Hu et al., 2005). However, unlike members of the SERK family proteins, SHR4z lacks the kinase domain typically responsible for the phosphorylation activity in SERKs. Therefore, SHR4z may serve as a decoy that interferes or competes with the interaction of one or more members of the SERK family with VuPOB1 but, by the phosphorylation /activation capacity of a SERK, is not able to activate the downstream pathway leading a HR (Figure 6). Molecular mimicry modulating plant host responses to microbial pathogens and nematodes is well documented (Ronald and Joe, 2018).

E3 ligases (of which VuPOB1 is a homolog) have been known for some time to be common interactors with RKs or RLKs. The Arabidopsis Plant U-Box E3 ubiquitin ligases PUB12 and PUB13 have been shown to be BAK1/SERK3 phosphorylation targets that lead to the degradation of FLS2 in order to attenuate Pathogen Associated Molecular Pattern (PAMP)-triggered immunity (PTI) in a

flg22-dependent manner (Lu et al., 2011). The RING-type E3 ligase XB3 is required for the accumulation of the receptor-like kinase (RLK) protein XA21 and promotes downstream R-mediated HR against bacterial blast *Xanthomonas oryzae* pv. Oryzae (*Wang et al., 2006*). Two Plant U-Box (PUB) family of putative E3 ubiquitin ligases (Os08g01900 and Os01g66130) were found to interact with four phylogenetically distant defense-related RLKs (Os04g38480, Os07g35580, Os07g35260, and Os08g03020) in rice (Ding et al., 2009). E3 ligases regulate as well as are regulated by RLKs at all steps of plant immune responses and constitute the central of plant defense signaling pathways (Duplan et al., 2014). As a result they have become the targets of pathogenic effectors. The effector AVR3a from the oomycete *Phytophthora infestans* targets and stabilizes the U-box-type Ub-ligase CMPG1/PUB20 which controls *P. infestans* elicitin INF1 triggered cell death (Bos et al., 2010). Effector AvrPiz-t from the rice blast fungus *Magnaporthe oryzae* interacts and inhibits the rice RING-type Ub-ligase APIP6 which is normally a positive regulator of PTI. The binding of AvrPiz-t and APIP6 results in degradation of both proteins in *N. benthamiana* and suppresses flg22-induced ROS production (Park et al., 2012).

In tobacco, the VuPOB1 homolog NtPOB1/NbPOB1 was recently found to target the U-box E3 ligase PUB17 for degradation suppressing Cf-mediated HR-PCD (Yang et al., 2006; Orosa et al., 2017). In Arabidopsis, there are 49 members in PUB E3 ligase group (Azevedo et al., 2001; Mudgil et al., 2004; Mazzucotelli et al., 2006) and several of these members have been shown to be involved in plant immunity. PUB12 and PUB13, for example, attenuate PTI by ubiquitinating the flg22 receptor FLS2 (Lu et al., 2011), and PUB 22, 23, and 24 act in combination to negatively regulate plant immunity by suppressing oxidative burst, the MPK3 activity, and transcriptional activation of defense regulatory genes (Trujillo et al., 2008). Since VuPOB1 appears to be a positive regulator of HR, additional studies are clearly needed to determine its interaction with one or more members of the PUB E3 ligase family and how this interaction functions in immunity.

It is clear that the HR response in B301 requires the expression of a number of components acting downstream of effector recognition by RSG3-301(Figure 6). Previous studies from our group (Huang et al., 2012; Mellor, 2013) have shown that RNAi silencing of the transcriptional activator VuGRF

leads to a loss of the SG3-induced HR. Similarly, silencing the expression of genes involved in cell wall modification (e.g., peroxidases, galacturonases, etc.), general defense (e.g., narbonin, oxylipin biosynthesis, etc.) and ROS release also decreases SG3-activated HR and associated abiosis leading to parasite death post-attachment (Mellor, 2013).

