Phenotype and Cellular Response of Wheat Lines Carrying *Cre* Genes to *Heterodera avenae* Pathotype Ha91

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Abstract

The cereal cyst nematode (CCN, *Heterodera avenae*), a major limiting factor for wheat production worldwide, is widespread in most wheat-growing regions in China. Accordingly, screening and characterization of resistant (R) wheat sources against *H. avenae* are very important. In this study, we screened 51 wheat lines, collected from the International Wheat and Maize Improvement Center (CIMMYT), carrying various *Cre* genes (*Cre1*, *Cre2*, *Cre3*, *Cre5*, *Cre7*, *Cre8*, *CreR*, and *Pt*). From that screen, we identified one immune (M) cultivar (with no adult females produced) and five resistant cultivars (with fewer than five females) to *H. avenae* pathotype Ha91. The *Cre3* gene conferred the most effective resistance against *H. avenae* pathotype Ha91 in both field and greenhouse assays. Conversely, the *Cre1* and *CreR* genes conferred the poorest effective resistance. Using Pluronic F-127 gel and a staining assay, juvenile nematodes invading wheat roots were observed, and nematode development was analyzed. Compared with

R and M roots, those of the susceptible (S) wheat cultivar Wenmai19 were more attractive to *H. avenae* second-stage juveniles (J2s). We observed the retardation of nematode development in R cultivars and tiny white female cysts protruding from the R cultivar VP1620. Nematodes in M roots either disintegrated or remained J2s or third-stage juveniles (J3s) and failed to complete their life cycle. Molting was also suppressed or delayed in R and M genotypes. For both S and R cultivars, syncytia were characterized by cell wall perforations and dense cytoplasm in hypertrophied syncytium component cells. Syncytial size increased gradually with nematode development in S cultivars. Moreover, an incompatibility reaction occurred in M wheat roots: the syncytium was disorganized, exhibiting disintegration and condensed nuclei. These sources of genetic resistance against CCN can potentially be planted in severely infested fields to reduce economic loss or can be used for introgression in breeding.

The cereal cyst nematode (CCN) *Heterodera avenae* is a destructive pest of cereal crops worldwide and causes significant yield losses in many countries (Cui et al. 2017; Nicol and Rivoal 2008; Smiley et al. 2011). For example, more than 50% of fields in major cereal-growing areas in Europe are infected by this nematode, with staggering annual yield losses reaching £3 million (Nicol and Rivoal 2008). In China, more than 4 million ha in major wheat-producing regions are infected by CCN, with losses estimated at 1.9 billion RMB per year (Peng et al. 2007, 2009). Moreover, yield losses of 16 to 55% have occurred in China when the CCN density exceeded 10 eggs per g of soil (Hou et al. 2012). CCN was first reported in Hubei Province in 1989, and subsequent surveys have indicated that this species is widely distributed, being found in more than 16 provinces in China to date (Cui et al. 2015). To address the high CCN prevalence, multiple control measures, such as resistance breeding, crop rotation, nematicide application, soil plowing, and amendments, have been adopted (Nicol and Rivoal 2008; Peng et al. 2009). Peng and Cook (1996) reported that pathotypes of *H. avenae* populations in China might differ considerably from those in other regions and that there are at least three CCN pathotypes in China. Yuan et al. (2010) reported a new pathotype (Ha43) from Xushui and Xingyang, near Zhengzhou. Cui et al. (2015) classified the Daxing and Huangyan populations as belonging to another new pathotype and named it Ha91.

However, the use of resistant (R) cultivars is the most cost effective and promising method for CCN control (Nicol et al. 2011). Sources of resistance in barley and wild wheat relatives have been identified and verified, and genes conferring resistance to some CCN populations have been introgressed into various cultivars (Ogbonnaya et al. 2001). Many genes underlying resistance to CCNs, including *Cre* (1–8, 3S,

R) genes, *Ha* (1–4) genes, the *R1* gene, and many other dominant or minor genes, have been identified using traditional mapping-based cloning strategies. The *Cre1* and *Cre8* genes were identified directly in *Triticum aestivum*, AUS 10894/AUS 4930, and Festiguay (de Majnik et al. 2003; Safari et al. 2005). In contrast, the *Cre2*, *Cre5*, and *Cre6* genes were derived from *Aegilops ventricosa* (Jahier et al. 2001; Ogbonnaya et al. 2001; Rivoal et al. 2001) and *Cre3* and *Cre4* from *A. tauschii* (de Majnik et al. 2003; Rivoal et al. 2001). In addition, the *Cre3S* and *Cre7* genes originated from *A. peregrina* and *A. triuncialis*, respectively (Jahier et al. 1998; Romero et al. 1998). *CreR* was identified in *Secale cereale* (Taylor et al. 1998), and *CreX* and *CreY* were derived from *A. variabilis* (Barloy et al. 2007). Furthermore, seven resistance genes, *Rha1*, *Rha1+*, *Rha2*, *Rha2+*, *Rha3+*, *Rha4*, and *RhaE*, were derived from barley (Barr et al. 1998; Seah et al. 2000; Smiley et al. 2011). The resistance gene *R1* and three dominant genes (*A*, *B*, and *C*), as well as many minor genes, have also been identified in wild oat (*Avena sterilis*) (Clamot and Rivoal 1984; Smiley et al. 2011). Further, the *Pt* gene identified from the bread wheat line GS50a, showing good resistance to *Pratylenchus thornei*, has been used in wheat breeding programs (Thompson et al. 1999; Zwart et al. 2004).

Pluronic F-127 gel has been used as a medium for studying nematodes because this transparent gel allows nematodes to travel freely, allowing three-dimensional assays (Wang et al. 2009). For example, Sasaki-Crawley et al. (2012) successfully applied Pluronic F-127 gel studies to compare *Globodera pallida* development in *Solanum tuberosum* and *S. sisymbriifolium*. The attraction of *H. filipjevi* or *H. avenae* juveniles toward root tips of wheat strains differing in resistance to CCN has also been investigated using Pluronic F-127 gel as a medium (Cui et al. 2012; Hu et al. 2015). Sedentary endoparasitic phytonematodes have acquired the ability to generate feeding sites in host cells to facilitate nutrient uptake (Mitchum et al. 2013). Dissolution of localized cell walls and protoplast fusion cause the syncytium to progressively enlarge until it ultimately incorporates up to 200 neighboring cells (Lilley et al. 2005). Nonetheless, there are large differences in syncytial composition between CCN R and susceptible (S) wheat cultivars (Aditya et al. 2015). Similarly, roots of both S and R cultivars are easily invaded by *H. avenae* J2s, although reduced

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numbers of white female cysts and decreased *H. avenae* egg density in soil are observed with R cultivars (Marshall and Smiley 2016).

