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DNA markers associated with low Fusarium head blight incidence and narrow flower opening in wheat

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Abstract Fusarium head blight (FHB) of wheat, caused by *Fusarium graminearum*, is an important fungal disease in many wheat-growing areas of the world. The objectives of this study were to determine the relationship between width and duration of flower opening and incidence of FHB in wheat, and to identify DNA markers associated with narrow flower opening and low FHB incidence. It was hypothesized that wheat lines whose flowers open briefly and narrowly have a reduced risk of infection. To test the hypothesis, we crossed wheat cultivars Patterson and Goldfield to generate a population of 100 random F_2-derived recombinant inbred lines (RILs). Florets of Patterson open wide; florets of Goldfield tend to stay closed. The population of RILs was characterized for FHB incidence and flower opening width (FOW) and duration in the $F_{7:9}$ and $F_{7:10}$ generations. Of the 305 simple sequence repeat primer pairs screened on the parents, 79 amplified polymorphic DNA bands. Pooled DNA from each of the two bulks was tested with these 79 SSR primer pairs. Four markers were found to have significant marker-trait association with low FHB incidence and narrow flower opening. The major QTL effect associated with narrow flower opening and low FHB incidence was found between the map interval *Xbarc200–Xgwm210*, explaining 29% of the phenotypic variation for FHB incidence averaged over six replicated tests in Indiana in 2002 and 2003. This adds credence to the hypothesis that narrow flower opening is responsible for low FHB incidence in this population. Breeding wheat lines for both morphological avoidance, such as narrow flower opening, and physiological resistance to FHB may be valuable in future breeding research to reduce crop production and grain quality losses in wheat due to FHB.

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Introduction

Fusarium head blight (FHB) of wheat is a devastating disease in the humid and semi-humid wheat (*Triticum aestivum* L.) growing areas of the world (McMullen et al. 1997). In North America and Asia the predominant causal agent is *Fusarium graminearum* Schwabe (teleomorph: *Gibberella zeae*). FHB causes premature death or blighting of the spikes and often substantially reduces grain yield and quality (Bai and Shaner 1994). Fusarium-infected kernels are often shriveled, lightweight, and contaminated with mycotoxins such as deoxynivalenol (DON). Deoxynivalenol is known to cause vomiting and feed refusal in non-ruminant animals and poses a threat to other animals and humans if exposure levels are high (Snijders 1990). The most effective strategy for controlling FHB in wheat is through the development of resistant cultivars.

Mechanisms of resistance to *Fusarium* in wheat are classified as morphological (passive) or physiological (active) (Rudd et al. 2001). Morphological features include traits such as height, awnedness, and width of flower opening during anthesis. Physiological resistance presumably involves a biochemical pathway that produces compounds that inhibit the pathogen after infection, while morphological resistance allows the plant to escape infection during its most susceptible stage (Wiese 1987). Therefore, morphological resistance would be better termed as morphological ''avoidance''. Schroder and Christensen (1963) were the first to describe two types of physiological resistance to FHB in wheat. Type-I resistance is resistance to initial infection, while type-II resistance inhibits the spread of the pathogen within the

spike after infection. Type-I resistance and morphological avoidance can easily be confused in the field because both cause a low incidence of the disease.

To date, most research efforts have focused on developing wheat lines with type-II resistance (Rudd et al. 2001). However, these lines have provided only partial resistance. Resistance to FHB exhibits quantitative variation, and its inheritance involves several loci on different chromosomes (Kolb et al. 2001). Nearly every chromosome in wheat has been reported to be associated with type-II resistance. Only chromosomes 1A, 6A, and possibly 1D have not been reported to be associated with FHB resistance (Kolb et al. 2001). More recently, quantitative trait loci (QTLs) for what is believed to be type I-resistance have been found on chromosomes 3A and 5A in wheat cv. Frontana (Steiner et al. 2004). The best approach to enhance the resistance and durability of wheat to FHB would be to combine morphological features, which cause a lower incidence of the disease, with physiological resistance.

It has been hypothesized that wheat plants which exhibit a narrow flower opening and/or a decrease in the duration of flower opening will have a lower incidence of FHB by reducing the area and time in which *Fusarium* spores can enter the floret and initiate infection. Although the degree of flower opening is considered to be a heritable trait, environmental conditions at anthesis, such as moisture stress, light intensity, and air movement, can greatly influence it.

