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Wheat *Mds-1* encodes a heat-shock protein and governs susceptibility towards the Hessian fly gall midge

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Gall midges induce formation of host nutritive cells and alter plant metabolism to utilize host resources. Here, we show that the gene *Mayetiola destructor susceptibility-1* (*Mds-1*) on wheat chromosome 3AS encodes a small heat-shock protein and is a major susceptibility gene for infestation of wheat by the gall midge *M. destructor*, commonly known as the Hessian fly. Transcription of *Mds-1* and its homoeologs increased upon insect infestation. Ectopic expression of *Mds-1* or induction by heat shock suppressed resistance of wheat mediated by the resistance gene *H13* to Hessian fly. Silencing of *Mds-1* by RNA interference conferred immunity to all Hessian fly biotypes on normally susceptible wheat genotypes. *Mds-1*-silenced plants also showed reduced lesion formation due to infection by the powdery mildew fungus *Blumeria graminis* f. sp. *tritici*. Modification of susceptibility genes may provide broad and durable sources of resistance to Hessian fly, *B. graminis* f. sp. *tritici*, and other pests.

Plant parasites, including many insects, nematodes, and microbes manipulate plants in order to utilize host resources. Hessian fly, a member of a large group of gall-inducing insects called gall midges, is a destructive insect pest of wheat, and a model organism to study plant-insect interactions[1,2]. Hessian fly larvae live between leaf-sheaths near the base of wheat seedlings. Even though it does not induce the formation of a typical outgrowth gall, a Hessian fly larva is able to convert the whole susceptible plant into a gall by inducing the formation of nutritive cells at the feeding site and by inhibiting plant growth while maintaining the infested plant alive as a source for nutrients[2,3]. The infested susceptible plant dies eventually after the insect ceases feeding on the plant. Successful infestation is accompanied by increased epidermal permeability of sheath cells, an indicator of nutritive cell formation[4]. Hessian fly larvae also suppress plant defense and reprogram metabolic pathways of susceptible plants during

compatible interactions[5]. In plants carrying a resistance (R) gene directed against Hessian fly infestation, a larva is unable to establish a permanent feeding site and dies within 3-5 days after hatching. Resistant plants resume normal growth after some initial growth deficit[6]. Thirty-two major R genes to Hessian fly have been identified in wheat or wheat relatives[7]. All known R genes have a typical gene-for-gene relationship with Hessian fly avirulence as observed in many plant – pathogen systems. Many R genes have been deployed to the field and are initially highly effective in protecting wheat from Hessian fly damage[1,2]. However, resistance mediated by R genes is short-lived and usually overcome by the insect within 3-6 years after the deployment of a resistant cultivar due to the appearance of new virulent Hessian fly biotypes[8]. All known R genes in wheat are also temperature-sensitive and lose resistance to Hessian fly above 28°C[9]. Further insights into the molecular aspects of the wheat – Hessian fly interaction are therefore needed to improve wheat resistance.

Expression profiling of nearly isogenic susceptible and resistant wheat cultivars revealed that many host genes are up- or down-regulated following Hessian fly infestation[5]. Based on the gene annotation, the changes in susceptible wheat are consistent with the conversion of the sheath cells to a nutrient sink with concomitant increases in carbohydrate metabolism, amino acid biosynthesis, and nutrient transport. The changes in gene expression in susceptible plants are also consistent with the dramatic shift from carbon-containing compounds to nitrogen-containing compounds that occurs during compatible interactions[10]. Some of the specifically induced genes with the greatest expression are predicted to be involved in stress responses, likely as the consequence of developmentally inappropriate alteration to host cell physiology or the hijacking of the stress responses for the benefit of Hessian fly larvae[5]. Here, we present a functional analysis of a predicted heat-shock protein gene that was represented by the EST

CD453475, named here *Mayetiola destructor susceptibility gene-1* (*Mds-1*). *Mds-1* gene showed the highest level of induction in wheat seedlings during compatible interactions to Hessian fly infestation[5]. We examined the impact of silencing and ectopic expression of *Mds-1* in susceptible and resistant wheat genotypes, respectively, on Hessian fly larval survival and development.

Results

Hessian fly infection induces *Mds-1*. Based on the EST CD453475 sequence, a full-length cDNA and the gene (GenBank Accession Code JN162442) were cloned from the susceptible wheat Newton by RACE-PCR and PCR (Supplementary Fig. S1). *Mds-1* encodes a protein of 151 amino acid residues and has 96% identity with a previously characterized heat-shock protein (HSP), HSP16.9, a member of a group of proteins with the α-crystallin domain[11] (Fig. 1a). Without infestation, very low levels of *Mds-1* transcript were detected in wheat leaf-sheaths (Fig. 1b), the feeding site for Hessian fly larvae. Higher transcript levels were found in developing grains. Both the transcript and protein levels increased in plants during compatible interactions following Hessian fly infestation, but no apparent increase in the transcript or protein levels was observed in plants during incompatible interaction with the wheat cultivar Molly (Fig. 1c), which carries the R gene *H13*[12].

Silencing of *Mds-1* inhibits Hessian fly development. Expression of *Mds-1* was reduced by virus-induced gene silencing (VIGS)[13] to determine the requirement for *Mds-1* expression during Hessian fly infestation. VIGS treatment of seedlings of the susceptible wheat Newton prevented the induction of *Mds-1* in Hessian fly-infested plants and rendered the susceptible genotype immune to Hessian fly infestation (Fig. 2a). Seven Hessian fly populations

representing at least 30 biotypes were tested, and all biotypes were incompatible on *Mds-1*-silenced wheat seedlings (Supplementary Table S1). The effect of *Mds-1* silencing was also assessed in stable transgenic plants. Twenty-four independent transgenic lines of the wheat cultivar Bobwhite, a Spring wheat with high efficiency for genetic transformation, were generated with a construct expressing double-stranded RNA for *Mds-1* silencing. Twenty of the transformants showed suppression of *Mds-1* induction by Hessian fly, whereas the remaining four did not have the suppression effect (Supplementary Table S2). The transgenic plants and their progeny with suppressed *Mds-1* lost susceptibility to Hessian fly biotype *GP* (Fig. 2b, Supplementary Table S2, Supplementary Fig. S4). Transgenic lines T1630, T1639, T2095, and T2357 were advanced to T2 generation (equivalent to F2 offspring from F0), and all these transgenic lines were resistant to all biotypes tested so far (Supplementary Table S1).

High levels of *Mds-1* expression confer R-gene plants susceptibility. Due to the hexaploid nature, a wheat gene usually has at least three homoeologs from A, B, and D genomes that share high nucleotide sequence similarity. RNA interference (RNAi) in transgenic and VIGS-treated plants may have reduced the abundance of *Mds-1* and related transcripts. The silencing strategy did concomitantly reduce levels of *Mds-1* related transcripts (Supplementary Figs. S5a, S5b, S6). To determine if *Mds-1* homoeologs are involved in wheat susceptibility to Hessian fly, we mapped *Mds-1* to wheat chromosome 3AS using Chinese Spring wheat ditelosomic and deletion lines (Supplementary Figs. S7a1, S7a2, S7b)[14]. The homoeologs of *Mds-1* on chromosomes 3B and 3D were identified by searching genomic sequences derived from these two chromosomes specifically (Supplementary Fig. S8)[15,16]. The two homoeolous genes on B and D genomes, named as *HB* and *HD*, respectively, were mapped to the similar distal regions of chromosomal 3B and 3DS using gene-specific primer pairs (Supplementary Figs.