Finally, it is worth noting that the discovery of a secreted effector involved in mediating the interaction of *S. gesnerioides* with its cowpea host raises questions about the generality of this strategy among other parasitic weeds. Examinations of the haustorial transcriptomes of other parasitic members of the Orobanchaceae (Yang et al., 2015) revealed presence of homologs of SHR4z in the transcriptomes of *S. hermonthica* and *Phelipanche aegyptiaca*, the former being a close relative to *S. gesnerioides* and the latter being much more evolutionarily diverged. Like SHR4z these homologs are also highly expressed in haustorial stages coincident with host contact. Whether these homologs are functional and acting in a manner similar to SHR4z has been addressed by Clarke et al. (C.R. Clarke, S.-Y. Park, Z. Yang, X. Jia, E. Wafula, L. Honaas, C. Yang, C.W. dePamphilis, J.H. Westwood, personal communication) who tested *P. aegyptiaca* secreted proteins for the ability to alter innate plant immunity. These investigators uncovered a set of five candidate effector proteins that suppress known plant defense pathways, including two LRR SERK-like proteins that are similar to that identified here. Despite years of research, wide-scale effective control methods for *Striga* based on breeding and selection of resistant cultivars and use of modified agronomic practices remain elusive. Understanding the molecular mechanism of *Striga* virulence and the evolutionary changes in the parasite that contribute to breaking host resistance enhance our abilities to better solve this riddle. Thus our findings here on the nature of secreted effectors could potentially contribute to the development of novel strategies for controlling *Striga* and other parasitic weeds and thereby enhancing plant productivity and food security worldwide.

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Tables and Figures

Table 1. Transcriptome assembly and gene capture statistics.

Transcriptome Metrics	Value
Transcriptome Size (Mbp)	115
Transcript/Contig Number	145407
Trinity component number	114231
Medium length (bp)	475
Contig N50 length (bp)	1218
Number of N50 contigs	27168
PlantTribe gene capture number	899
PlantTribe gene capture percentage	93.74%
COSII gene capture number	197
COSII gene capture percentage	89.55%
UCOs gene capture number	353
UCOs gene capture percentage	98.88%

Figure 1. Differential response of cowpea cultivars B301 and California Blackeye No. 5 to parasitism by different races of *Striga gesnerioides* races from West Africa.

(A) Representative photographs illustrating the phenotypic response of B301, a multirace resistant cultivar, and California Blackeye No. 5 (BE) a susceptible cultivar, to parasitism by different races of *S. gesnerioides*. Shown are the appearance of 2 day-old germinated SG4, SG4z and SG3 seedlings prior to host contact (0 days post-inoculation (dpi)) and the roots of cowpea cultivars B301 and BE at 3 dpi and 10 dpi. Scale bar represents 200 μm.

(B) Measured frequency of each different phenotypic event category during the interaction of the three *S. gesnerioides* races, SG3, SG4, and SG4z with resistant (B301) cowpea roots at 10 dpi. The abbreviation of the phenotypic event categories are as follows: AT, Attachment; HR, hypersensitive response; TS, tubercle swelling; and CE, cotyledon expansion. The interaction event ratio for each category was obtained by counting the number of each event category and dividing by the total number of phenotypic events occurring on each host plant. Statistical analysis was done using the ANOVA with a total of 10 independent host plants inoculated with equivalent amounts of the different races. The pairwise comparison p-value was determined by Tukey's honest significance (Tukey HSD) test following GLM. Bars represent ± SD of the biological replicates. An asterisk (*) indicates a p value < 0.05.

Figure 2. Expression difference of effector candidates between SG4 and SG4z.

(A) Heatmap of expression change of effector candidates over development. The expression level (the mean of log2TPM) is represented by color intensity from low (red) to high (blue) in heatmap. Upregulated effector candidates in SG4z (red bar) and upregulated effector candidates in SG4 (green bar) were grouped into different clusters. The blue bar includes the candidate effectors highly expressed in SG4z at the early attachment stage, while upregulated in SG4 at late attachment stages. Hierarchical clustering was performed based on pairwise correlation between contigs.

(B) Shown is the relative abundance of the SGall_040908.1 transcript in germinated seedlings and haustoria of the three different races of *Striga* races (SG3: blue; SG4: green; and SG4z: red) at 3 days-post inoculation (dpi) and 10 dpi as determined by qRT-PCR. Relative expression was normalized to endogenous control PP2A and calibrated relative to expression in SG3 at 0 dpi. Data shown are means ± SD of three technical replicates. The quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) expression trend confirmed RNA-seq result.

Figure 3. Ectopic overexpression of SHR4z suppresses cowpea host innate immunity.

(A) Representative photographs showing the subcellular localization of SHR4z- and SHR4zΔSP-mCherry fusion proteins in transgenic cowpea B301 roots at 20 days post-transformation as viewed by confocal microscopy. B301 plants were transformed with *A. rhizogenes* containing the effector (pK7WG2D-SHR4z-mCherry and pK7WG2D-SHR4z**Δ**SP-mCherry) and control (pK7WG2D-mCherry) gene constructs. The roots of pK7WG2D-SHR4z**Δ**SP-mCherry transgenic plants were incubated with 100 μM MG132 for 6 h before confocal imaging to prevent the rapid proteolytic degradation of truncated protein. Visualization of mCherry fluorescence was performed with a 564-630 nm filter range and GFP fluorescence with a 497-544 nm filter range. The scale bar is 10 μm.

