Development of an Evaluation System for *Fusarium* Resistance in Wheat Grains and Its Application in Assessment of the Corresponding Effects of *Fhb1*

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Abstract

Fusarium head blight (FHB) caused by *Fusarium* species is a globally important wheat disease. Host resistance to FHB is composed of multiple mechanisms, including resistance to initial infection (type I), disease spread (type II), toxin accumulation (type III), kernel infection (type IV), and yield loss (type V), of which the last three have been less studied. Traditionally, the *Fusarium*-damaged kernel rate (FDK; percentage of *Fusarium*-infected grains) from point- or spray-inoculated experiments was used as the parameter for type IV resistance, which may be problematic because of the influence of type II resistance. Here we propose a new definition for type IV resistance: that is, the resistance against *Fusarium* infection expressed in wheat grains that have the same chance in contact with the pathogen, under favorable temperature and humidity for infection. *Fhb1* confers strong type II resistance, leading to significantly reduced FHB severity and FDK. To investigate the role of *Fhb1* in type IV resistance, a pair of near-isogenic lines, R22W (*Fhb1* carrier, resistant in terms of type II resistance) and S22V (non-*Fhb1*, susceptible), along with eight wheat genotypes differing at *Fhb1* were inoculated at different

grain development stages with *Fusarium* macrospores both in vivo and in vitro. The in vivo experiments with all florets inoculated demonstrated a significant reduction in thousand kernel weight (TKW) in inoculated grains, regardless of their *Fhb1* status and developmental stages. Surprisingly, R22W showed more TKW reduction than S22V, which was supported by the scanning electron microscopy observation that confirmed the more severe degradation of starch granules in R22W grains. The in vitro experiments demonstrated that grains from both R22W and S22V promoted fungal colonization, but no significant difference was found between the two lines. In summary, our results indicated that the proposed type IV evaluation system is effective in determining different grain resistance levels, providing novel tools for FHB resistance breeding. The finding that *Fhb1* is not associated with type IV resistance enriches our understanding of this gene.

Keywords: evaluation method, *Fhb1*, *Fusarium* head blight, grain resistance, *Triticum aestivum*

Fusarium head blight (FHB) in wheat, mainly caused by the *Fusarium graminearum* species complex and *F. culmorum*, is a globally important disease. *F. avenaceum*, *F. langsethiae*, and *F. sporotrichioides* are also causal agents of FHB. The pathogens causing FHB are primarily type A and type B tricothecene producers (Foroud et al. 2019). Based on different host reactions to FHB infection, five resistance mechanisms have been proposed: type I for resistance to initial infection, type II for resistance to disease spread within spikes, type III for resistance to toxin accumulation in grains, type IV for resistance to grain infection, and type V for tolerance to yield loss (Mesterházy 1995). Of these, type II is the best characterized, followed by type I, whereas types III, IV, and V have not been well studied (Buerstmayr et al. 2009; Liu et al. 2009). The *Fusarium*-damaged kernel rate (FDK) in spray- or point-inoculated experiments

has been associated with type IV resistance and evaluated in many studies (Ágnes et al. 2014; Goral et al. 2019; Szabó-Hevér et al. 2012; Tamburic-Illincic 2012), but knowledge on the resistance of wheat grain to FHB, as well as its relationship with other resistance types (especially type II resistance), is insufficient.

The evaluation of type IV resistance is currently based mostly on FDK, which is calculated from grain samples harvested from single spikes, plants, or plots in spray- or point-inoculated experiments as the percentage of *Fusarium*-infected or affected grains. In most studies, quantitative trait loci (QTLs) for FDK coincided with those for type I and/or II resistance (Buerstmayr et al. 2009; Schweiger et al. 2016; Yang et al. 2005), implying that FDK may not be a good parameter for type IV resistance (equivalent to “grain resistance” in this article), which should be independent from type I and II resistance. Based on its definition, type IV resistance should reflect the resistance of grain per se upon *Fusarium* infection: that is, the resistance level expressed in wheat grains that have the same chance in contact with the pathogen, under favorable temperature and humidity for infection. Therefore, it is inappropriate to use FDK as the parameter for type IV resistance in spray- or point-inoculated experiments.