The objectives of this study were 1) to screen international sources of R wheat carrying *Cre* genes that could be transferred to locally adapted cultivars via introgression and deployed to control CCNs in China; 2) to use Pluronic F-127 to study the phenotype in response to CCN infection of wheat varieties carrying *Cre* genes; and 3) to assess host cellular responses of different R wheat varieties carrying *Cre* genes to *H. avenae* pathotype Ha91.

Materials and Methods

Nematode collection. CCNs in soil were collected from infested wheat fields in Daxing, Beijing, China, at the end of the wheat-growing season. Cysts were extracted from the soil using Cobb's sieving gravity method (Persmark et al. 1992). As reported by Cui et al. (2015), the collected nematodes were identified as *H. avenae*, and the pathotype was identified as Ha91.

Resistance tests. *Field resistance test.* A CCN-infested field in the wheat-producing area of Daxing, Beijing, China, was used for field resistance tests during the 2012–13 season. According to previous experiments, the average density was 25 to 30 cysts per 200 g of soil (Hao et al. 2014). Fifty wheat lines collected from the CIMMYT (35 resistant wheat sources and 15 sources carrying different *Cre* genes [*Cre1*, *Cre2*, *Cre3*, *Cre5*, *Cre7*, *Cre8*, *CreR*, and *Pt*]) and one local control (Wenmai19) were tested. Twenty seeds were manually planted with uniform spacing in a 1-m × 2-m plot with four rows, and three replicates were included as randomized blocks. During Feekes growth stage 10.5.3 (Miller 1992), five wheat plants per plot were randomly selected, and newly formed white female cysts were counted (Marshall and Smiley 2016). The S wheat cultivar Wenmai19 was used as a local control. The resistance of cultivars was classified based on the number of cysts per root system: M = immune (0 females); R (0.1 to 5.0 females); MR = moderately R (5.1 to 10.0 females); MS = moderately S (10.1 to 15.0 females); S (15.1 to 25.0 females); and HS = highly S (>25.0 females) (Nicol et al. 2009).

Pot resistance test. To confirm resistance, greenhouse tests are generally more reliable than field tests because the field environment is variable (Yuan et al. 2011). Thus, we performed two greenhouse tests in 2013 and 2014. Seeds of each cultivar were germinated in Petri dishes and then transplanted into polyvinyl chloride tubes (3 cm in diameter, 30 cm in length) with 200 cm³ of a sterile soil mixture (loamy soil-compost-sand at 3:2:5). *H. avenae* cysts were hatched at 14 to 16°C; simultaneously, freshly hatched J2s were used to infect wheat roots (Cui et al. 2015). A total of 300 J2s were inoculated into each tube immediately after the seeds were planted, after which inoculations with 300 or 200 J2s were performed at 3-day intervals, resulting in a final inoculum density of 800 J2s per tube. Ten replicates were performed for each cultivar. The plants were successively grown in a greenhouse for 1 week at 14/18°C, 1 week at 16/20°C, 3 weeks at 18/25°C, and 2 weeks at 22/30°C, with an 8-h dark/16-h light cycle under normal plant growth conditions, including irrigation, fertilization, and control of diseases and insects. Seven weeks later, after the last inoculation, plants and soil samples were washed over a 250-μm sieve using a gentle stream of tap water. White females that were attached to roots and in the surrounding soil were collected and counted under a stereoscopic microscope, and the mean number of white females per plant was calculated. The resistance of each cultivar was classified as described above.

Attraction ability test. According to the results of resistance classification described above, the S cultivar Wenmai19, the R cultivar VP1620, and the M cultivar CROC-1/AE.SQUARROSA (224)//OPATA*B (Croc B) were selected for attraction tests, as described by Wang et al. (2009). To produce 23% (w/v) Pluronic F-127 gels (Sigma Aldrich, St. Louis, MO), 23 g of Pluronic F-127 powder was first added to sterile water at 2 to 4°C and dissolved by stirring overnight in a final volume of 100 ml. The dissolved gel was then stored at 4°C prior to use. Next, 1 ml of gel containing approximately 800 J2s and one sterile 3-day-old wheat root tip (1 cm in length) were added to wells of 12-well tissue culture plates. The culture plates were maintained in a plant growth chamber (Jiangnan Instrument

Co., Ltd, Ningbo, China) at 14 ± 1°C. The attracted J2s surrounding the wheat tips within 0.3 cm were counted at 2, 4, 6, 12, and 24 h after the experiment began, and photographs were obtained using a Leica dissecting microscope (Leica M165C, Wetzlar, Germany). Five replicates were performed for every cultivar, and each assay was performed three times.

Juvenile development analysis. Analysis of differences in juvenile nematode invasion, development, and reproduction can provide information about active resistance mechanisms (Pariyar et al. 2016). To identify the resistance mechanisms involved in different wheat-nematode interactions, we tested cultivars carrying different resistance genes. A pot experiment was conducted by counting the numbers of J2s, J3s, and fourth-stage juveniles (J4s) in the roots of the above-mentioned S, R, and M wheat cultivars at 5, 15, and 25 days after inoculation (DAI). Five wheat plants were randomly selected, and the roots were then carefully washed and stained with acid fuchsin, as described by Bybd et al. (1983). Juvenile CCNs at different developmental stages were examined for morphology and counted using a Leica dissecting microscope (Leica M165C, Wetzlar, Germany). Five replicates were performed for each cultivar, and each assay was performed three times.