S7a3, S7a4, S7b, S8). Transcript abundance of all these three homoeologs was induced by Hessian fly infestation and by heat stress (Supplementary Figs. S7c, S7d). The ditelosomic lines of Chinese Spring wheat missing *Mds-1* or either one of its two homoeologs on B and D chromosomes are still susceptible to Hessian fly infestation, indicating that each of the homoeologous genes is able to confer wheat susceptibility to Hessian fly infestation.

To determine whether *Mds-1* expression alone is sufficient for wheat susceptibility to Hessian fly, we took advantage of the lack of induction of *Mds-1* by Hessian fly in the resistant wheat cultivar Molly. *Mds-1* was ectopically expressed at high levels in Molly through stable transformation (Supplementary Fig. S9). Although normally resistant to Hessian fly, Molly plants became susceptible to the insect when *Mds-1* was ectopically expressed (Fig. 3a). Seven independent transgenic lines with ectopic *Mds-1* expression were produced to avoid positional or other undesirable effects (Supplementary Table S2). All seven transgenic lines with ectopic *Mds-1* expression were susceptible to Hessian fly infestation. In addition to ectopic expression, heat stress was also adapted to induce *Mds-1* expression since *Mds-1* encodes a heat shock protein. When Molly seedlings were stressed at 37°C and then allowed to recover at 20°C, high levels of *Mds-1* transcript were observed (Fig. 3b), and the plants with high levels of *Mds-1* expression became susceptible (Fig. 3c). Heat-shock studies with wheat cultivars containing other R genes gave similar results (Supplementary Fig. S10). Heat-induced wheat susceptibility was not due to a general stress response since susceptibility did not occur in *Mds-1*-silenced, transgenic plants (Fig. 4).

***Mds-1* silencing suppresses metabolic changes associated with susceptibility.** The impact of *Mds-1* silencing and ectopic expression on epidermal permeability (an indicator of nutritive cell formation)[4], plant-growth inhibition, and expression of nutrition-related host genes

were examined in wheat with or without Hessian fly infestation. Hessian fly infestation induced strong epidermal permeability, as measured by the uptake of neutral red dye, in control Bobwhite (Fig. 4a), as compared with background staining of uninfested control Bobwhite (Fig. 4a1, UCB). Uninfested, *Mds-1* silenced Bobwhite plants displayed neutral red uptake comparable to untreated control Bobwhite plants, and Hessian fly infestation failed to induce epidermal permeability in *Mds-1* silenced Bobwhite plants. Heat stress treatment also had no effect on permeability in either uninfested or infected *Mds-1* silenced Bobwhite plants. *Mds-1* silencing also prevented the plant growth inhibition that is observed after Hessian fly infection of control Bobwhite plants (right panel). Heat treatment could not induce susceptibility in the silenced plants either. On the other hand, high levels of ectopic *Mds-1* expression allowed the normally avirulent biotype *GP* to induce strong epidermal permeability in the otherwise resistant wheat cultivar Molly (Fig. 4b). High level of *Mds-1* expression induced by heat stress also allowed biotype *GP* to induce strong epidermal permeability of Molly cells. High levels of *Mds-1* expression through either heat stress or ectopic expression allowed the normally avirulent Hessian fly biotype to inhibit plant growth. *Mds-1* silencing also limited the ability of Hessian fly to up-regulate nutrient production-related genes and down-regulate defense genes in the susceptible host Bobwhite, a phenomenon normally observed only in resistant plants during incompatible interactions (Supplementary Fig. S11).

***Mds-1* expression is correlated with powdery mildew susceptibility.** To determine whether *Mds-1* is specific to Hessian fly or a common susceptibility gene to other biotrophics as well, transgenic plants with *Mds-1* silenced were tested against two wheat biotrophic pathogens, the powdery mildew caused by the fungus *Blumeria graminis* f. sp. *tritici*; and the leaf rust caused by the fungus *Puccinia triticina*. Inoculation of wheat seedlings with the isolate KS-5 of

159 *B. graminis* f. sp. *tritici* revealed that all tested transgenic Bobwhite lines with *Mds-1* silenced
160 exhibited reduced lesion size in response to the pathogen, whereas control Bobwhite plants
161 displayed full lesion development that is consistent with susceptibility (Fig. 5a, Supplementary
162 Table S2). *B. graminis* f. sp. *tritici* infection strongly upregulated *Mds-1* in powdery mildew-
163 susceptible cultivars Molly, Newton, and Bobwhite, but only slightly affected *Mds-1* transcript
164 abundance in the powdery mildew-resistant cultivar Duster (Fig. 5b), which contains the R gene
165 *Pm3*[17]. Conversely, high levels of *Mds-1* transcript in Duster, as induced by heat stress (Fig. 5c),
166 were correlated with a phenotype switch from resistant to susceptible to *B. graminis* f. sp. *tritici*
167 (Fig. 5d). On the other hand, inoculation of wheat seedlings with the PRTUSS4 strain of the rust
168 pathogen *Puccinia triticina* revealed that *Mds-1* silencing does not affect wheat resistance or
169 susceptibility to the rust fungal pathogen (Supplementary Fig. S12).
170
171 **Discussion**
172 Our data with RNAi suppression and ectopic expression of *Mds-1* indicate that *Mds-1* is
173 sufficient to confer wheat susceptibility to Hessian fly infestation. However, the susceptibility of
174 Chinese Spring deletion lines lacking *Mds-1* or one of its homoeologs on 3BS and 3DS suggests
175 that both *Mds-1* and its homoeologs can confer wheat susceptibility to Hessian fly. This is not
176 surprising considering the fact that diploid goatgrass (with D genome only) is also a host of
177 Hessian fly, and the fact that the coding sequences of *Mds-1* and its the homoeologs are highly
178 conserved (Supplementary Fig. S8). Interestingly, the promoter and 3'-noncoding regions are
179 highly diversified among *Mds-1* and related genes. The difference in the promoter and
180 noncoding regions is consistent with significant variation in the expression levels among *Mds-1*
181 homoeologous genes in response to Hessian fly infestation and heat stress (Supplementary Fig.

S7)[5]. Therefore the functions of these highly conserved proteins may be differentiated through differential expression under different conditions. *Mds-1* is among the most induced by Hessian fly infestation as well as under heat-stress (Supplementary Figs. S5, S7)[5], suggesting its involvement in wheat susceptibility to Hessian fly in the wheat lines we studied. Indeed, *Mds-1* is sufficient for inducing a state of susceptibility as evidenced by the conversion of the resistant cultivar Molly to susceptibility by ectopic expression of *Mds-1* alone in multiple independent transgenic lines.