Wheat grain is mainly composed of endosperm, which in turn is mainly composed of starch and protein. Two types of starch granules (large and small) were found in wheat endosperm, with diameters of 10 to 30 µm for the former and <10 µm for the latter (Parker 1985). Upon *Fusarium* spp. infection, grains become shriveled and bleached, with poor grain filling and reduced grain weight, owing to the decomposition of grain starch and protein caused by *Fusarium* spp. The pathogen first infects the exocarp and then the endocarp and finally destroys the amyloplasts and protein in the endosperm (Jansen et al. 2005), leading to a significantly reduced protein content in infected grains. With scanning electron microscopy (SEM), Packa et al. (2008) observed a large amount of hyphae on the surface and in the

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endosperm of infected grain, with small starch granules disappearing and large starch granules being severely damaged.

The *Fhb1* gene initially identified in Sumai3 is the most stable and effective resistant gene against FHB. It has been reported that wheat genotypes with *Fhb1*, such as Sumai3, Ning7840, and Wangshuibai (WSB), showed a significantly lower percentage of infected spikelets and FDK than those without *Fhb1* (Balut et al. 2013; Clark et al. 2016). However, it is unclear whether the reduced FDK was caused directly by grain resistance or indirectly by low FHB severity or whether *Fhb1* confers grain resistance. The main objectives of this study were to develop an evaluation system for *Fusarium* resistance in wheat grain and to apply it in the assessment of the role of *Fhb1* in grain resistance via both in vivo and in vitro experiments on wheat genotypes differing at *Fhb1*.

Materials and Methods

Plant materials. Chinese FHB-resistant cultivar Ning7840, U.S. FHB-susceptible cultivar Clark, and two near-isogenic lines (NILs) contrasting in *Fhb1* alleles (R22W and S22V) were used in this study. Ning7840, an *Fhb1* carrier, has a pedigree of Anhui11/Aurora/Sumai3 and consistently shows low FHB severity (<15%) under point-inoculated experiments. Clark does not have *Fhb1* and consistently shows a high level of FHB severity (up to 100%) under point-inoculated experiments. The two isogenic lines were derived from the cross Ning7840/Clark*7 and generated via marker-assisted selection, of which R22W has *Fhb1* and S22V does not. In addition to these four genotypes, several other genotypes were also included in the SEM experiment: (i) *Fhb1* carriers, including a Japanese variety Tokai66, a Chinese wheat variety Sumai3, and a Chinese wheat landrace WSB; and (ii) non-*Fhb1* carriers, including a Chinese wheat landrace Haiyanzhong (HYZ), a breeding line S42 that has good type II resistance, and a U.S. wheat cultivar Bobwhite susceptible to FHB.

Fungal isolates and inoculum preparation. A virulent *F. graminearum* isolate of the 15-acetyl-deoxynivalenol (15ADON) chemotype, Fg0865 (kindly provided by Dr. Hongxiang Ma, Jiangsu Academy of Agricultural Sciences, Nanjing, China), was used in both in vivo and in vitro experiments. An additional *F. graminearum* isolate of the 15ADON chemotype, FgW (kindly donated by Dr. Saihua Chen, Yangzhou University, Yangzhou, China), was used only in in vitro experiments.

Pure *F. graminearum* isolates were taken with an inoculation loop from a marginal colony on slant medium and were then transferred onto potato dextrose agar (PDA), which was incubated for 5 to 7 days under 26°C and was then kept in a refrigerator under 4°C for subsequent use.

Eight to ten fungal plugs were taken from the PDA medium and transferred into a flask with 80 ml of autoclaved liquid mung bean medium, which was then put on a shaker and shaken at 180 rpm under 26°C for 72 to 120 h. The spore concentration was measured with a hemacytometer and adjusted to a final concentration of 1×10^5 spores/ml.

The in vivo experiment. Field experiments and sampling. All wheat materials were sown in the experimental field of Yangzhou University. Each genotype was planted in two replications, each having five rows of 15 plants. The middle florets of a wheat spikelet are usually smaller and less developed than the lateral florets; therefore, at anthesis, all middle florets were manually removed to retain only two lateral florets in each spikelet to ensure that no florets were missed when inoculation was performed. Ten microliters of inoculum was injected into each of the remaining florets of a spike at three developmental stages: 5, 10, and 15 days post-anthesis (dpa), respectively. At 15 days after inoculation, the inoculated spikes were harvested, threshed, and stored at -20°C for subsequent experiments. Uninoculated control spikes had the same treatments except for inoculation, and samplings were carried out at 20, 25, and 30 dpa, respectively, corresponding to the sampling timepoints of their inoculated counterparts.