The objectives of the investigation reported here were to (1) determine whether or not there is relationship between width of flower opening and incidence of FHB, and (2) identify DNA markers associated with low FHB incidence and narrow flower opening.

Materials and methods

Plant materials

A population consisting of 100 wheat (*Triticum aestivum* L.) recombinant inbred lines (RILs) from a single cross of the cultivars Patterson and Goldfield was developed from 100 random F_2 plants by single-seed descent. Patterson (Ohm et al. 1998) has a wide flower opening, while Goldfield (Ohm et al. 2000) has a narrow flower opening. Each of the 100 RILs in the $F_{7:9}$ and $F_{7:10}$ generations plus the two parents were seeded into 1-m-long head rows in the field in both 2002 and 2003 and evaluated for heading date and FHB incidence. All field tests were arranged as a randomized complete block design. Wheat seeds for all plots were seeded into soil with corn residue on the surface to provide *F. graminearum* inoculum for infection. Indiana test locations in 2002 were at West Lafayette (eight replications) and Evansville (four replications), while in 2003 they were at West Lafayette (eight replications), Vincennes (two replications) and Evansville (two replications). In both years, there were two separate, spacially isolated tests of four replications each at West Lafayette; one test was mist-irrigated approximately 3 weeks prior to and 3 weeks post-flowering on non-rainy days from 6 a.m. to 8 a.m. and/or from 6 p.m to 8 p.m., and the other test was not misted.

Data on flower opening width (FOW) and its duration of the 100 RILs plus the parents were obtained from four tests in the greenhouse in 2002 and 2003 and one test in the field in 2003. The number of replications (plots were the primary spike of a plant) for each RIL plus the parents for each test was as follows: greenhouse tests one and two included three replications in each test; greenhouse test three had five replications; greenhouse test four had four replications; the field test had six replications. All tests were arranged in a randomized complete block design. For all greenhouse experiments, seedlings were first vernalized at 4°C for 65 days in a cold chamber and then transplanted to 10-cm-diameter pots in the greenhouse. All pots were randomly placed on greenhouse benches with uniform lighting provided by 400 W lamps placed 1.5 m apart and 1.5 m above the greenhouse bench. Six seedlings of each RIL and the parents, Goldfield and Patterson, were transplanted as hill plots into the field in mid-March, 2003. This was sufficiently early in the season to achieve normal plant growth under field conditions and late enough and to avoid possible winter cold damage to certain RILs.

Heading date and disease evaluation procedure

Heading date was the date in May on which at least half of the spikes had 50% emerged from the flag leaf sheath. As heading date was recorded as a possible confounding factor in distinguishing significant differences in FHB incidence among lines, we reported heading dates instead of flowering dates because one can accurately estimate heading date 1 or 2 days before or after the actual heading date on each visit to the plots. All entries initiated flowering on the third to fourth day after heading, depending on temperature. In 2002, FHB incidence of each line was calculated by visually estimating the number of diseased spikes per 1-m row, whereas in 2003 FHB incidence was calculated by counting the diseased spikes among 100 random primary spikes per head row. Disease incidence was determined at 21–26 days after heading.

Evaluation of flower opening width and duration

In the greenhouse, plants were selected 1–2 days before flowering and placed in a completely randomized arrangement in an area of the greenhouse that had uniform lighting and temperature. Plants were not watered until the entire primary spike had completed anthesis. The degree of flower opening was determined by measuring with a standard metric ruler the distance to the nearest 0.5 mm between the tip of the palea and lemma

when the floret had obtained its maximum opening during anthesis. Flower opening duration was the time elapsed (minutes) between when the flower first began to open and when it was completely closed again (less than 1 mm open). The FOW and duration observations were recorded for three to five florets, each in a different spikelet of the primary spike. The mean of the three to five observed florets was calculated and recorded as the observation for each plant (replication). Most observations were recorded from 8 a.m to 10 a.m., when most flowering occurred.