The mechanism for *Mds-1* in wheat susceptibility remains to be determined. The inability of Hessian fly larvae to induce *Mds-1* in the presence of an effective R gene suggests that the R protein blocks *Mds-1* induction by virulent effectors from Hessian fly (Fig. 6). The conversion of resistant plants into susceptibility under the condition of high levels of *Mds-1* expression suggests that MDS-1 either suppresses plant defenses, activates susceptibility pathways, or a combination of both. Our data points to the possibility that MDS-1 suppresses host defense as suggested by the up-regulation of defense genes in plants with an effective R gene and in *Mds-1*-silenced transgenic plants (Supplementary Fig. S11), and activates susceptibility pathways as indicated by up-regulation of nutrient metabolic pathways and the formation of nutritive cells (Fig. 4, Supplementary Fig. S11). During compatible interactions, *Mds-1* is likely induced by Hessian fly or fungal effectors through interactions with *Mds-1* regulatory elements directly or indirectly[18], or pathways that lead to activation of heat-shock transcription factors (Fig. 6)[19,21]. The MDS-1 protein may then suppress plant defenses, and activate, directly or indirectly, wheat susceptibility pathways, or serve as a component to allow effectors from Hessian fly and *B. graminis* f. sp. *tritici* to activate wheat susceptibility pathways. The activation of wheat susceptibility pathways leads to metabolic changes in the host[5,10],

205 resulting, in the case of Hessian fly, in the formation of nutritive cells and plant susceptibility[3].
206 During incompatible interactions, the prevention of *Mds-1* induction by Hessian fly due to an
207 intervention from a specific R – AVR protein recognition inhibits the induction of *Mds-1* and
208 thus nutritive cell formation. The lack of nutrients and the activation of defense pathways result
209 in plant resistance.

210 Hessian fly induces cells in the wheat sheath to develop into nutritive cells for the
211 nourishment of fly larvae, which involves the conversion of host sheath cells to a nutritional sink.
212 The process also induces a variety of stress related genes, which initially were construed to
213 reflect the host response to infection. However, the evidence presented here indicates that the
214 Hessian fly specifically exploits the heat-shock protein gene *Mds-1* and other related genes for
215 the benefit of larval growth. Our results added another dimension to the remarkable and ancient
216 small HSP family proteins, including MDS-1, which are involved in a wide range of functions
217 from construction of the animal eye lens to stress responses[22]. The proliferation of small *HSP*
218 genes in plants has been postulated to be an adaptation to dynamic environmental changes,
219 including heat stress. Our results indicate that the Hessian fly and *B. graminis* f. sp. *tritici*
220 exploit a heat shock-like response that leads to host susceptibility.

221 Remarkably, the *Mds-1* silenced plants were observed to be poor hosts for the powdery
222 mildew fungus *B. graminis* f. sp. *tritici*. Measurements of *Mds-1* expression during infection of
223 normal wheat plants revealed that *Mds-1* is also induced to higher levels of expression during *B.*
224 *graminis* infection. The wheat variety Duster is resistant to many strains of *B. graminis* f. sp.
225 *tritici* including the KS-5 isolate, and very low *Mds-1* expression was detected in Duster plants
226 challenged by the fungus. Ectopic expression studies of *Mds*-1 were not conducted in the Duster
227 variety. Nonetheless, heat stress of Duster led to both elevated levels of *Mds-1* and the loss of

resistance. The possibility exists that *B. graminis* f. sp. *tritici* specifically exploits the heat shock pathway to suppress host immunity responses. The effect of *Mds-1* silencing on susceptibility, however, did not extend to the leaf rust pathogen *Puccinia triticina* as silenced plants were equally susceptible to rust infection as normal plants (Supplementary Fig. S12).

The possible negative impact of *Mds-1* silencing on wheat remains to be determined. Initial examination of apparent phenotypic abnormalities in *Mds-1* silenced wheat lines include partial sterility, smaller grain weight, reduced plant height, and low seed germination rates (Supplementary Table S4). However, abnormalities in plants with *Mds-1* silenced vary from plant to plant and may have been caused by positional effects due to different integration sites of the *Mds-1* construct into the wheat genome or by tissue culture. For practical application, potential negative impact needs to be eliminated or reduced to minimum for economic benefit. One way to reduce potential negative impact is to use tissue-specific promoters for gene silencing[23]. The rice S gene, *Pi21* that encodes a transporter-like protein and is highly conserved among monocots, has been engineered for broad resistance to rice blast[24]. Unlike R genes that have similar structures and possibly similar action modes[25], S genes exhibit greater variations in structures and functional mechanisms[26-30]. The variation in S genes provides us opportunities for fundamental research to reveal mechanisms of plant susceptibility and resistance as well as for practical applications to develop plants with improved resistance for pest management.

The effect of *Mds-1* expression in wheat on resistance to Hessian fly and the loss of resistance to both Hessian fly and powdery mildew upon heat stress also provided insight into the resistance mechanisms. Numerous plant species with single major R genes lose resistance to herbivores under heat stress, suggesting a possible role of *Hsp* genes in plant susceptibility[9, 31-34]. The observations that a bacterial pathogen injects an HSP-like protein into host cells for

virulence, and elevation in HSP70 levels induced by heat stress makes plants susceptible to an otherwise avirulent pathogen also support a role of HSPs in plant susceptibility[35]. However, various HSPs including HSP90, HSP70, an HSP-like protein, and a small HSP have been found to interact with disease resistance protein complexes and are required for disease resistance[36-43]. The basis for the role of MDS-1 and possibly other HSPs as well in plant susceptibility and the role of HSPs in plant resistance remains to be revealed. In a similar situation, receptor-like kinase genes are required for both fungal susceptibility and resistance in different plant – pathogen systems[26,27]. The dominant effect of *Mds-1* and possibly other temperature-dependent susceptibility genes under elevated temperatures pose a threat to the effectiveness of plant resistance to Hessian fly and other pests under scenarios of global climate change[32,44-46]. A better understanding of the molecular mechanisms of *Mds-1* and other temperature-dependent, dominant susceptibility genes is needed to preserve plant resistance in the face of global warming.

Methods

Wheat (*Triticum aestivum*) genotypes. Bobwhite is a spring wheat cultivar susceptible to Hessian fly and powdery mildew (*Blumeria graminis* f. sp. *tritici*). Bobwhite is widely used for producing transgenic plants because of its high efficiency for genetic transformation, and was used for generating RNA interference (RNAi) transgenic plants. Newton, a winter wheat with no Hessian fly R gene, was used as susceptible control. Molly and Iris, two near-isogenic lines of Newton, contain *H13* and *H9* R genes, respectively[12]. WGRC42 contains R gene *Hdic*[47]. Duster is powdery mildew resistant with R gene *Pm3c*[17].

Seedlings were grown in pots, 10 cm in diameter, in Pro-Mix 'BX' medium (Hummert, Topeka, KS) in growth chambers (Model AR-66L, Percival, Perry, IA) with a 16/8h light/dark cycle at 275 µmol/m^2/s under 20/18°C day/night. Heat stress was delivered by adjusting temperature with other conditions unchanged.

Infestation and sample collection. Hessian fly biotypes: *GP* avirulent to all known resistance (R) genes, *vH9* virulent to *H9*, *vH13* virulent to *H13*[48,49]. Hessian fly populations with mixed biotypes were also used (Supplementary Table S1)[50]. Wheat seedlings were infested with ~15 eggs/plant by confining flies with a mesh screen cage. The time when neonates just reached the feeding site was taken as the initial infestation time (monitored by dissecting extra infested plants). Wheat tissues were collected 12 h to 96 h later from the initial infestation time. Plants were phenotyped 2 weeks later with resistance defined as normal wheat growth and insect death, and susceptibility defined as stunted wheat growth and normal insect development. Leaf-sheath tissue of 10-15 mm at the feeding site was cut out, insects removed, and used for various analyses. Each sample contained a pool of 10 plant tissues. Samples were frozen immediately in liquid nitrogen and stored in -80°C.