(B) Representative images of HR and tubercle swelling (TS) on composite plants. B301 roots from composite plants (pK7WG2D-SHR4z**Δ**SP construct) were inoculated with pre-germinated SG4 and SG4z *S. gesnerioides* seedlings at 30 days after transformation. Interactions (HR, hypersensitive response; TS, tubercle swelling; and CE, cotyledon expansion) were examined under light and fluorescence microscopy. The transgenic roots were visualized by GFP labeling. The scale bar is 200 μm. **(C-E)** Effects of ectopic overexpression of SHR4zΔSP on suppression of B301 innate immunity. *Ex vitro* composite B301 plants were generated by ectopically overexpressing the SHR4zΔSP transcript. Transgenic and non-transgenic roots were inoculated with two-day germinated SG4 (C) SG4z (D) and SG3 (E) seedlings, and the phenotypic responses of the roots were scored at 10 d post inoculation (dpi), 20 dpi, and 30 dpi. The interaction event ratio for each category was obtained by counting the number of each event category and dividing by the total number of phenotypic events occurring on transgenic (green) and non-transgenic (white) roots of each host plant. Statistical analysis was done using the paired t-test on more than 10 independent host plant replicates (SG3-10 dpi: N=22; SG3-20 dpi: N=13; SG3-30 dpi: N=11; SG4-10 dpi: N=14; SG4-20 dpi: N=14; SG4-30 dpi: N=14; SG4z-10 dpi: N=12; SG4z-20 dpi: N=12 dpi; SG4z-30 dpi: N=11). Boxplots indicate the median (horizontal lines), 25th and 75th percentile range (boxes) and up to 1.5 X IQR (whiskers). An asterisk (*) indicates a p value < 0.05.

Figure 4. Identification of VuPOB1, a BTB-BACK E3 ligase, as the cowpea host target of SHR4z.

(A) Shown are the results of yeast-2-hybrid assays indicating that SHR4z without signal peptide (SHR4zΔSP) interact with VuPOB1. The various indicated proteins were expressed as AD- and BD-fusions in MaV203 yeast cells. Transformed MaV203 cells were grown on selective media (Leu-, Trp-, Ura-) and X-gal media to test for interacting partners and on nonselective media (Leu-, Trp-) to test for the transformation efficiency. BD-FLAG and AD-FLAG fusion proteins were used as negative controls for interaction in yeast.

(B) Bimolecular fluorescence complementation (BiFC) assay demonstrating the *in planta* interaction SHR4zΔSP with VuPOB1 in cytoplasm. Confocal microscopy was used to visualize the interaction of SHR4zΔSP with VuPOB1 following transient co-expression of plasmids containing YN-VuPOB1 and YC-SHR4z**Δ**SP in *N. benthamina* leaves. The scale bar is 25 μm.

(C) Co-immunoprecipitation analysis indicates that myc-SHR4zΔSP interacts with HA-VuPOB1 *in planta*. Myc- SHR4zΔSP and HA- VuPOB1 fusions were transiently expressed in *N. benthamina* leaves following Agrobacterium-mediated transformation. Transformed tissue were subsequently infiltrated with 100 μM MG132 or H_2O as control 6 h before harvesting. Total protein was extracted 3 d after initial infiltration and incubated with anti-myc beads to immunoprecipitate myc-SHR4zΔSP. Both protein extract (3% input) and immunoprecipitates (IP) were analyzed by immunoblot (IB) using anti-HA and anti-myc antibody.

Figure 5. VuPOB1 functions as a positive regulator of plant defense. (A) Shown is the relative transcript abundance (level of expression) of VuPOB1 in the roots of B301 and BE cowpea before parasite attachment and 3 d post inoculation (dpi) and 10 dpi with *Striga* races SG4 and SG4z. The relative expression was normalized with endogenous control 18S and calibrated in relative to expression in BE at 0 dpi. Statistical analysis was done using unpaired t-test with three technical replicates. Error bar represents ± standard deviation (SD). **(B)** Effect of RNAi-silencing of VuPOB1 on SG4-triggered host resistance response. *Ex vitro* composite B301 plants were generated by expressing constructs capable of RNAi silencing of VuPOB1 expression. Transgenic (green) and non-transgenic (white) roots were inoculated with two-day germinated SG4 seedling, and at 10 dpi, 20 dpi, and 30 dpi the phenotypic responses of the roots were scored. The abbreviation of the phenotypic event categories are as follows: HR, hypersensitive response; TS, tubercle swelling; and CE, cotyledon expansion. The interaction event ratio for each category was obtained by counting the number of each event category and dividing by the total number of phenotypic events occurring on each host plant. Statistical analysis was done using the paired t-test with more than 10 independent host plant replicates (10 dpi: N=16; 20 dpi: N=15; 30 dpi: N=15). An asterisk (*) indicates a p value < 0.05.