Sample photography. Photographs of the inoculated and check wheat spikes were captured with a Canon G12 camera, and those

of the corresponding grains were taken with an MVX10 macro-zoom fluorescence microscope. For SEM observation, wheat grain samples were fixed in 2.5% glutaraldehyde under 4°C for 12 h, washed three to four times with 0.1 M of phosphate-buffered saline, and then dehydrated with gradient concentrations of ethanol (30 → 50 → 70 → 80 → 90 → 95 → 100%) and a step of anhydrous Na₂SO₄, with each step lasting 10 to 15 min. The samples were then dried in a CPD-300 Critical Point Dryer, crosscut, attached to the sample stage with double-sided tape, and finally sprayed with gold nanoparticles in an SCD500 ion sputter coater. Observation of grain starch granules was carried out with SEM (Philip XL-30ESM), and photos were taken at the same magnification.

The in vitro experiment. Mature grain samples were disinfected with 75% of ethanol for 1 min, washed three times with sterile water, treated with sodium hypochlorite solution for 40 min, and washed three to five times with sterile water for the in vitro assay.

The disinfected grains were arranged on PDA medium in three circles spaced 2 cm apart, each having six grains. A fungal plug of *F. graminearum* was placed onto the center of the PDA medium, which was then sealed and cultivated under 26°C. A PDA plate with only a fungal plug in the center was used as the control. Fungal colonies were photographed every 24 h after 36 h of initial cultivation in order to record their growth speed. Colony diameter was measured with a vernier, and the time for a colony to cover the entire medium surface was also recorded.

Results

Changes in spikelets and grains after inoculation. Artificial point-inoculation on all of the remaining lateral florets of each spikelet provided equal chances for every developing grain to contact *Fusarium* inoculum. Compared with the checks, all of the inoculated florets showed FHB symptoms. Regardless of their allelic status at *Fhb1*, the inoculated spikes were thinner than their respective checks, especially those inoculated at 5 dpa (Supplementary Fig. S1).

Compared with the checks, all grains from the inoculated spikes were markedly shriveled, especially those from spikes inoculated at 5 dpa, whereas the shrinkage became less pronounced when inoculated at later stages (Fig. 1). No significant difference was found between *Fhb1* carriers (Ning7840 and R22W) and non-*Fhb1* carriers (Clark and S22V) (Fig. 1).

Over the course of grain development, the impact of *Fusarium* infection kept decreasing in the tested lines, as reflected in the change rate of grain length, width, and thousand kernel weight (TKW). Overall, the most significant change was found for TKW, especially when inoculated at later stages (15 dpa), followed by grain width and grain length (Table 1). No significant difference was observed for grain length among the four tested lines compared at the same developmental stages. Notably, TKW reduction in R22W was significantly greater than that in S22V, demonstrating the negative effect of *Fhb1* on grain resistance to *Fusarium* infection, which is in sharp contrast to its role in type II resistance.

Changes in grain starch granules after *Fusarium* inoculation. Compared with the uninoculated grains, the inoculated grains showed degradation of starch granules at various degrees (i.e., the more developed the grains, the less degraded the starch granules) (Figs. 2, 3, and 4). At the same developmental stages, however, the tested lines showed different degrees of degradation of starch granules. Clear changes were observed in inoculated grains, including degradation in the kernel protein matrix, reduction in the number of small starch granules, and the pits or cavities on the surface of large starch granules, especially in the equatorial groove region.

When inoculated at 5 dpa, significant degradation of starch granules was observed in all tested materials, reflected in sharp shrinkage of and numerous cavities on starch granules compared with the checks (Fig. 2). Among the FHB-resistant cultivars with *Fhb1*, Ning7840 showed the least degradation in starch granules. Of the FHB-susceptible cultivars, Bobwhite showed the most degradation and almost no starch granules could be observed, whereas Clark exhibited relatively less degradation. Between the two NILs, R22W

showed markedly more degradation than S22V in which only small pits appeared on the starch granules.