Powdery mildew. Isolate KS-5 of *B. graminis* was used for infection. A conidia suspension was uniformly sprayed onto plants, and the plants were placed in a chamber for symptom development. Phenotype was scored 8 days post inoculation. Disease evaluation was based on a 0 to 4 scale as described previously[51].

DNA and RNA extraction and blot analysis. Genomic DNA was extracted from wheat tissues following CTAB procedure[47]. Plasmid DNA was extracted using a QIAprep Miniprep Kit (Qiagen). Total RNA was extracted using TRI reagent (Molecular Research Inc. Cincinnati, OH). RNA samples were further purified through an RNease kit (Qiagen). DNA and RNA were

297 quantified with a NanoDrop-1000. Northern blot analysis was carried out as described
298 previously[5].

299 ***Mds-1* cloning.** Based on EST sequence, a full cDNA was cloned using a SMART™
300 RACE Kit (Clontech, Mountain View, CA) with primers in Supplementary Table S3. The
301 RACE-PCR product was gel-purified using a GeneClean® Turbo for PCR Kit (Qbiogene,
302 Carlsbad, CA), and cloned directly into the vector pCR® II-TOPO® (Qbiogene). Positive
303 clones were sequenced using M13 primers contained in the vector.

304 Full length *Mds-1* gene was cloned by PCR with DNA from Newton using primers Mds-
305 1Lb and Mds-1R (Supplementary Table S3). Sequence alignments were produced using
306 ClustalW2[53] and printed using BoxShade.

307 **Western blot analysis.** A full length recombinant protein was produced and used for a
308 polyclonal antibody in rabbits by GenScript (Piscataway, NJ). The antibody was affinity-
309 purified. Protein extracts were prepared by homogenizing wheat tissues in Tris-HCl buffer (pH
310 8.0) containing protease inhibitor cocktail (Sigma, St. Louis, MO). Protein concentration was
311 measured using a bicinchoninic acid kit (Sigma). About 180 µg of protein extract was loaded
312 onto a 12% gel and separated using Xcell surelock electrophoresis cell (Invitrogen). Proteins
313 were transferred to PVDF membrane (Millipore, Danvers, MA). The membrane was blocked
314 using 5% (w/v) milk in Tris-buffered Saline (100 mM Tris, pH7.4, 150 mM NaCl) with 0.1%
315 Tween 20 (TBST) for 1 h at 4°C. The membrane was then incubated overnight at 4°C with
316 primary antibody-HRP conjugation (0.5ug/ml of 2% milk/TBST). The membrane was then
317 washed three times in TBST and incubated for 1 min with HRP chemiluminescent detection
318 reagents (Invitrogen). The membrane was exposed to film for 4 h before development.

VIGS treatment. Barley stripe mosaic virus (BSMV)[54] was used for VIGS[13,55-58]. A 338 bp 3'-fragment of *Mds-1* was amplified by PCR with primers Mds-1La and Mds-1R (Supplementary Table S3). The PCR fragment was ligated into the γ genome at antisense orientation.

Infectious RNA transcripts were synthesized using a mMessage mMachine T7 transcription kit (Ambion, Austin, TX) from linearized α, β, and γ target plasmids[13]. The silencing BSMV inoculum was made by combining an equal molar ratio of α, β, and γ transcripts with excess inoculation buffer containing a wounding agent (FES). The first leaf of 10 day old seedlings was inoculated and plants were then infested with Hessian fly after apparent visual symptom 8 days post-BSMV inoculation.

***Mds-1* knockdown construct.** An RNAi-based construct was made according to the Gateway system[57,58]. The 338-bp *Mds-1* fragment was amplified as in VGIS. PCR product was directionally inserted into pENTR™/ D-TOPO (Invitrogen). The final RNAi construct was made by recombination from an LR clonase reaction using a Gateway® LR clonase enzyme mix between the entry vector carrying the *Mds-1* fragment and the pANDA-mini vector[57], from which the identical *Mds-1* fragment was inserted into both sides of the 920-bp GUS linker in antisense and sense orientations. The transcribed RNA contains a hairpin that forms dsRNA.

***Mds-1* expression construct.** Full *Mds-1* coding region was amplified by PCR with primers Mds-1Lc and Mds-1R (Supplementary Table S3). Mds-1Lc contains the start codon and an added sequence with a BCL I restriction site (TGATCA). An internal BCL I site exists in the 3'-UTR. PCR product was digested with BCL I, and the resulting DNA fragment was ligated into pAHC17 at the BamHI site[59,60]. The resulting construct contains full *Mds-1* coding region under a maize ubiquitin promoter (*Ubi-1*).

Transgenic plants. For silencing, the pANDA construct and pAHC20[59,60] containing the bar gene were co-bombarded with 1:1 ratio into embryogenic calli as described by Altpeter[61] and modified by Ayella et al.[62]. Briefly, embryos (2-5 mm) were then excised from immature seeds and plated on CM4 media to initiate the formation of somatic embryo for 2-7 days. Somatic embryos were selected for highly embryogenic calli and were co-bombarded with pAHC20 and *GOI* plasmids at 1:1 ratio by using the particle inflow gun. After 5 days wheat calli were placed on CM4 medium containing 5 mg/L glufosinate for two wks. Cultures were transferred twice to CM4 medium with 10 mg/L glufosinate for 2 wks each. The growing embryogenic tissues were transferred to shoot production medium (MSP) with 5 mg/L glufosinate selection until green shoots were observed. The cultures were then re-transferred to elongation and rooting medium (MSE) containing 5 mg/L glufosinate but not 2,4-D for 2-3 wks. Shoots that developed roots were then transferred to potting soil. Recovered plants were screened for herbicide resistance with 0.2% Liberty (AgEvo, Pikeville, NC). Plants survived are presumably transformed with pAHC20, which usually indicates a high probability of co-transformation with the target construct. Herbicide resistant plants were analyzed for *Mds-1* construct presence with PCR.

Epidermal permeability. Neutral red stain (Sigma, St. Louis, MO) was used to determine epidermal permeability as described[4].

Real-time PCR (qPCR). qPCR was performed as described previously[52]. Primers were designed using the software package Beacon Designer 7(Supplementary Table S3). RNA was collected from three biological replicates. Relative fold-changes for transcripts were calculated using the comparative $2^{-\Delta\Delta CT}$ method[63] and normalized to actin control.

Statistical test. Three biological replicates were carried out for each qPCR analysis. Data were subjected to analysis of variance (ANOVA), and Tukey's honestly significant

365 difference (HSD) multiple comparisons were conducted using ProStat software Version 5.5(Poly
366 Software International Inc., Pearl River, NY, USA). Tukey's 95% simultaneous confidence
367 intervals were used to separate data into groups. The lower case letters were used to represent
368 different groups with statistically significant difference at $P \leq 0.05$.

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AUTHOR CONTRIBUTIONS: X.L. and M.C. conceived the study. X.L., C.K., J.L., L.H., and G.A. conducted the experimental work. H.N.T. generated the transgenic plants. X.L., C.K., J.L., H.N.T., G.R.R., G.A., F.F.W. and M.C. analyzed the data. X.L., H.N.T., B.S.G., G.R.R., F.F.W. and M.C. wrote the paper.