(C) Effects of ectopic overexpression of VuPOB1 on SG4z mediated suppression of B301 innate immunity. *Ex vitro* composite B301 plants were generated by ectopically overexpressing the full length VuPOB1 transcript. Transgenic (green) and non-transgenic (white) roots were inoculated with two-day germinated SG4z seedlings, and at 10 dpi, 20 dpi, and 30 dpi the phenotypic responses of the roots were scored. The abbreviation of the phenotypic event categories and scoring of phenotypic categories are as above. Statistical analysis was done using the paired t-test with more than 10 independent host plant replicates (10 dpi: N=13; 20 dpi: N=10; 30 dpi: N=10). Boxplots indicate the median (horizontal lines), 25th and 75th percentile range (boxes) and up to 1.5 X IQR (whiskers). An asterisk (*) indicates a p value < 0.05.

Figure 6. Proposed model of activation and suppression of cowpea host HR by different races of *S. gesnerioides*. Parasitism of cowpea host B301 roots by *Striga* races SG3 or SG4 elicits a

hypersensitive response (HR) at the site of attachment. The intracellular NLR protein RSG3-301is required for the response and may work directly or indirectly upon recognition of parasite effectors with members of the cowpea SERK family of proteins to bring about activation of the positive regulator of HR, VuPOB1. VuPOB1 then initiates activation of previously identified components of the HR pathway (see Huang et al., 2012, Mellor 2013) possibly involving interaction with PUB E3 ligases. In contrast, apoplastically localized SHR4z secreted from the SG4z haustorium enters B301 host root cells via an unknown transport mechanism, where it interacts with VuPOB1 resulting in their subsequent ubiquitination and degradation and loss of HR in the host root.

Supporting Information

Figure S1. Map of West Africa showing the general distribution of the various races of *S. gesnerioides*.

Figure S2 Overall similarity of global gene expression profile. Heatmap showing the relationship of gene expression changes in comparisons of two cowpea cultivars.

Figure S3 Differential expression analysis between SG4 and SG4z.

Figure S4 The workflow of predicting effector candidates in *S. gesnerioides* using transcriptome data.

Figure S5 Differentially expressed candidate effectors and qRT-PCR verification of selected effector candidates.

Figure S6 Gene and protein structure of SHR4z.

Figure S7 Verification of SHR4z and VuPOB1 overexpression and RNAi silencing VuPOB1 in transgenic roots and non-transgenic roots.

Figure S8 B301 roots expressing the full length SHR4z and control did not show difference between transgenic and non-transgenic roots.

Figure S9 Protein structure and phylogenetic relatedness of cowpea VuPOB1.

Figure S10 Effect of RNAi-silencing of VuPOB1 on SG4z-triggered host responses.

Figure S11. Phylogenetic relationship among SHR4z and SERK homologs in Arabidopsis and cowpea.

Methods S1

Table S1 Summary of differential expressed gene statistics from between race comparisons at differential stages of the host-parasite interaction.

Table S2 Summary of contigs identified at different stages of the discovery process in the identification of effector candidates.

Table S3 List of oligonucleotide primers used in this study.