Cellular response analysis. Andres et al. (2001) reported that infection of R wheat lines with *H. avenae* results in a hypersensitive reaction with deterioration of syncytial cells, whereas the syncytia of S wheat cultivars continue to enlarge. To study the underlying resistance mechanism, the roots of infected S, R, and M plants were harvested at 5, 10, 15, and 25 DAI and then stained using the acid fuchsin method. The samples were rinsed in distilled water twice for 3 min each, and the roots were subsequently incubated for 2 h in 1% acid fuchsin solution at 37°C and rinsed in distilled water. Root samples 1 cm in length were collected from each test plant, immersed overnight in 3% glutaraldehyde, and dehydrated by passage through a graded ethanol series (10 to 100%). The samples were then infiltrated and embedded in paraffin at 60°C, after which they were sectioned using a JB-4 microtome. The sections were stained for 5 min with 1% toluidine blue in 1% borax solution (TAAB) at 40°C as described by Kong et al. (2016). The syncytial sizes of Wenmai19, VP1620, and Croc B were measured at 5, 15, and 25 DAI as previously described (Siddique et al. 2012). For each wheat plant, 10 syncytia were randomly selected and examined; the structures were outlined, and their areas were calculated by using software with a Leica M165C microscope (Kong et al. 2016).

Statistical analysis. Statistical analysis was performed on the number of white female cysts identified in the greenhouse pot trials and field tests, the number of attracted J2s in Pluronic F-127, and the numbers of juveniles of different developmental stages per root as well as on the growth indices of syncytial areas. Analysis of variance (ANOVA) was performed individually for each experiment. Significant differences ($P \leq 0.05$) among treatments were determined by using the least significant difference (LSD) test using SPSS 12.0 software (SPSS Inc.).

Results

Resistance test. To identify valuable genetic resources for wheat breeding programs, we evaluated 51 wheat cultivars carrying *Cre* genes, all obtained from CIMMYT. In the field test (2012), 14 cultivars were found to be HS, 16 S, 12 MS, four MR, and four R; Croc B was M (Table 1). In pot tests (2013 and 2014), the results from 2013 were similar to those from 2014. In 2013, 35 cultivars were HS, seven S, three MS, and three MR; VP1620 (*Cre3*) and Croc A were R, and Croc B was M (Table 1). In 2014, 37 cultivars were HS, seven S, and four MR; VP1620 (*Cre3*) and Croc A were R, and Croc B was M; no cultivars were MS (Table 1). Based on the three years of screening results, Croc B was consistently M to *H. avenae* pathotype Ha91 in both field and greenhouse tests. Wheat cultivar VP1620, which carried the *Cre3* resistance gene, was highly effective in suppressing *H. avenae* reproduction. Lines derived from the same variety exhibited an identical or similar resistance level, and Croc A was also R to this population; however, the resistance gene has yet to be identified in Croc A and Croc B (Table 2). In the field trial, prevention of

Table 1. Evaluation of 51 cultivars from CIMMYT carrying *Cre* genes in the presence of *H. avenae* pathotype Ha91 in field and greenhouse trials from 2012 to 2014