When inoculated at 10 dpa, starch granules of the tested genotypes showed various degradation with pits or cavities on the surface, resulting from the invasion of mycelia, whereas those of the checks were intact with a smooth surface (Fig. 3). Of the *Fhb1* carriers, Ning7840 was the least affected, whereas Sumai3 showed more severe degradation of starch granules. Of the non-*Fhb1* carriers, HYZ was the least affected and performed better than the *Fhb1* carriers, followed by S42, which was worse than Ning7840 but better than the other *Fhb1* carriers. Bobwhite was the worst performer in terms of starch granule degradation, followed by Clark. Of the two NILs, S22V showed similar starch granule degradation as HYZ, whereas R22W exhibited much more severe degradation.

When inoculated at 15 dpa, starch granules of HYZ and S22V showed a smooth surface without obvious pits or cavities. Obvious degradation of starch granules was still observed in Bobwhite, whereas only small pits were found on starch granules of the other genotypes (Fig. 4).

When the results from different treatments were taken together, Ning7840 always showed the least degradation of starch granules among the *Fhb1* carriers, whereas HYZ and S22V were the least affected among the non-*Fhb1* carriers, in contrast to the severe infection in Bobwhite. Between the two NILs, the *Fhb1* carrier R22W always exhibited more severe degradation of starch granules than the non-*Fhb1* carrier S22V. These results demonstrated a clear difference in grain resistance to *Fusarium* infection among the tested genotypes, of which Ning7840, HYZ, and S22V showed good grain resistance, indicating the independence of type IV from type II resistance and a lack of obvious effect of *Fhb1* on grain *Fusarium* resistance.

Influence of wheat grains on the growth rate of *F. graminearum* strains. Compared with the check, fungal colonies of all treatments with wheat grains grew much faster; however, no

significant difference was found among the three treatments with the same *F. graminearum* strain, implying that all wheat grains, despite their origin from the resistant or the susceptible NIL, can stimulate mycelium growth (Fig. 5; Supplementary Fig. S2). However, under the same treatment, the growth rate of the Fg0865 strain was slower than that of FgW. In terms of time requirements for the fungal colonies to cover the entire Petri dish, 108 h was needed for Fg0865 compared with only 84 h for FgW when wheat grains were added, whereas 132 h was needed for Fg0865 compared with only 108 h for FgW when grains were not added.

Discussion

Of the FHB resistance mechanisms, types I and II proposed by Schroeder and Christensen (1963) and type III by Mesterházy (1995) and Mesterházy et al. (1999) had clear definitions and have been widely accepted; however, type IV resistance was not clearly defined by Mesterházy (1995), and no specified evaluation method was proposed either. Thereafter, researchers and breeders usually took FDK as the parameter for type IV resistance, for which grains harvested from point- or spray-inoculated spikes were evaluated to calculate or estimate the percentage of shriveled, bleached, or scabbed grains. In most published studies, the percentage of scabbed spikelets (PSS) as a parameter for type II resistance exhibited a significantly positive correlation with FDK, and most QTLs for PSS coincided with those for FDK (Abate et al. 2008; Bonin and Kolb 2009; Islam et al. 2016; Yang et al. 2005), indicating that FDK is just another parameter for type II resistance instead of type IV resistance, which should be independent from the former. Here we proposed a new definition for type IV resistance: that is, the resistance against *Fusarium* infection expressed in wheat grains that have the same chance in contact with the pathogen, under favorable temperature and humidity for infection. Based on this definition, we investigated the relationship between *Fhb1* and grain resistance.

Fhb1 is a well-known FHB resistance QTL, consistently showing major effects on type II resistance in different genetic backgrounds.

Fig. 1. Phenotypic variation of genotypes inoculated at different developmental stages. All genotypes were inoculated at 5, 10, and 15 days post-anthesis (images 1, 2, and 3, respectively) and grains were all sampled at 15 days postinoculation. A1, A2, and A3, R22W. B1, B2, and B3, S22V. C1, C2, and C3, Ning7840. D1, D2, and D3, Clark. Fg-I, *Fusarium graminearum* inoculated; CK, uninoculated check.