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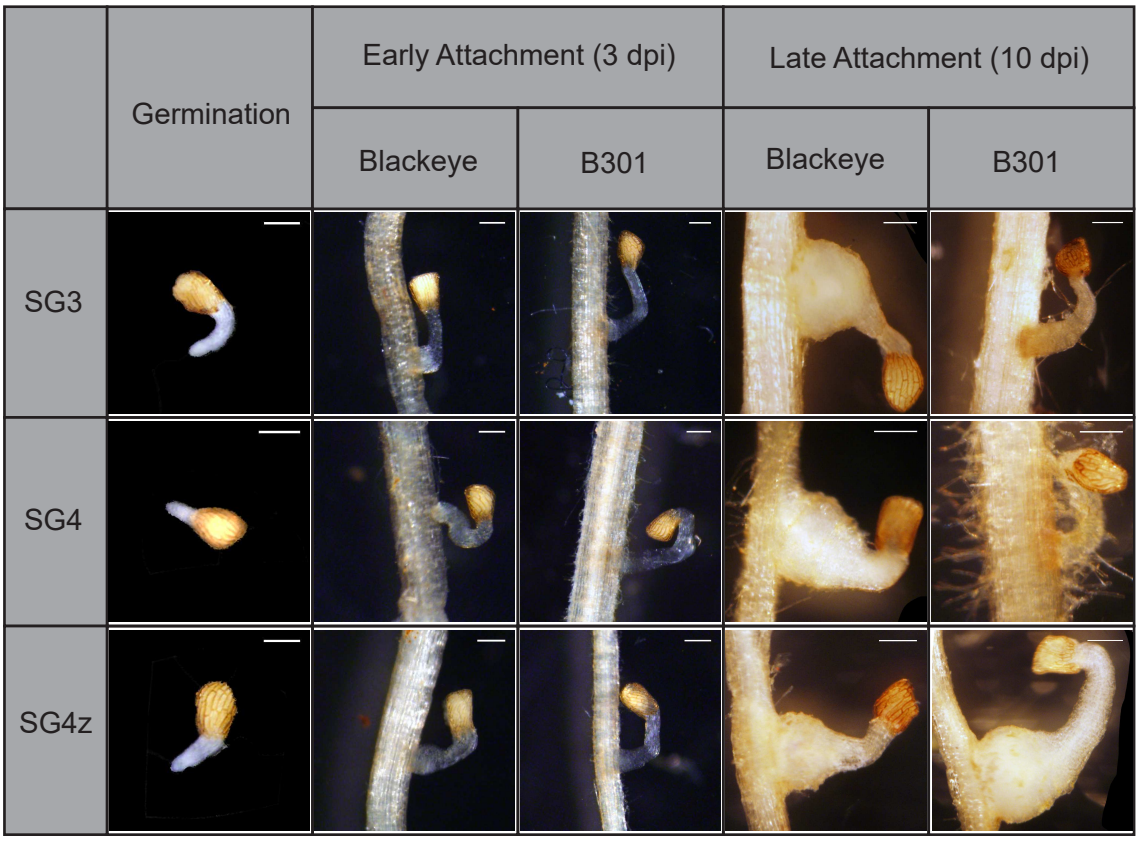

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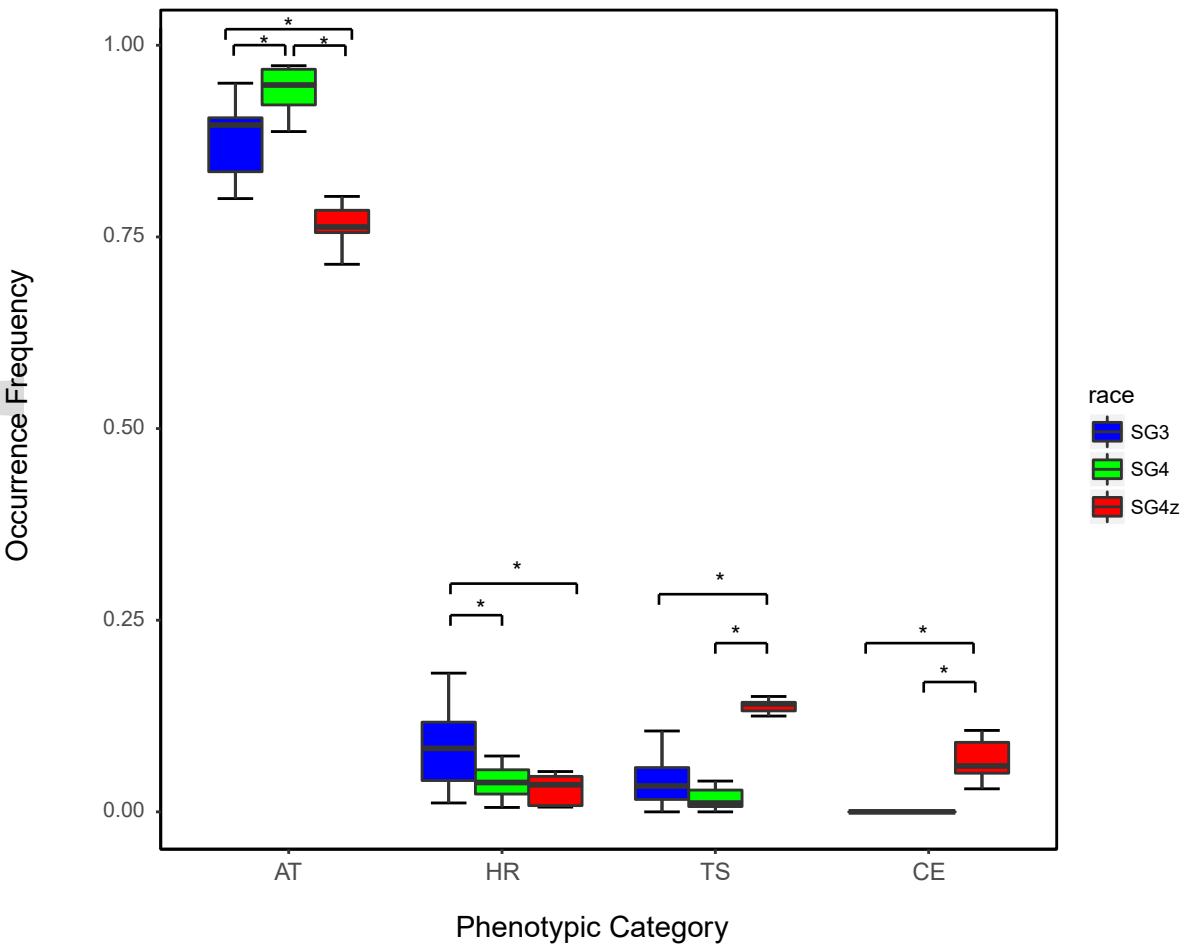