Competing Financial Interest

The authors declare no competing financial interests.

Coordinates for *Mds-1* have been deposited in the GenBank database under accession code JN162442

Figure Legends

Figure 1

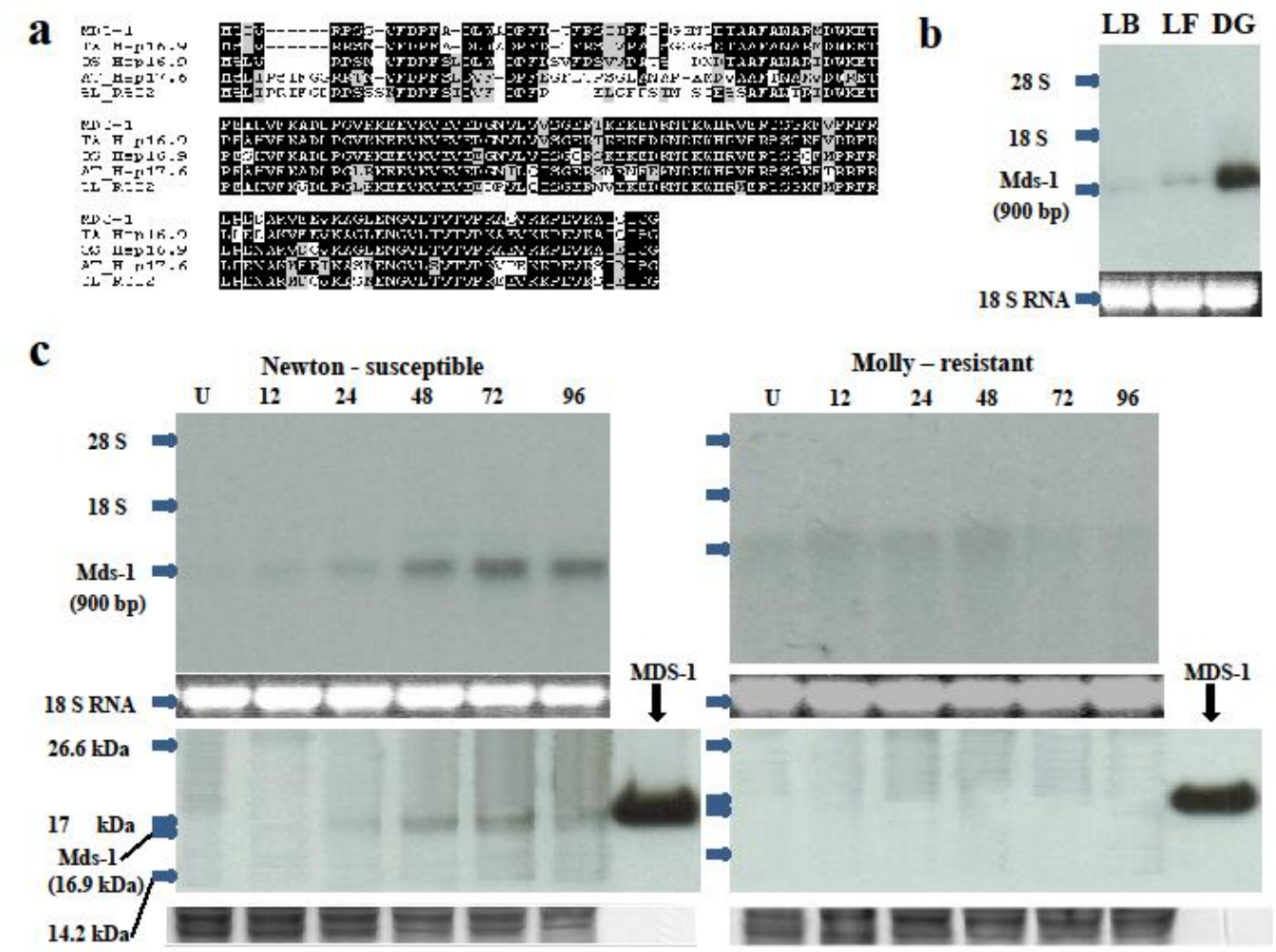


Fig. 1. *Mds-1* encodes a small heat shock protein induced by Hessian fly. (**a)** An alignment of MDS-1 and related proteins from various plant species. The names of the proteins (Genbank accession codes) are as follows: TA_Hsp16.9 (1GME_A); OS_Hsp16.9 (P27777); AT_Hsp17.6 (CAA34208); SL_RSI2 (AF123255). TA, OS, AT, and SL represent *Triticum aestivum*, *Oryza sativa*, *Arabidopsis thaliana*, and *Solanum lycopersicum*, respectively. *Mds-1* was cloned from the susceptible wheat cultivar Newton. (**b)** *Mds-1* transcript levels in uninfested susceptible

Newton wheat leaf-blades (LB), leaf-sheaths (LF), and developing grains (DG). 28 S, 18 S, and Mds-1 (900 bp) along with arrows on the left of the northern blot represent the locations of 28 S rRNA, 18 S rRNA, and *Mds-1* mRNA. An 18 S rRNA image of the gel is given below the blot as loading control. (**c)** *Mds-1* is upregulated during Hessian fly infestation in susceptible Newton, but not in resistant Molly wheat. Northern (upper panels) and western (lower panel) blot analyses of *Mds-1* transcript and protein with samples from susceptible Newton plants (left panels) and resistant Molly plants (right panels) at 0 (U), 12 to 96 h, respectively, after infestation with Hessian fly biotype *GP*. Plants were infested by confining mated females in pots with screens. Females deposit eggs on leaf blades. Neonates migrate into a plant and live between leaf sheaths next to the base. The time point 0 was taken right before neonates reach the feeding site. 28 S, 18 S, Mds-1 (900 bp), and 18 S rRNA are as described in b. The positions of protein size markers and the location of the Mds-1 protein are given on the left of the western blots along with arrows. MDS-1 represents a recombinant protein control (0.116 µg per lane). A coomassie-blue gel image is given under each western blot as loading control.