Line name ^u	Gene ^v	2012 ^w		2013 ^x		2014 ^x	
		Mean $\pm$ SD ^y	Reaction ^z	Mean $\pm$ SD ^y	Reaction ^z	Mean $\pm$ SD ^y	Reaction ^z
DAGDAS CHECK		51.6 $\pm$ 6.77	HS	68.1 $\pm$ 3.75	HS	63.6 $\pm$ 3.72	HS
MAYOOR/TKSN1081/AE.SQUARROSA (222)		47.0 $\pm$ 8.39	HS	38.7 $\pm$ 4.13	HS	43.5 $\pm$ 3.03	HS
ES84-24/DYNASTY		44.0 $\pm$ 5.83	HS	40.0 $\pm$ 4.24	HS	66.5 $\pm$ 3.63	HS
Silverstar	<i>Cre1</i>	38.2 $\pm$ 2.59	HS	45.9 $\pm$ 4.95	HS	44.1 $\pm$ 3.48	HS
BILINMIYEN96.7		33.4 $\pm$ 6.22	HS	44.5 $\pm$ 3.27	HS	51.0 $\pm$ 3.74	HS
LOCAL CHECK		33.6 $\pm$ 5.22	HS	40.6 $\pm$ 4.14	HS	58.8 $\pm$ 4.23	HS
LOV41/LI7/LE2062		33.2 $\pm$ 3.83	HS	62.0 $\pm$ 3.43	HS	72.7 $\pm$ 4.42	HS
GS50A	<i>Pt</i>	31.8 $\pm$ 5.40	HS	53.6 $\pm$ 4.48	HS	38.9 $\pm$ 2.51	HS
2-49		29.6 $\pm$ 3.91	HS	39.4 $\pm$ 4.09	HS	34.6 $\pm$ 3.84	HS
CPI133859		28.2 $\pm$ 4.43	HS	35.8 $\pm$ 3.99	HS	42.0 $\pm$ 2.98	HS
Chara	<i>Cre1</i>	28.8 $\pm$ 3.56	HS	16.2 $\pm$ 2.69	S	17.7 $\pm$ 2.83	S
AUS 10894	<i>Cre1</i>	28.2 $\pm$ 3.89	HS	36.1 $\pm$ 3.95	HS	35.1 $\pm$ 4.23	HS
SUNCO		25.2 $\pm$ 1.92	HS	36.4 $\pm$ 3.63	HS	37.1 $\pm$ 2.60	HS
CPI133872		25.1 $\pm$ 3.80	S	54.8 $\pm$ 7.19	HS	57.4 $\pm$ 5.58	HS
SABUF/7/ALTAR84		23.2 $\pm$ 4.55	S	59.4 $\pm$ 5.21	HS	61.3 $\pm$ 3.62	HS
VP5053	<i>Cre8</i>	23.2 $\pm$ 2.48	S	63.5 $\pm$ 4.14	HS	33.2 $\pm$ 3.88	HS
KRC66/SERI/4/YMH/TOB/MCD/3/LIRA		22.4 $\pm$ 3.51	S	35.5 $\pm$ 5.19	HS	43.2 $\pm$ 1.93	HS
Potch92		22.0 $\pm$ 4.52	S	34.6 $\pm$ 3.72	HS	67.5 $\pm$ 3.02	HS
SABUF/3/BCN/CETA/AE.SQUARROSA (895)		21.0 $\pm$ 3.08	S	46.5 $\pm$ 6.34	HS	82.3 $\pm$ 3.77	HS
ALTAY 2000		20.8 $\pm$ 4.15	S	37.5 $\pm$ 5.25	HS	37.1 $\pm$ 2.92	HS
ID-2150	<i>Cre2</i>	20.2 $\pm$ 3.70	S	39.3 $\pm$ 4.59	HS	64.7 $\pm$ 2.75	HS
GS50A T41.1	<i>Pt?</i>	19.2 $\pm$ 3.89	S	41.8 $\pm$ 6.14	HS	16.5 $\pm$ 2.17	S
AUSGS50AT34/SUNCO/CUNNINGHAM-98		19.0 $\pm$ 2.74	S	39.2 $\pm$ 4.64	HS	36.3 $\pm$ 2.83	HS
GATCHER		18.2 $\pm$ 3.27	S	38.5 $\pm$ 3.17	HS	25.8 $\pm$ 2.85	HS
VL411R		16.4 $\pm$ 2.88	S	36.9 $\pm$ 3.93	HS	45.6 $\pm$ 2.67	HS
6R(6D)	<i>CreR</i>	16.2 $\pm$ 2.38	S	36.9 $\pm$ 3.73	HS	37.6 $\pm$ 3.53	HS
SUNCO/2*PASTOR		15.4 $\pm$ 1.67	S	36.7 $\pm$ 2.75	HS	36.3 $\pm$ 3.19	HS
F130L1.12/ATTILA		15.2 $\pm$ 1.92	S	29.2 $\pm$ 3.71	HS	52.8 $\pm$ 4.26	HS
BURBOT-6		15.4 $\pm$ 1.87	S	27.2 $\pm$ 4.31	HS	30.6 $\pm$ 2.98	HS
SONMEZ		14.4 $\pm$ 1.52	MS	59.5 $\pm$ 5.85	HS	33.8 $\pm$ 3.96	HS
AUS4930.7	<i>Cre1?</i>	14.0 $\pm$ 3.16	MS	16.5 $\pm$ 3.24	S	20.7 $\pm$ 3.06	S
T-2003	<i>Cre7</i>	13.8 $\pm$ 1.78	MS	23.4 $\pm$ 3.37	S	24.2 $\pm$ 1.87	S
BATAVIA		13.8 $\pm$ 1.64	MS	31.3 $\pm$ 3.53	HS	37.1 $\pm$ 3.66	HS
ZANDER-44		12.0 $\pm$ 1.58	MS	22.3 $\pm$ 3.40	S	18.2 $\pm$ 2.65	S
WARIGAL		11.2 $\pm$ 0.19	MS	27.8 $\pm$ 1.87	HS	29.2 $\pm$ 2.61	HS
GOLDMARK		11.0 $\pm$ 2.73	MS	20.5 $\pm$ 2.37	S	28.6 $\pm$ 2.50	HS
ES84.24/GRK		10.8 $\pm$ 2.05	MS	24.4 $\pm$ 2.67	S	27.4 $\pm$ 3.09	HS
AUS4930.7/2*PASTOR	<i>Cre1?</i>	10.8 $\pm$ 1.92	MS	18.8 $\pm$ 3.48	S	36.3 $\pm$ 2.83	HS
SERI		10.6 $\pm$ 2.30	MS	25.9 $\pm$ 2.51	HS	53.5 $\pm$ 3.34	HS
ALTAR 84/AEGILOPS SQUARROSA (TAUS)/OPATA		10.2 $\pm$ 1.92	MS	42.2 $\pm$ 2.53	HS	40.9 $\pm$ 2.76	HS
Frame	<i>Cre8</i>	10.2 $\pm$ 3.27	MS	13.5 $\pm$ 3.87	MS	8.4 $\pm$ 0.97	MR
IWA 8604765(PI 628144)		8.2 $\pm$ 1.48	MR	11.1 $\pm$ 0.88	MS	23.9 $\pm$ 2.77	S
IWA 8608077(PI 621458)		8.0 $\pm$ 2.24	MR	14.7 $\pm$ 0.95	MS	18.2 $\pm$ 2.86	S
MILAN	<i>Cre5?</i>	7.4 $\pm$ 1.14	MR	8.2 $\pm$ 3.96	MR	6.6 $\pm$ 1.71	MR
KATEA-1		6.2 $\pm$ 1.92	MR	5.5 $\pm$ 2.67	MR	9.1 $\pm$ 1.52	MR
VP1620	<i>Cre3</i>	4.6 $\pm$ 1.52	R	4.8 $\pm$ 1.23	R	4.1 $\pm$ 1.37	R
VENTURA(SUN376G)		3.6 $\pm$ 0.89	R	5.3 $\pm$ 1.63	MR	7.0 $\pm$ 1.49	MR
AUS4930 6.5/GS50A	<i>Pt?</i>	3.4 $\pm$ 1.14	R	28.2 $\pm$ 2.04	HS	33.2 $\pm$ 2.44	HS
CROC1/AE.SQUARROSA (224)/OPATA*A		0.2 $\pm$ 0.84	R	1.6 $\pm$ 1.43	R	0.9 $\pm$ 0.87	R
CROC_1/AE.SQUARROSA (224)/OPATA*B		0	M	0	M	0	M
Wenmai19		45.0 $\pm$ 7.52	HS	62.8 $\pm$ 1.87	HS	66.5 $\pm$ 3.03	HS

^u Source from CIMMYT; Wenmai19 was used as the local susceptible control; CROC1/AE.SQUARROSA (224)/OPATA*A= CrocA, CROC_1/AE.SQUARROSA (224)/OPATA*B= CrocB.

^v Gene information was obtained from the Soil Borne Pathogens Program of CIMMYT-Turkey, further information supported by Nicol and Rivoal (2008), Nicol et al. (2009), and Smiley 2014; *Cre* gene is reported against CCN species, *Pt* gene is resistant to *Pratylenchus thornei*, *Cre1?*, *Cre5?* and *Pr?* mean the source have wild progenitors of wheat resistant gene and possess partial resistance to CCN or *Pt*.

^w Wheat cultivar susceptibility to *H. avenae* in 2012 was evaluated in fields in Daxing, Beijing.

^x Wheat cultivar susceptibility to *H. avenae* in 2013 and 2014 was evaluated in the greenhouse.