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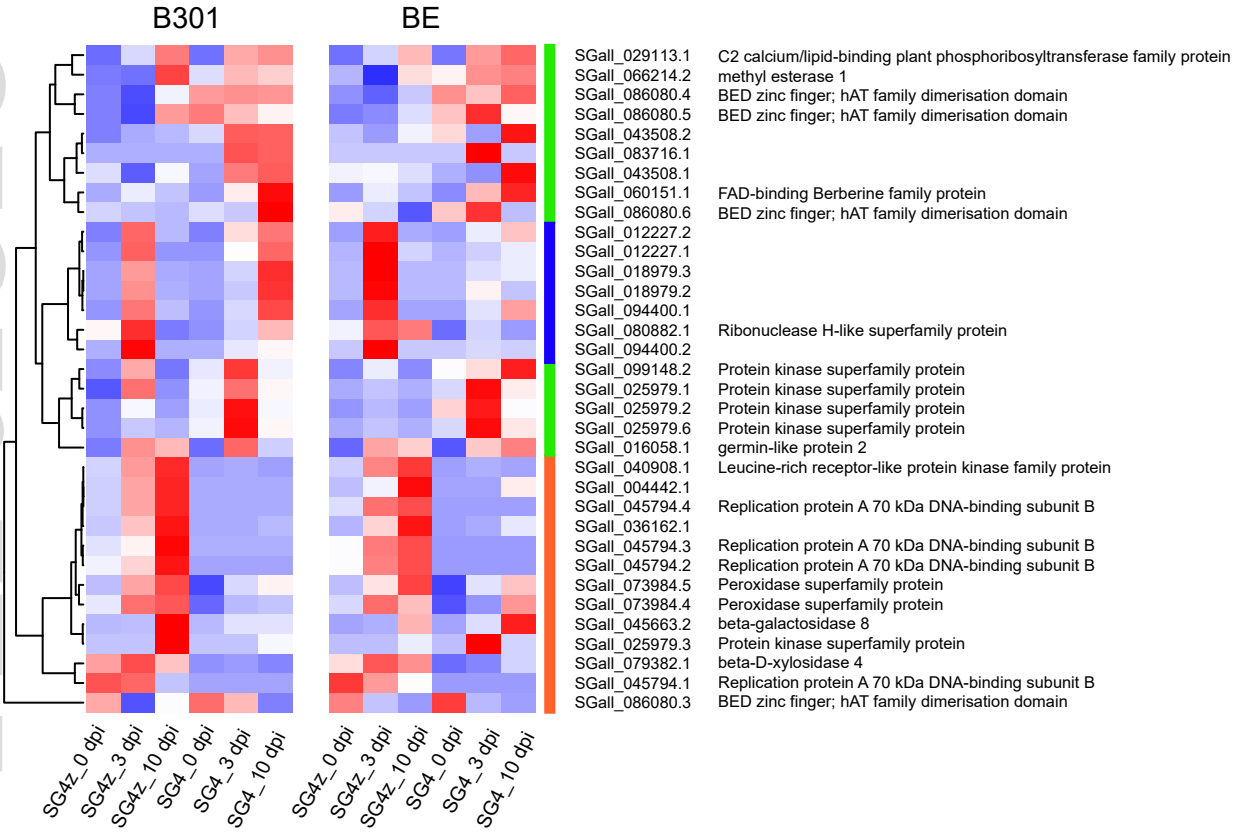