Fhb1 is located on chromosome arm 3BS and present in Sumai3 and some Chinese and Japanese landraces (Bai et al. 2018; Su et al. 2019). Several studies proposed its role in reducing toxin accumulation, which is still in dispute (Audenaert et al. 2013; Hofstad et al. 2016; Lemmens et al. 2008; Walter et al. 2015). Three articles have been published on the cloning of *Fhb1* (Li et al. 2019; Rawat et al. 2016; Su et al. 2019); however, none of them revealed its molecular mechanism for the various FHB resistance components, and there have been controversies regarding the candidate genes and their possible roles in FHB resistance. As stated earlier, FDK is a parameter for type II resistance. Thus, it is no wonder that FDK can be significantly reduced by *Fhb1* as a gene for type II resistance; however, whether this gene provides type IV resistance still remains unclear.

Table 1. Relative change rates of grain length (GL), grain width (GW), and thousand kernel weight (TKW) in wheat lines inoculated at different developmental stages^y

Timing of inoculation ^z	Accession	GL	GW	TKW
5 dpa	R22W	-22.37 a	-40.00 ab	-89.09 a
	S22V	-20.27 a	-47.70 a	-87.80 b
	Ning7840	-18.97 a	-35.09 b	-86.50 c
	Clark	-15.18 a	-39.22 ab	-87.11 bc
10 dpa	R22W	-6.91 a	-37.78 a	-77.24 ab
	S22V	-10.05 a	-36.96 a	-75.48 b
	Ning7840	-5.90 a	-37.18 a	-78.50 a
	Clark	-3.44 a	-27.38 b	-76.93 ab
15 dpa	R22W	0.68 a	-29.45 a	-63.62 a
	S22V	2.95 a	-29.83 a	-58.49 c
	Ning7840	4.26 a	-20.46 a	-49.73 d
	Clark	2.90 a	-23.89 a	-60.36 b

^y Relative change rates are given in percentages and were calculated via $(V_t - V_{ck})/V_{ck} \times 100$, where V_t and V_{ck} represent trait values of the treatment and the check, respectively. Different letters following the trait values indicate significance at $P < 0.05$.

^z dpa, days post-anthesis.

In this study, a pair of NILs for *Fhb1* and genotypes with a wide range of FHB resistance were used in in vivo and in vitro experiments, in which grain resistance was investigated in different aspects and the results clearly showed that *Fhb1* does not confer grain resistance.

In the in vivo experiment, we developed a new methodology for evaluating type IV resistance, in which the middle florets as well as the underdeveloped spikelets were eliminated to retain only the well-developed lateral florets. This method ensures the homogeneity in inoculation, providing an equal opportunity for each grain in contact with the inoculum and making the results among grains more comparable. It is well acknowledged that grains become shriveled and of low TKW after *Fusarium* infection, which was witnessed in our experiments. Based on the changes in grain length, grain width, and TKW after inoculation, we found that grain resistance was influenced by grain developmental stages, with more developed grains having better resistance to *Fusarium* species, and this was irrelevant to the allelic status of *Fhb1*. Of the two NILs, R22W with *Fhb1* constantly exhibited higher TKW reduction compared with S22V without *Fhb1*, which was unexpected. Mesterházy (1995) also proposed type V resistance as a measurement for tolerance to yield reduction after *Fusarium* infection, but few studies to date have targeted this type of resistance. Based on our results, TKW was the only significantly changed trait in relation to grain resistance, which has no relation to the other two key elements of yield (i.e., spikes per unit area and grain number per spike). Therefore, we propose that type V be merged into type IV resistance because they essentially reflect the same resistance mechanism, and the reduction in TKW under the grain resistance assay proposed in this study could be used as the evaluation parameter for type IV resistance. This simple and feasible methodology can be easily adopted by breeders and researchers.