Figure 2

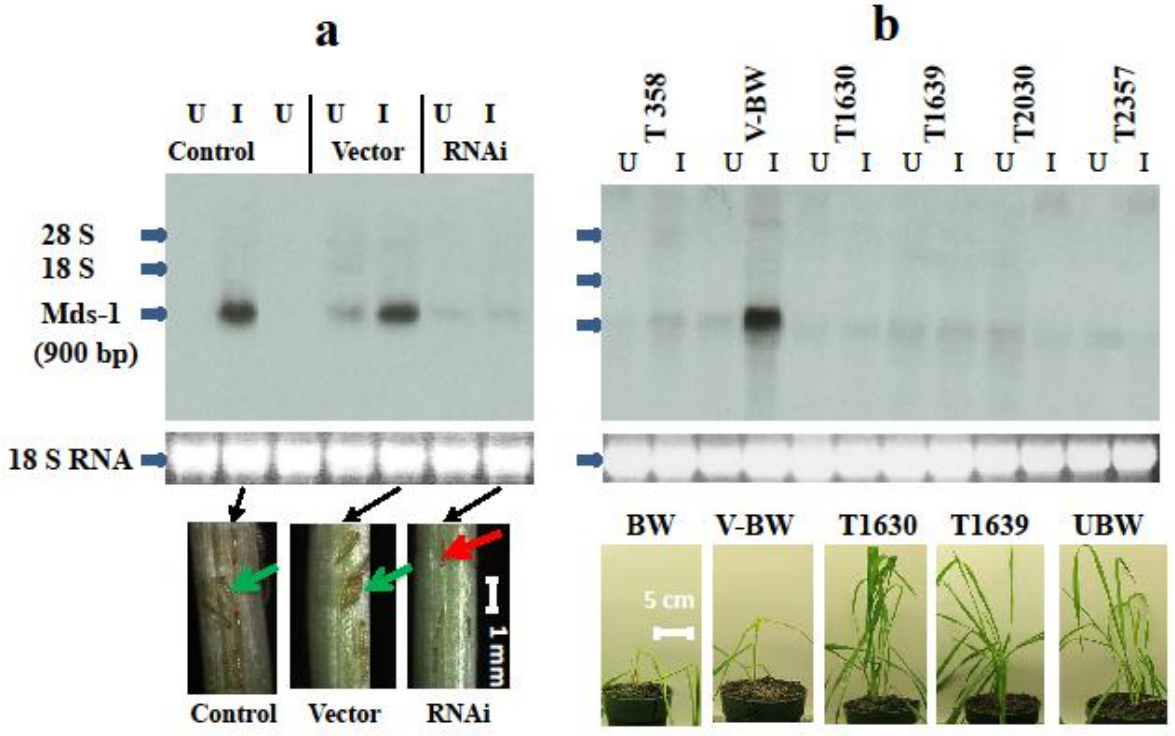
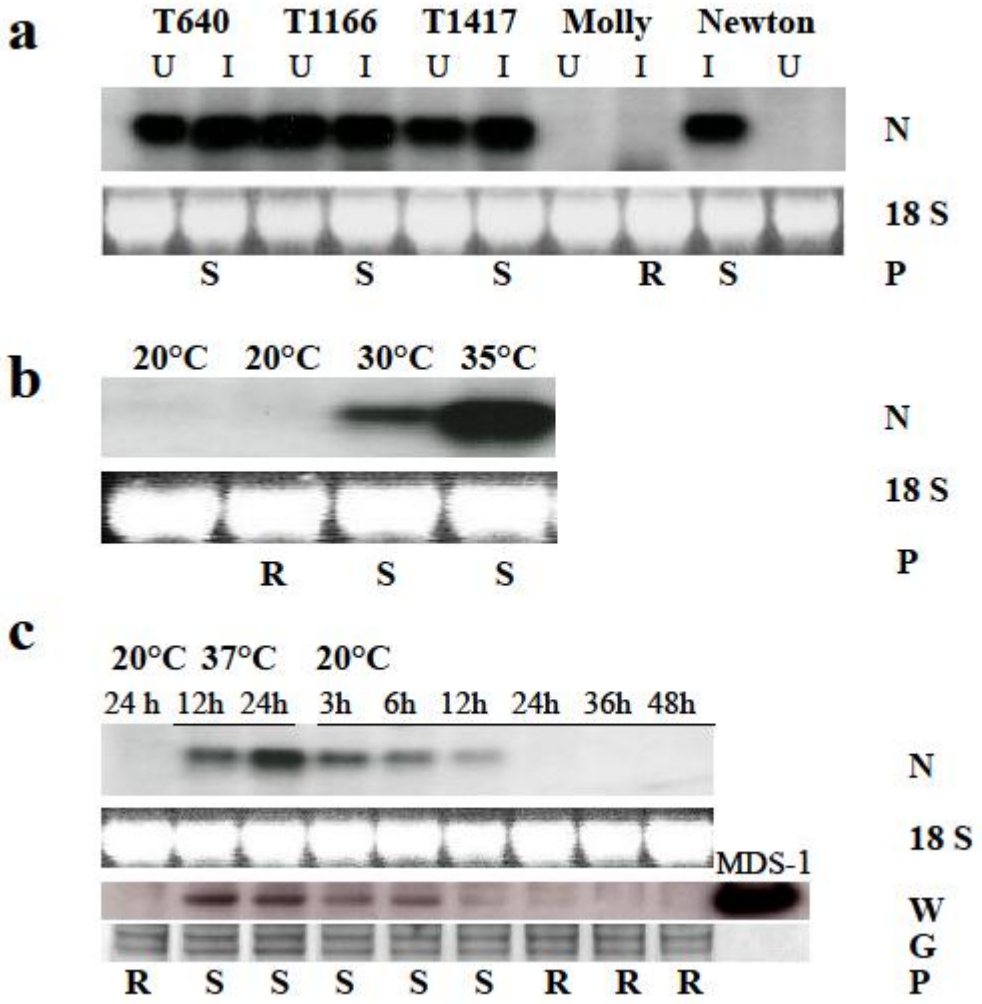


Fig. 2. Silencing of *Mds-1* in wheat confers immunity to susceptible plants. (**a)** Transient silencing of *Mds-1* in the susceptible genotype Newton confers immunity to Hessian fly biotype *GP*. Newton is a winter wheat and the susceptible recipient parents of several isogenic lines including Molly and Iris that contain different R genes[12]. Northern blot analysis of *Mds-1* transcript in plants of untreated control, treated with the original virus (Vector), or treated with the modified virus that carried the 338 bp fragment of *Mds-1* (RNAi) (Supplementary Fig. S2a). U, uninfested; I, infested plants. 28 S, 18 S, and Mds-1 (900 bp) along with arrows on the left of the northern blot represent the locations of 28 S rRNA, 18 S rRNA, and *Mds-1* mRNA. An 18 S

rRNA image is given under the blot as loading control. Phenotypes of Hessian fly larvae on the control, vector- and RNAi-treated plants are given in the lower panel. Green arrows point to live larvae, the red arrow points to a dead larva. (**b)** Suppression of *Mds-1* expression in transgenic Bobwhite plants confers immunity to Hessian fly biotype *GP* infestation. Bobwhite is a spring wheat line with high efficiency for genetic transformation. Bobwhite is susceptible to Hessian fly infestation. The upper panel is a Northern blot of independent transgenic Bobwhite lines T385, T1630, T1639, T2030, T2357, produced with an RNAi construct (Supplementary Fig. S2b); and an empty vector-transformed Bobwhite wheat, V-BW, with (I) and without (U) Hessian fly infestation. Other denotations are the same as in a. The lower panel shows phenotypic differences among a non-transgenic Bobwhite plant (BW), an empty vector-transgenic plant (V-BW), and the *Mds-1*-silenced plants T1630 and T1639 after Hessian fly infestation. Growth of infested BW and V-BW was inhibited, but growth of infested T1630 and T1639 plants was comparable to that of uninfested control Bobwhite (UBW) plants. Resistance was observed in both seedling and adult transgenic plants.