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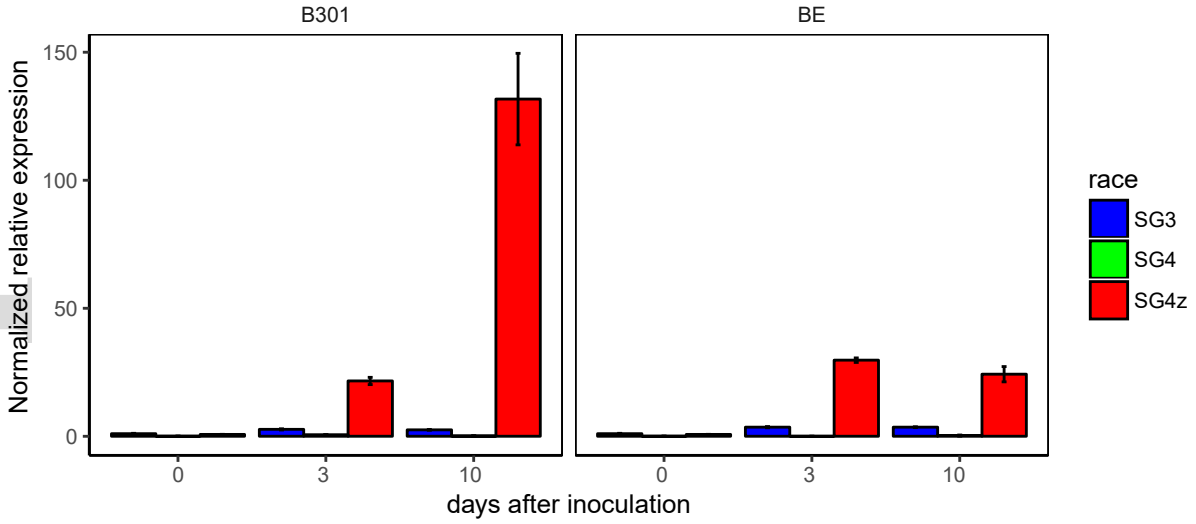