^y Mean $\pm$ standard deviation of the mean from 10 wheat root replicates.

^z Reaction: M = immune (0 cysts), R = resistant (0.1 to 5.0 cysts), MR = moderately resistant (5.1 to 10.0 cysts), MS = moderately susceptible (10.1 to 15.0 cysts), S = susceptible (15.1 to 25.0 cysts), and HS = highly susceptible (>25.0 cysts).

H. avenae reproduction was observed for MILAN wheat cultivars carrying the *Cre5* gene. AUS4930 6.5/GS50A, which carried the *Pt* gene, as well as IWA 8604765 (PI 628144), IWA 8608077 (PI 621458), KATEA-1, and VENTURA (SUN376G), were MR (Table 2). The resistance reactions in the present study were almost in accordance with the report by Li et al. (2013) (Table 3). Wheat cultivars carrying the *Cre1* and *CreR* genes exhibited the lowest effective resistance to *H. avenae* pathotype Ha91, findings that were obviously different from the results of Smiley (2014), Smiley et al. (2011), Yuan et al. (2011), Nicol and Rivoal (2008), and Nicol et al. (2009) (Table 3). *Cre7* and *Cre8* resistance genes were also ineffective at suppressing *H. avenae* reproduction, with reactions comparable to those reported by Li et al. (2013), Yuan et al. (2011), Nicol and Rivoal (2008), and Nicol et al. (2009), but not by Smiley (2014) or Smiley et al. (2011) (Table 3).

Attraction test. In the present study, *H. avenae* J2s were quickly attracted to wheat roots in Pluronic F-127 gel, and those that traveled within 3 mm of the roots were counted. We found that most J2s directly and rapidly migrated toward roots, whereas others traveled in a more curved fashion, with their head and tail relatively close together. The nematodes that traveled in a curved fashion moved slowly forward or backward in circles and displayed almost no change in position (Fig. 1). In the Pluronic F-127 gel, *H. avenae* J2s traveled toward wheat roots within the first 2 h after initiation of the attraction test. The number of J2s in contact with roots increased over time. By 4, 6, 12, and 24 h after initiation, the numbers of J2s surrounding the root tip had increased to 63.3, 99.3, 137.7, and 143.7 for Wenmai19; 48.0, 84.3, 106.7, and 114.0 for VP1620; and 39.7, 51.0, 59.7, and 60.3 for Croc B, respectively (Fig. 2). The roots of the S wheat cultivar Wenmai19 were more attractive to *H. avenae* J2s than were R and M roots. Although the roots became dehydrated after approximately 24 h, and the ability of the wheat roots to attract decreased over time, the number of J2s increased slowly, peaking at 24 h. We also counted the number of J2s 48 h after initiation, but no changes were observed relative to 24 h. Overall, 143.7, 114.0, and 60.3 J2s were observed surrounding Wenmai19, VP1620 (*Cre 3*), and Croc B roots, respectively (Fig. 2).

Juvenile development analysis. We assessed the effects of different cultivars on juvenile CCNs by counting the numbers of J2s, J3s, and J4s in wheat roots. The roots of S, R, and M cultivars were stained at 5, 15, and 25 DAI. At 5 DAI, the numbers of established nematodes in R and M roots were significantly lower than that in S roots, with an average of 248 J2s being observed in cv. Wenmai19 roots but only an average of 56 in VP1620 roots and 31 in Croc B roots. The number of penetrated CCN juveniles decreased gradually over time (Table 2). At 15 DAI, the total numbers of CCN juveniles in the roots were 135, 20, and 10 in Wenmai19, VP1620, and Croc B, respectively. At 15 DAI, the proportion of CCN juveniles that had become J3s was more than 90% in Wenmai19, whereas it was decreased to 71% in VP1620 and only 26% in Croc B. In Wenmai19

roots at 25 DAI, the overwhelming majority of the penetrated J2s were able to develop into J4s. In contrast, we found that nematodes did not develop into J4s in the M cultivar Croc B. CCN juveniles in VP1620 were developmentally retarded, with only 40% developing into J4s (Fig. 3). At 35 DAI, white female cysts were observed protruding from the roots of the S cultivar but not from those of the M and R cultivars. The nematodes in Croc B roots either disintegrated or remained J2s or J3s and failed to complete their life cycle. However, in VP1620, tiny white female cysts protruded nearly 10 days later than in the S cultivar.

Cellular response analysis. How nematodes alter root cell development to produce and maintain syncytia is a question of great interest. In root slices of the S cultivar, most nematodes had penetrated within 48 h and then migrated to the stelar regions, where the feeding sites (syncytia) had been initiated. However, syncytia were initiated later in M and R roots; in M roots, syncytia were initiated at 5 DAI. We selected four time points (5, 10, 15, and 25 DAI) for analysis in accordance with CCN development into J2s, J3s, and J4s (Fig. 4). At 5 DAI, necrosis had initiated adjacent to the nematode lip region in M and R roots (Fig. 4A, E). At 10 DAI, extreme nuclear hypertrophy was always observed in the syncytium of the S host. In cv. Wenmai19, a major portion of the syncytium stretched over the vascular tissue (Fig. 4J). In contrast, syncytia in VP1620 formed inside the vascular tissue, starting at the endodermis and enclosing xylem elements (Fig. 4F), and in Croc B, syncytia were located in cortical cells, adjacent to the endodermis, and outside of the central area of the vascular bundle (Fig. 4B). By 15 DAI, a major change occurred in the appearance of the syncytia in the S cultivar: the syncytia were highly active, with hypertrophied nuclei and fewer large vacuoles (Fig. 4K). In contrast, the syncytia of the R cultivar were still dominated mainly by large vacuoles with weakly stained cytoplasm, indicating low activity, and the vascular elements were forced into irregular arrangements to avoid lateral roots and large syncytia (Fig. 4J). An incompatibility reaction occurred in roots of the M cultivar: the syncytium was disorganized, exhibiting disintegration and appearing to be inactive and nonfunctional (Fig. 4C). Continuous syncytial development was observed over a period of 25 DAI. The syncytia of the S cultivar exhibited dense cytoplasm with numerous subcellular organelles (Fig. 4L). In contrast, the cytoplasm in the syncytia of the R cultivar was reduced to a thin layer around the cell walls and wall fragments. After nuclear degeneration, the cytoplasm also appeared to undergo degradation (Fig. 4H). At 25 DAI, the syncytia of the M cultivar exhibited complete tonoplast breakdown and may have been completely nonfunctional. No structurally recognizable nuclei were observed in these syncytia, indicating that the nuclei had likely degenerated (Fig. 4D). Over the same period, the syncytial cells of Wenmai19 were considerably larger than those of the R and M cultivars (Fig. 5).