596 **Figure 3**



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599 **Fig. 3. Ectopic expression and heat induction of *Mds-1* in resistant wheat.** (**a)** Ectopic
600 expression of *Mds-1* correlates with a phenotype switch of Molly plants from resistance to
601 susceptibility to Hessian fly biotype *GP* infestation. Northern blot analysis of *Mds-1* transcript
602 in samples from transgenic Molly lines T640, T1166, and T1417 (Supplementary Table S2,
603 Supplementary Fig. S9); non-transgenic, resistant Molly; and the susceptible wheat Newton with
604 (I) and without (U) Hessian fly (biotype *GP*) infestation. 28 S, 18 S, and Mds-1 (900 bp) along

with arrows on the left of the northern blot represent the locations of 28 S rRNA, 18 S rRNA, and *Mds-1* mRNA. An 18 S rRNA image is given as loading control. Interaction phenotypes are given under the 18 S rRNA images with: R, resistant; S, susceptible. (**b)** Heat stress induces *Mds-1*. Northern blot and phenotypic analyses of resistant genotype Molly seedlings that were either unstressed (20°C) or stressed at 30 or 35°C. (**c)** The decay of heat-induced *Mds-1* transcript and protein correlates with restoration of Molly resistance to Hessian fly. Northern (upper panel), western (lower panel), and phenotypic analyses on Molly plants were conducted during recovery after stress. Seedlings were stressed at 37°C for 12 or 24 h, and some 24 h-stressed plants were allowed to recover at 20°C for 3 to 48 h. Denotations for northern blot and phenotypic analysis are the same as in a. The positions of protein size markers and the location of the Mds-1 protein are given on the left of the western blot. A coomassie-blue stained gel image is give under the blot as loading control. Plants were infested with Hessian fly biotype *GP*.

Figure 4

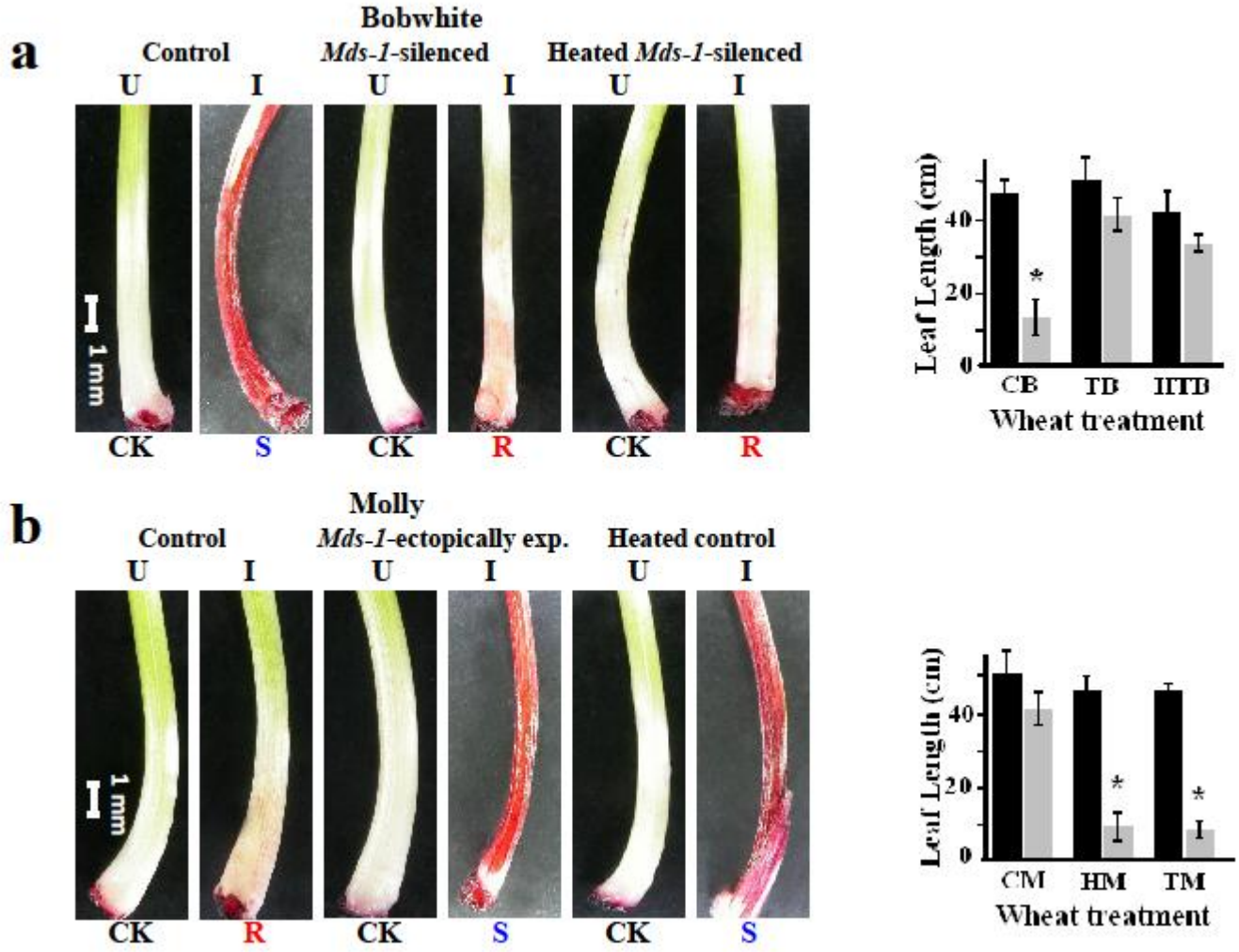
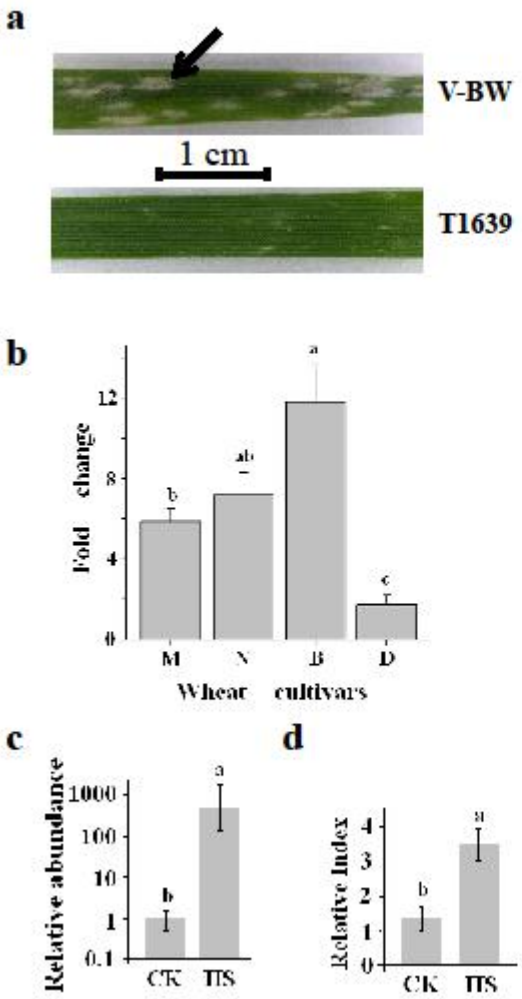


Fig. 4. *Mds-1* expression levels affect Hessian fly infestation. Hessian fly biotype *GP* was used for all infestation. **(a)** *Mds-1* silencing inhibits Hessian fly-mediated nutritive cell formation and plant-growth suppression. The left panel shows epidermal permeability as indicated by neutral red staining in control, *Mds-1*-silenced, and heat-stressed *Mds-1*-silenced Bobwhite. The letters U and I indicate uninfested and infested plants. Resistant (R) or susceptible (S) phenotypes of infested plants and uninfested controls (CK) are below the images. The right panel shows