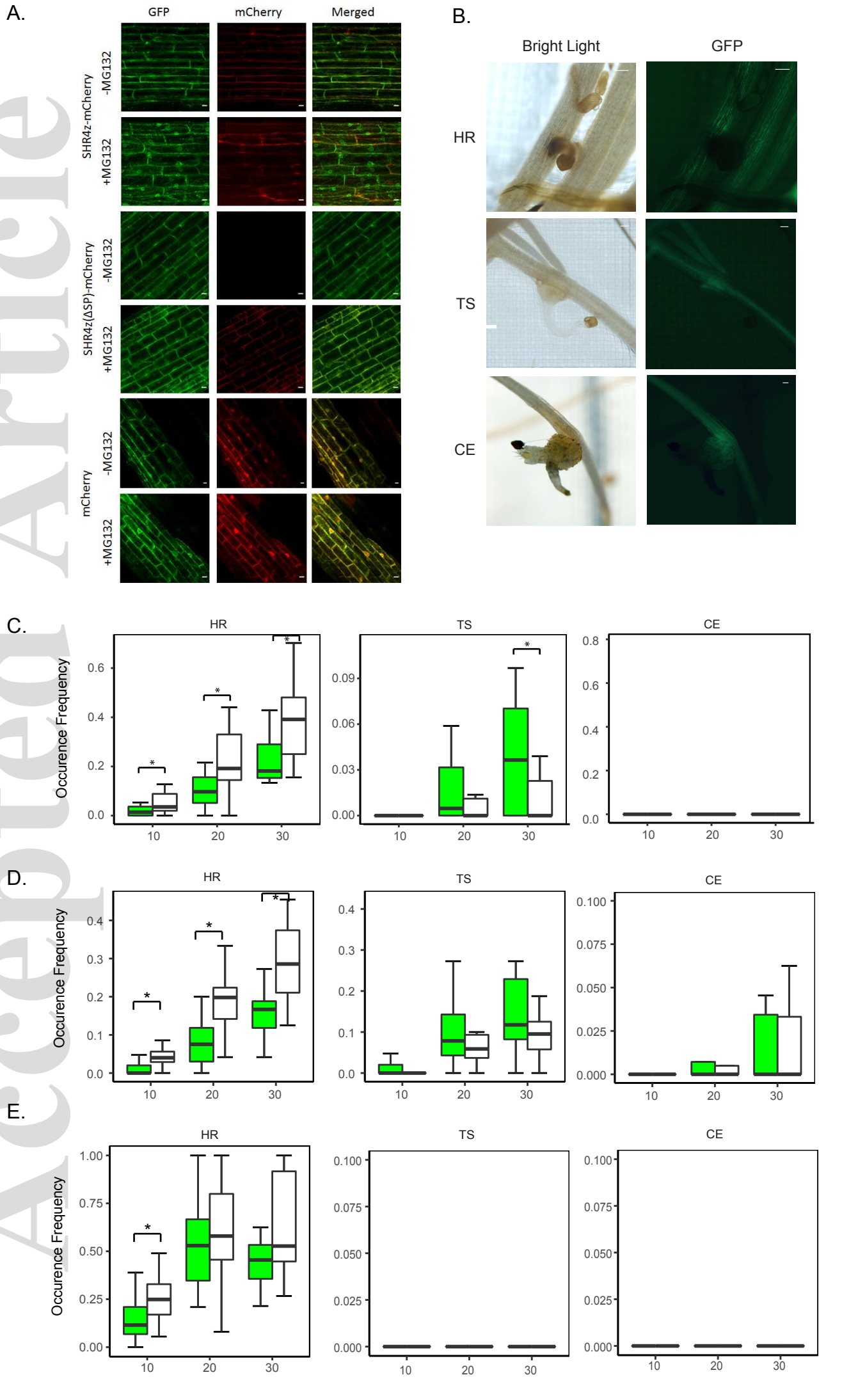

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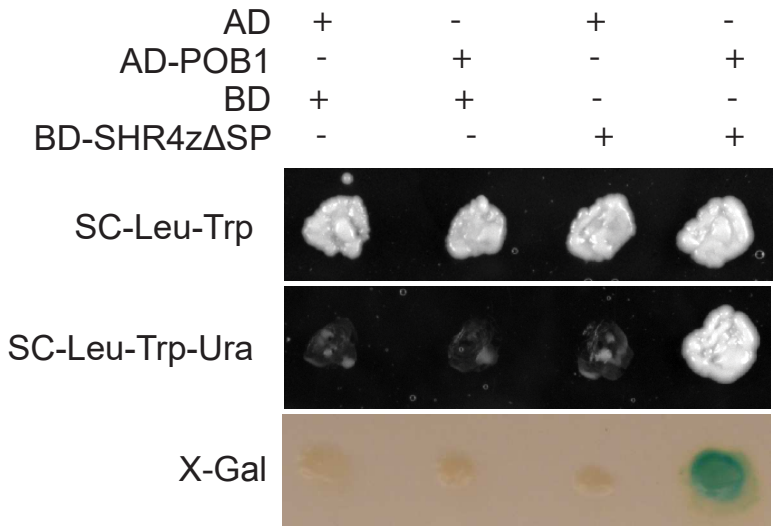

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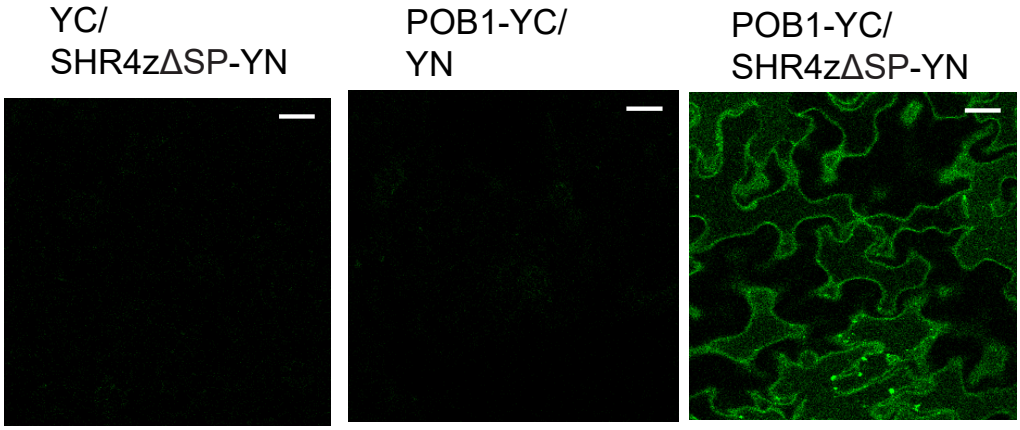

C.