Discussion

The sources of resistance to *H. avenae* identified to date have predominantly originated from wild relatives of wheat in the *Aegilops* genus and have already been transferred to hexaploid wheat backgrounds for breeding purposes via introgression (Nicol et al. 2011). Assessment of the relative resistance of genotypes includes growing plants in soil at sites of high infestation or in naturally infested soil transported to controlled environmental conditions (Eastwood et al. 1991). A comparison of tests conducted in the current study under field and greenhouse conditions revealed that 11 wheat cultivars showed higher susceptibility in the greenhouse than in the field, whereas none of the cultivars except for cv. Chara showed higher susceptibility in the field than in the greenhouse (Table 2). VP1620 (*Cre3*) exhibited significantly greater resistance than was associated with any of the other *Cre* genes in the tested group (Table 2). *Cre3* has also been shown to be moderately effective against *H. avenae* populations in Hebei, China, but not against *H. avenae* and *H. filipjevi* populations in Henan (Nicol et al. 2009) (Table 3). Safari et al. (2005) reported that in Australia, the most marked reduction in the number of female cysts was produced by *Cre3*, followed by *Cre1* and *Cre8*. In addition, *Cre3* and *Cre6* provide better resistance than *Cre1* against pathotype Ha13 but are susceptible to pathotypes Ha11 and Ha12 (Ogbonnaya et al. 2001).

Table 2. Numbers of different *H. avenae* developmental stages observed within susceptible, resistant, and immune wheat roots.

Time ^x	Cultivars ^y	No. of different stages of juveniles ^z		
		J2s	J3s	J4s
5 DAI	Wenmai19	248.0 ± 16.09 a	0	0
	VP1620	56.0 ± 9.17 b	0	0
	Croc B	30.7 ± 4.16 c	0	0
15 DAI	Wenmai19	12.3 ± 1.53 a	122.3 ± 5.13 a	0
	VP1620	5.7 ± 1.15 b	14.0 ± 3.46 b	0
	Croc B	7.6 ± 1.53 b	2.7 ± 0.58 c	0
25 DAI	Wenmai19	0	4.7 ± 1.15 a	53.3 ± 4.51 a
	VP1620	1.3 ± 1.52 a	4.3 ± 2.08 a	3.7 ± 3.06 b
	Croc B	2.3 ± 0.57 a	1.4 ± 0.57 b	0

^x DAI, days after inoculation.

^y Wenmai19, susceptible wheat host; VP1620, resistant wheat host; Croc B, immune wheat host.

^z Mean ± standard deviation of the mean from five wheat root replicates. Different letters denote significant differences ($P \leq 0.05$). J2, second-stage juvenile; J3, third-stage juvenile; J4, fourth-stage juvenile.

A previous study clearly demonstrated that the presence of the resistance genes *Cre2*, *Cre3*, *Cre5*, *Cre7*, or *Cre8* in wheat does not adequately suppress *H. avenae* reproduction in soil from the U.S. Pacific Northwest, whereas wheat cultivars carrying *Cre1* are highly effective at suppressing *H. avenae* reproduction (Smiley 2014;

Smiley et al. 2011) (Table 3). Nicol and Rivoal (2008) indicated that the effects of certain *Cre* genes depend on both the CCN species and pathotype. *Cre2* confers a high level of resistance to populations of *H. avenae* pathotypes Ha71 and Ha11 as well as Ha12 and Ha41 but is ineffective against Hg1 to HgIII and Ha13 (Delibes et al.

Table 3. Resistance reactions of wheat from CIMMYT carrying *Cre* genes to CCN species and populations according to the present study and previous reports^w

Line name	Gene	Present study ^x			Li et al. ^y	Smiley et al. ^y	Yuan et al. ^z	Nicol et al. ^z
		2012	2013	2014	2013	2011 and 2014	2011	2008 and 2009
DAGDAS CHECK		HS	HS	HS	HS	*	HS	N
MAYOOR//TKSN1081/AE.SQUARROSA (222)		HS	HS	HS	S	*	HS	N
ES84-24/DYNASTY		HS	HS	HS	HS	*	HS	N
Silverstar	<i>Cre1</i>	HS	HS	HS	HS	M	HS	MR
BILINMIYEN96.7		HS	HS	HS	*	*	*	*
LOCAL CHECK		HS	HS	HS	*	*	*	*
LOV41//LI7/LE2062		HS	HS	HS	HS	*	*	N
GS50A	<i>Pt</i>	HS	HS	HS	S	*	HS	N
2-49		HS	HS	HS	MS	*	HS	N
CPI133859		HS	HS	HS	*	S	*	MS
Chara	<i>Cre1</i>	HS	S	S	S	R	HS	MR
AUS10894	<i>Cre1</i>	HS	HS	HS	*	M	*	*
SUNCO		HS	HS	HS	S	*	*	N
CPI133872		S	HS	HS	*	MR	MS	*
SABUF/7/ALTAR84		S	HS	HS	*	*	S	*
VP5053	<i>Cre8</i>	S	HS	HS	HS	R	HS	S
KRC66/SERI/4/YMH/TOB//MCD/3/LIRA		S	HS	HS	HS	*	*	N
Potch92		S	HS	HS	S	*	HS	N
SABUF/3/BCN//CETA/AE.SQUARROSA (895)		S	HS	HS	S	*	HS	N
ALTAY 2000		S	HS	HS	*	*	HS	*
ID-2150	<i>Cre2</i>	S	HS	HS	S	S	*	MS
GS50A T41.1	<i>Pt?</i>	S	HS	S	S	*	*	*
AUSGS50AT34/SUNCO//CUNNINGHAM-98		S	HS	HS	S	S	S	P
GATCHER		S	HS	HS	S	*	HS	N
VL411R		S	HS	HS	HS	HS	HS	S
6R(6D)	<i>CreR</i>	S	HS	HS	HS	HS	R	MR
SUNCO/2*PASTOR		S	HS	HS	HS	*	HS	N
F130L1.12/ATTILA		S	HS	HS	S	S	*	N
BURBOT-6		S	HS	HS	S	*	*	N
SONMEZ		MS	HS	HS	S	S	*	N
AUS4930.7	<i>Cre1?</i>	MS	S	S	S	*	HS	P
T-2003	<i>Cre7</i>	MS	S	S	MS	R	HS	MS
BATAVIA		MS	HS	HS	S	*	HS	N
ZANDER-44		MS	S	S	S	*	HS	N
WARIGAL		MS	HS	HS	HS	*	HS	N
GOLDMARK		MS	S	HS	S	*	HS	N
ES84.24/GRK		MS	S	HS	HS	*	HS	N
AUS4930.7/2*PASTOR	<i>Cre1?</i>	MS	S	HS	MR	R	HS	N
SERI		MS	HS	HS	R	*	HS	N
ALTAR84/AEGILOPSSQUARROSA (TAUS)//OPATA		MS	HS	HS	MS		*	N
Frame	<i>Cre8</i>	MS	MS	MR	MS	MR	HS	P
IWA 8604765(PI 628144)		MR	MS	S	*	*	*	*
IWA 8608077(PI 621458)		MR	MS	S	*	*	*	*
MILAN	<i>Cre5?</i>	MR	MR	MR	MS	MR	HS	P
KATEA-1		MR	MR	MR	MS	HS	HS	N
VP1620	<i>Cre3</i>	R	R	R	MR	HS	HS	S
VENTURA(SUN376G)		R	MR	MR	R	*	HS	N
AUS4930 6.5/GS50A	<i>Pt?</i>	R	HS	HS	*	*	*	N
CROC-1/AE.SQUARROSA (224)//OPATA*A		R	R	R	R	R	MR	S
CROC_1/AE.SQUARROSA (224)//OPATA*B		M	M	M	*	*	*	*
Wenmai19		HS	HS	HS	HS	*	HS	*