628 growth inhibition of cultivar Bobwhite by Hessian fly. Solid bars, uninfested; gray bars, infested
629 plants of control Bobwhite (CB), *Mds-1*-silenced Bobwhite (TB), or heat-stressed, *Mds-1*-
630 silenced Bobwhite (HTB). Four replicates were carried out for each analysis. Data were
631 subjected to analysis of variance (ANOVA) using ProStat software (Poly Software International
632 Inc., Pearl River, NY, USA). '*' indicates significance level at $P \leq 0.05$. Standard error is given
633 in each graph. (**b)** High levels of *Mds-1* expression in wheat confer avirulent insects (biotype
634 *GP*) the ability to manipulate normally resistant Molly plants. The left panel shows neutral red
635 staining of leaf-sheaths. *Mds-1*-ectopically exp. indicates Molly plants with *Mds-1* ectopically
636 expressed. Other denotations are the same as in a. The right panel shows growth inhibition of
637 Molly seedlings by biotype *GP*. Solid bars, uninfested; gray bars, infested plants of control
638 Molly (CM), heat-stressed Molly (HM), or Molly with *Mds-1* ectopic expression (TM). Four
639 replicates were carried out for each analysis and standard error is given in the figure.
640

641 **Figure 5**



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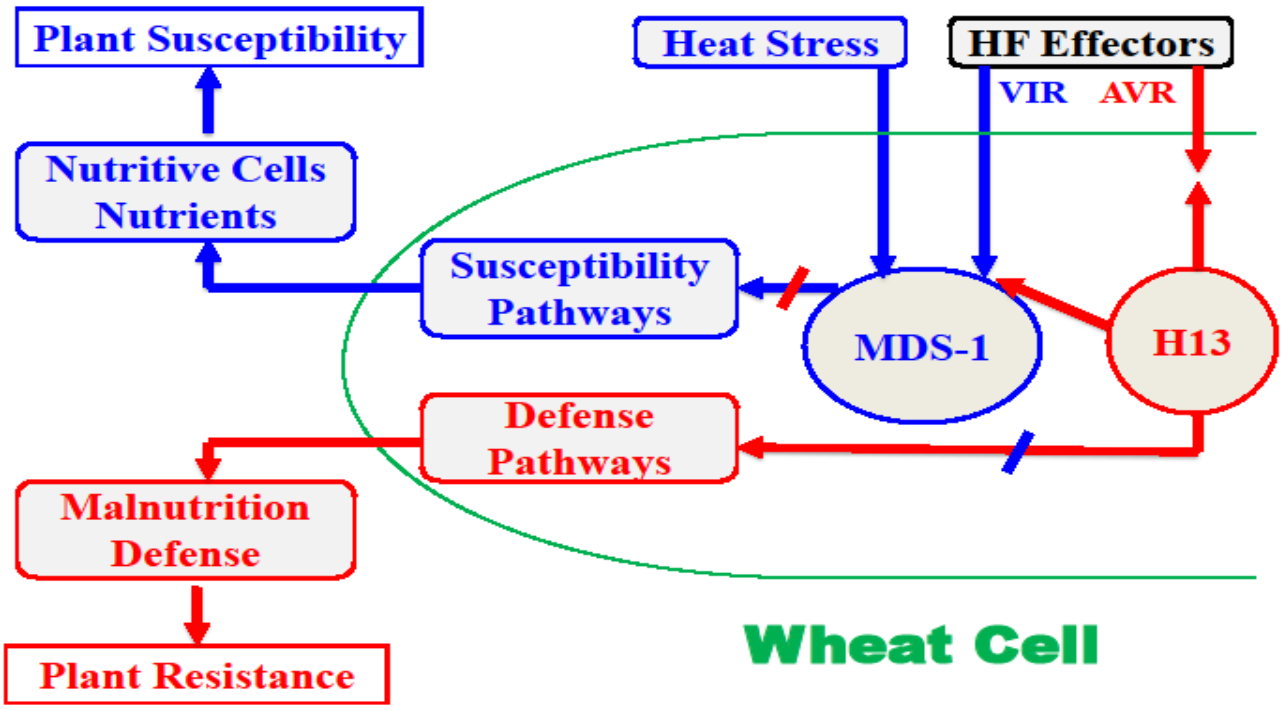
644 **Fig. 5. Levels of *Mds-1* expression affect development of *B. graminis* f. sp. *tritici*. (a)** *Mds-1*
645 silencing inhibits virulence of *B. graminis* f. sp. *tritici* on the susceptible wheat Bobwhite. Large
646 lesions (arrow) on a vector-transformed, Bobwhite plant (V-BW), small lesions on the *Mds-1*-
647 silenced plant T1639. **(b)** *Mds-1* transcript increases following *B. graminis* f. sp. *tritici* infection
648 in susceptible cultivars Molly (M), Newton (N), and Bobwhite (B), but not in the resistant
649 cultivar Duster (D) based on real-time PCR (qPCR) results of samples 24 h after inoculation.

650 Three biological replicates were carried out for qPCR. Data were subjected to ANOVA analysis.
651 Tukey's pairwise comparisons based on Student's range statistics were then conducted. Tukey's
652 95% simultaneous confidence intervals for pair-wise comparisons were used to separate data into
653 groups with significant differences. Lower case letters indicate different groups at $P \leq 0.05$.
654 Standard error is given in the figure. (**c)** Heat stress (HS, at 35°C for 24 h) induces *Mds-1*
655 expression over 100 fold in the resistant cultivar Duster compared with control plants (CK)
656 determined by qPCR. Three replicates were carried out and standard error is given in the figure.
657 (**d)** Heat stress compromises the resistance of Duster to *B. graminis* f. sp. *tritici*. The relative
658 disease index was 1.3±0.5 on control plants (CK), but climbed to 3.6±0.4 on plants stressed at
659 35°C for 24 h (HS) on a scale of 0 to 4.
660

661 **Figure 6**



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664 **Fig. 6. A model for *Mds-1* involvement in plant susceptibility.** Blue arrows between objects
665 indicate conditions leading to plant susceptibility through *Mds-1* up-regulation. Red arrows
666 between objects indicate conditions leading to plant resistance through the prevention of *Mds-1*
667 induction. The arrows between MDS-1 and H13 R protein indicate possible interactions between
668 them. During compatible interactions in susceptible plants, virulent (VIR) effectors from
669 Hessian fly upregulate *Mds-1* expression either directly by targeting *Mds-1* regulatory elements
670 or indirectly through activating heat-shock transcription factor(s). High levels of *Mds-1*
671 expression result in activation of susceptibility pathways, leading to the reprogramming of
672 metabolic pathways, suppression of plant defense, and the formation of nutritive cells at the

673 feeding site of host plants. During incompatible interactions in plants with R gene *H13*, the
674 corresponding Avirulence (AVR) effector from Hessian fly is recognized by H13 R protein,
675 leading to blockage of *Mds-1* upregulation and activation of defense pathways. The suppression
676 of high level *Mds-1* expression inhibit nutritive cell formation and possibly other susceptibility
677 events, leading to Hessian fly larval death due to the lack of nutrition and possibly increased
678 toxicity.