Parker (1985) demonstrated that large starch granules appear in 4 to 6 dpa in endosperm cells, and the plasma membrane of the starch granules begins to form vesicles or protrusions toward the cell matrix at 12 to 14 dpa; the latter were finally isolated from the large starch granules, forming small starch granules, which are able to proliferate

Fig. 2. Scanning electron microscopy of endosperm structures of inoculated genotypes sampled at 20 days post-anthesis. **A and a**, R22W. **B and b**, Ning7840. **C and c**, Tokai66. **D and d**, Sumai3. **E and e**, Wangshuibai (WSB). **F and f**, S22V. **G and g**, Clark. **H and h**, Bobwhite. **I and i**, Haiyanzhong (HYZ). **J and j**, S42. Fg-I, *Fusarium graminearum* inoculation done at 5 days post-anthesis (A through J); CK, uninoculated check (a through j).

Fig. 3. Scanning electron microscopy of endosperm structures of inoculated genotypes sampled at 25 days postanthesis. **A and a**, R22W. **B and b**, Ning7840. **C and c**, Tokai66. **D and d**, Sumai3. **E and e**, Wangshuibai (WSB). **F and f**, S22V. **G and g**, Clark. **H and h**, Bobwhite. **I and i**, Haiyanzhong (HYZ). **J and j**, S42. Fg-I, *Fusarium graminearum* inoculation done at 10 days post-anthesis (A through J); CK, uninoculated check (a through j).

Fig. 4. Scanning electron microscopy of endosperm structures of inoculated genotypes sampled at 30 days postanthesis. **A and a**, R22W. **B and b**, Ning7840. **C and c**, Tokai66. **D and d**, Sumai3. **E and e**, Wangshuibai (WSB). **F and f**, S22V. **G and g**, Clark. **H and h**, Bobwhite. **I and i**, Haiyanzhong (HYZ). **J and j**, S42. Fg-I, *Fusarium graminearum* inoculation done at 15 days post-anthesis (A through J); CK, uninoculated check (a through j).

Fig. 5. Growth rate of *Fusarium graminearum* strains **A**, Fg0865 and **B**, FgW on potato dextrose agar medium with different treatments over the time course. CK, uninoculated check.

themselves. After a successful infection, enzymes are secreted from hyphae to destroy starch granules as well as grain protein (Ortega et al. 2019; Wang et al. 2005). With the aid of SEM, Packa et al. (2008) reported that small starch granules disappeared and large starch granules were severely damaged in *Fusarium*-infected kernels, which agrees well with our observations. However, no comparison was made on grain resistance between different genotypes in their study. In our experiments, regardless of inoculation timepoints, S22V without *Fhb1* always showed less degradation in starch granules than R22W with *Fhb1*, implying that *Fhb1* might have negative effects, or at least neutral effects, on grain resistance.

We were also wondering whether grain resistance against *Fusarium* infection varied among genotypes with different type II QTLs, ranging from resistant genotypes with or without *Fhb1* to susceptible genotypes without any known type II QTLs. Unexpectedly, we found that the *Fhb1* carrier Tokai66 with superior type II resistance turned out to be susceptible in terms of type IV resistance, whereas the non-*Fhb1* carrier HYZ exhibited good type IV resistance. These results clearly demonstrate that grain resistance does vary among genotypes, and it is independent of *Fhb1* and other QTL components mediating type II resistance.

In the in vitro experiment, no inhibition zone was observed in any of the three treatments; on the contrary, fungal colonies in plates with grains grew faster than those of the check, and grain samples at later developmental stages resulted in faster colony growth rates, implying that the accumulated carbon and nitrogen sources may stimulate fungal growth. The absence of a significant difference among the three treatments demonstrated that *Fhb1* has no effect on in vitro grain resistance, in agreement with our in vivo experiments.

Conclusion

Here we proposed a new definition for grain resistance to *Fusarium* infection and developed an evaluation system for this type of resistance, including in vivo and in vitro experiments, in which different resistance levels were observed from the surface to the inner structure of grains. Our results demonstrated that grain resistance varies among wheat genotypes and is independent of type II resistance. We also demonstrated that *Fhb1* may have negative, or at least neutral, effects on grain resistance, further enriching knowledge on the function of this well-known QTL. We recommend merging type V into type IV resistance and using the reduction in TKW under the evaluation system proposed here as an indicator for type IV resistance, which simplified the FHB resistance types. However, whether our conclusions also apply to other FHB causal agents, such as *F. culmorum*, *F. avenaceum*, *F. langsethiae*, and *F. sporotrichioides*, remains to be clarified because only *F. graminearum* isolates of 15ADON chemotypes were used in this study.

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