^w * indicates not tested; gene and reaction are the same as in Table 1.

^x The present study was conducted in naturally infested fields in 2012 and in a greenhouse by artificial inoculation in 2013 and 2014.

^y Tested organism was *H. avenae*.

^z Tested organism was *H. filipjevi*; P = positive for resistance, N = negative for resistance.

1993; Ogonnaya et al. 2001, 2009). In addition, the genes *Cre5* and *Cre8* confer partial resistance to pathotype Ha13, and *Cre5* is partially effective against Ha12 and Ha41 (Jahier et al. 2001; Ogonnaya et al. 2001). Although *CreR* confers resistance or moderate resistance

to *H. filipjevi* (Nicol and Rivoal 2008; Nicol et al. 2009; Yuan et al. 2011), cultivars carrying this gene were found to be highly S to the *H. avenae* population used in the present study, similar to findings reported by Smiley et al. (2011), Smiley (2014), and Li et al. (2013)

Fig. 1. Attraction of *Heterodera avenae* (pathotype Ha91) juveniles toward immune, resistant, and susceptible wheat root tips in Pluronic F-127 gel. Scale bar = 1 mm. **A-E**, Observations at 2 h (**A**), 4 h (**B**), 6 h (**C**), 12 h (**D**), and 24 h (**E**) after the initiation of interaction between roots and juveniles in the immune wheat root group. **F-J**, Observations at 2 h (**F**), 4 h (**G**), 6 h (**H**), 12 h (**I**), and 24 h (**J**) after the initiation of interaction between roots and juveniles in the resistant wheat root group. **K-O**, Observations at 2 h (**K**), 4 h (**L**), 6 h (**M**), 12 h (**N**), and 24 h (**O**) after the initiation of interaction between roots and juveniles in the susceptible wheat root group.

Fig. 2. Differences in the attraction of *Heterodera avenae* (pathotype Ha91) juveniles in Pluronic F-127 gel toward wheat root tips with different resistance levels. Each point represents the mean of five replicates; the vertical bars indicate the standard deviation of the mean.

(Table 3). Moreover, Nicol et al. (2011) reported that both *CreR* and *CreI* were effective sources of resistance against *H. avenae* populations from Henan, China, whereas these genes were both compatible with *H. avenae* pathotype Ha91. Similarly, Nicol et al. (2011) observed VL411R to be the most useful cultivar in both Henan and Hebei, which was inconsistent with the results of Li et al. (2013). Varying degrees of resistance to a given plant disease are usually due to differences in the examined environments, populations, and methods, and we suggest that these differences were the reason for the differences observed in the present work.

Host resistance was examined in relation to nematode attraction toward and penetration of roots, as well as to nematode motility, maturation, and reproduction within roots (Linsell et al. 2014). The penetration of J2s into R and M cultivars was significantly reduced compared with S cultivars, and the nematodes were unable to develop into mature females, reflecting the resistance reaction of the host. In addition to migration, nematode multiplication was greatly inhibited by plant resistance. Infection assays have shown that R cultivars could hinder the development of soybean cyst nematodes and significantly reduce the final number of cysts (Liu et al. 2015). Indeed, only 10% of *Pratylenchus thornei* juveniles developed to the next stage in R roots compared with roots from S cultivars (Linsell et al. 2014). In the present study, the mean numbers of J2s, J3s, and J4s observed in the roots of the S cultivar Wenmai19 were 4.4-, 8.7-, and 14.4-fold higher than in the roots of the R cultivar VP1620 and 8.1-, 45.3-, and 53.3-fold higher than in the

roots of the M cultivar Croc B, respectively. These findings suggest that molting is also suppressed or delayed in R and M genotypes. This conclusion is supported by the results of Pariyar et al. (2016), who observed nematode development to be reduced in R and MR accessions. During CCN development, juveniles penetrating S and MR cultivars were able to successfully complete their life cycle, whereas this was not the case for the M cultivar. The invaded J2s gradually declined and failed to develop beyond J3s; furthermore, J2s were still found at 35 and 63 DAI, indicating that reproductive development was suppressed in the M cultivar. The numbers of white female cysts per plant ranged from 0.2 to 4.8 in the roots of the R cultivars, and the observed decrease in reproduction indicates that the nematodes either exited the R roots or failed to develop and reproduce.