SSR assay

DNA samples were prepared from $F_{7:10}$ seedlings of each RIL according to previously described protocols (Saghai-Maroof et al. 1984). Bulked segregant analysis (Michelmore et al. 1991) was used in this study. To construct the two bulks, equal amounts of DNA from eight lines consistently exhibiting low FHB incidence and narrow flower opening across tests in 2002 and 2003 were pooled, and eight lines exhibiting high FHB incidence and wide flower opening were pooled. Sequence information of the SSR primers was made available from Röder et al. (1998), Pestsova et al. (2000), and Ward et al (2003). A total of 305 SSR primer pairs were screened on the parents. Primers showing polymorphism between the parents were then screened on the bulks. The PCR conditions were as follows: 1× *Taq* buffer, 200 μM each dNTPs, 1.5 m M $MgCl_2$, 0.25 μM of each forward and reverse oligonucleotide primer, 40 ng DNA, and 1 U *Taq* polymerase in a 25-µl reaction volume. The reaction mixtures were denatured at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, either 60°, 55°, or 50°C for 40 s, and 72°C for 1 min, with a final 7-min extension at 72°C for 7 min. DNA products were resolved on 3% (w/v) agarose gel (1.5% Metaphore, 1.5% agarose) (Cambrex Corp, East Rutherford, N.J.). Markers that were polymorphic between the bulks were confirmed by genotyping the individuals from each bulk. If a significant marker-trait association was indicated by an F-test ($P < 0.05$), the whole population was genotyped. Segregation distortion was tested by Chi-square analysis.

Statistical analysis

A histogram and normal probability plot were constructed for each environment to check for normality of the field and greenhouse data using the mean FHB incidence and FOW . An "arcsin" transformation was performed on the FHB incidence data if variances among lines were significantly different. The significance of heading date on FHB incidence was tested using analysis of covariance; treating heading date as a covariate. To adjust for heading date variation between locations due to latitude differences, the earliest heading line at each location was given a score of 1, while the other lines were given a whole number value depending on how many days they headed after the earliest line. For example, if the earliest line headed on May 4th, and the latest on May 14th, the earliest line was given a score of "1" while the latest line was given a score of $(14-4+1) =$ "11".

Analysis of variance was performed to calculate the mean FOW and duration for each recombinant inbred line and to detect significant differences ($P < 0.05$) among lines and between tests. Simple linear regression was used to relate FHB incidence to FOW and relate FOW to duration of flower opening. Marker-trait association was tested by one-way analysis of variance. Broad-sense heritability for FHB incidence and flower opening based on entry means was calculated by the formula $H^2 = \sigma^2_g/(\sigma^2_g + \sigma_{g\times e}/r + \sigma^2_e/rn)$ where $\sigma^2_g$is the genetic variance component among the recombinant inbred lines, $\sigma^2_e$is the environment variance component (among experiments), $\sigma_{g \backslash times e}$ is the environment × genetic variance component, r is the number of experiments, and n is the average number of plants tested per line per experiment. Linkage analysis was performed using MAPMAKER/EXP 3.0B. The Haldane map function was used to estimate the distance between markers. A LOD score of five and a maximum distance of 25 cM and 50 cM were used in the determination of linkages. A 1,000 permutation test was carried out on the data to determine the proper LR threshold (Doerge and Churchill 1996). The presence and location of QTLs and the amount of phenotypic variation explained by the markers were calculated using Windows QTL CARTOGRAPHER.

Results

FHB field evaluations

Generally, in 2002, FHB incidence was lower than in 2003, but there were significant differences among lines and between the parents in the misted test at Lafayette and in the test at Evansville. Conditions for FHB development were more favorable in 2003, but not so severe that lines which typically display low FHB incidence were overcome with the disease. In 2003 there were significant differences among lines and parents in all four tests. Thus, the phenotyping of lines and parents for FHB incidence for marker identification is based on means over six replicated tests; two tests in 2002 and four tests in 2003, or a total of 20 observations per mean.

Incidence of FHB in the field was higher on Patterson than on Goldfield ($P < 0.001$), and the difference between Patterson and Goldfield for FHB incidence was greater in 2003 than in 2002 (Fig. 1). Mean FHB incidence of the RILs was distributed continuously in 2002 and 2003, but the incidence was greater in 2003, and in both years the population mean did not significantly