To achieve a successful parasitic interaction, nematodes must sense and respond precisely to a range of chemical signals (Reynolds et al. 2011). Host tropism is thought to involve root exudates that can stimulate the attraction of nematodes toward host roots either directly or via modification of the rhizosphere (Prot 1980). In our study, juveniles were found surrounding the root tips in Pluronic F-127 gel regardless of whether the wheat cultivar was M, R, or S. For compatible interactions, a plant must be attractive to specific nematodes (Curtis 2008), and Kaplan et al. (1980) reported suppressed motility in R roots and associated this response with the accumulation of phytoalexins, such as glyceollin. While ethylene is a key molecule in nematode attraction (Kammerhofer et al. 2015), the compounds responsible

Fig. 3. Penetration and developmental differences of *Heterodera avenae* (pathotype Ha91) in immune, resistant, and susceptible wheat roots. **A-D**, Observations of juveniles of different developmental stages in the immune wheat root group at 5 DAI (**A**, J2s have only penetrated the lateral root elongation region, and no J2s are found in the main roots), 15 DAI (**B**, J2s and a few developed J3s are present in the root tip), 25 DAI (**C**, J2s and J3s are present in the root tip), and 35 DAI (**D**, fewer J2s and J3s are present in the root tip). **E-H**, Observations of juveniles of different developmental stages in the resistant wheat root group at 5 DAI (**E**, many J2s have penetrated the lateral root elongation region, and few are present in the main roots), 15 DAI (**F**, many J3s and few J2s are present in the same plant roots), 25 DAI (**G**, several J4s and J3s and few J2s are present in the same plant roots), and 35 DAI (**H**, several J4s and few J2s and J3s are present in the same plant roots). **I-L**, Observations of juveniles of different developmental stages in the susceptible wheat root group at 5 DAI (**I**, many J2s have penetrated the lateral root elongation region and main roots), 15 DAI (**J**, J3s and few J2s are present in the same plant roots), 25 DAI (**K**, several J4s and few J3s are present in the same plant roots), and 35 DAI (**L**, many white females protrude from the roots).

for the attraction of *H. avenae* to Wenmai19, VP1620, and Croc B have not yet been identified. Andres et al. (2001) reported that infection of R wheat lines with *H. avenae* resulted in a hypersensitive reaction, with deterioration of syncytial cells. However, the syncytia of the S wheat cultivar grew considerably and were packed with dense cytoplasm, exhibiting wall ingrowths considered to be a specialization for promoting nutrient transport for developing nematodes (Andres et al. 2001; Siddique et al. 2012). The size of the syncytia increased gradually with nematode development in S cultivars, which is consistent with previous reports (Grundler et al. 1998). One previous study identified R lines as exhibiting delays and degeneration in syncytial formation in comparison with an S cultivar at several time points (Palomares-Rius et al. 2012). The results regarding the structure of the syncytia in the M wheat cultivar Croc B indicated late syncytial initiation, increased necrosis, and early syncytial degeneration as signs of a host resistance reaction. Williams and Fisher (1993) reported that syncytia in an S cultivar became highly active at 15 DAI, with an increased volume of cytoplasm with numerous organelles. In contrast, the syncytia of an R cultivar were dominated by coalescing large vacuoles with reduced cytoplasm. The syncytial

cytoplasm in the R cultivar had completely disintegrated at 33 DAI, which was consistent with our finding that the syncytia of the R cultivar degenerated at later *H. avenae* developmental stages.

Because CCNs are host specific, host resistance will continue to be the most profitable and easily applied management strategy (Rivoal and Nicol 2009). Indeed, the identification of R wheat and barley cultivars for production applications in China has become a priority grain project. However, few sources of genetic resistance against CCNs have been documented in bread wheat (*Triticum aestivum*). China is a large country with a variable climate, and the species and population pathotypes of CCNs present are quite complex; this situation increases the difficulty of screening R cultivars for common use. Despite the highly abundant resources for Chinese native wheat varieties, no R cultivars are yet available. Fortunately, an increasing number of nematologists have contributed to resistance screening studies over the years, and significant wheat variety resources in China have been obtained. Our results confirm five wheat cultivars as being R to *H. avenae* pathotype Ha91. We are currently studying the mechanism of resistance in these varieties in order to combine genetic breeding with plant allelopathy.

Fig. 4. Syncytial structure showing the development of the syncytium induced by *Heterodera avenae* (pathotype Ha91) in immune, resistant, and susceptible wheat roots. **A-D**, Observations in histological sections from the immune wheat root group at 5 DAI (**A**, showing necrosis near the nematode head and feeding site initiation in endodermal cells), 10 DAI (**B**, cell fusion), 15 DAI (**C**, showing syncytia in the roots), and 25 DAI (**D**, showing syncytial apoptosis) after the initiation of the interaction between roots and juveniles. **E-H**, Observations in histological sections from the resistant wheat root group at 5 DAI (**E**, showing feeding site initiation near endodermis cells), 10 DAI (**F**, showing a large syncytium and hypertrophied nucleus), 15 DAI (**G**, showing many larger vacuoles and lateral meristems), and 25 DAI (**H**, showing the syncytium restricted by xylem cells, lateral meristems, and the beginning of syncytium degeneration). **I-L**, Observations in histological sections from the susceptible group of wheat roots at 5 DAI (**I**, showing the initiation of the syncytium from central metaxylem cells, necrosis surrounding the nematode head and perforation of the cell walls), 10 DAI (**J**, showing a large syncytium stretching from the endodermis to the central xylem), 15 DAI (**K**, showing a large syncytium containing hypertrophied active nuclei and small vacuoles, phloem cells deformed by the syncytium, and adjacent metaxylem fusion), and 25 DAI (**L**, showing larger syncytia and syncytium coalescence) after the initiation of interaction between roots and juveniles. Scale bar = 50 μ m, N = nematode, S = syncytia, nec = necrosis.

Fig. 5. Sizes of the syncytia induced by immune, resistant, and susceptible wheat roots at different days after inoculation (dai). Each point represents the mean of 10 replicates; vertical bars indicate the standard deviation of the mean.

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