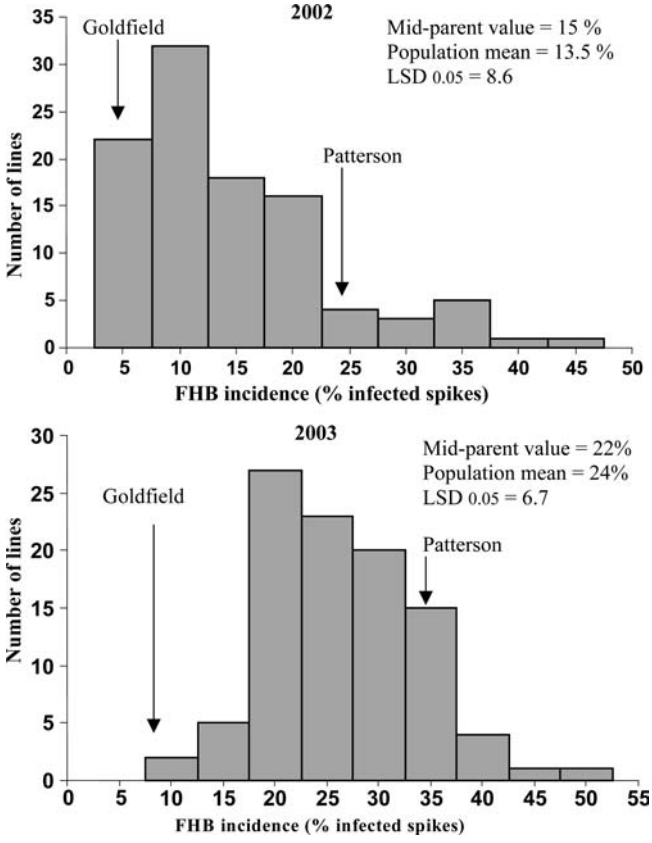

Fig. 1 Frequency distributions of Fusarium head blight incidence in a wheat recombinant inbred population from the cross Patterson × Goldfield. Data averaged over two replicated tests in 2002 and four replicated tests in 2003

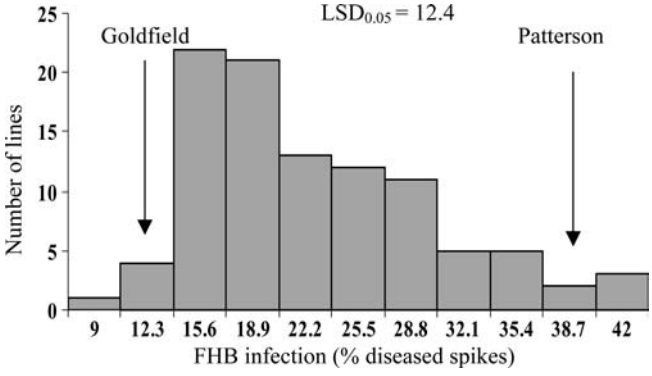

Fig. 2 Frequency distribution of FOW in a wheat recombinant inbred population from the cross Patterson × Goldfield. Data averaged over five tests

deviate from the mid-parent value (Fig. 1). At each location, heading of the RILs occurred over about a 10-day period, with Patterson typically heading 2–3 days before Goldfield. An ANOCOVA revealed significant variation ($P < 0.001$) in FHB incidence among the RILs at each location and suggested that FHB incidence was significantly correlated with heading date at both locations in 2002 ($P < 0.05$). In 2003, FHB incidence at only one out of the three field locations was significantly correlated with heading date, and when all three locations in 2003 were averaged, heading date was not significant ($P = 0.144$). Although variation in FHB incidence due to different environments and line × environment interaction was noted, the rankings of individual lines remained fairly consistent between 2002 and 2003 ($r = 0.73$). Broad-sense heritability for FHB incidence was 0.64.

Evaluations on flower opening

Flowers of the parent line Patterson opened wider and remained open longer than those of the parent line Goldfield ($P < 0.001$). An ANOVA revealed significant variation in FOW and duration of flowering among the RILs in each of the five evaluations ($P < 0.05$). The population mean for FOW did not differ from that of the mid-parent value (Fig. 2). FHB incidence and FOW were positively correlated (Fig. 3a). Although variation in flower opening due to environments and line × environment interaction was noted, there was significant positive correlation in FOW between experiments, including between the greenhouse and field experiments ($r = 0.41$, $P < 0.016$). A scatter-plot of the mean FOW versus the mean flower opening duration for the RILs show that as the degree of flower opening increased, so did the flower opening duration (Fig. 3b).

Bulked segregant analysis and marker-trait associations

Of the 305 SSR primer pairs screened on the parents, 79 amplified polymorphic DNA bands. Pooled DNA from each of the two bulks was tested with these 79 SSR marker pairs. Four of these pairs amplified polymorphic DNA between the two bulks (Fig. 4a) and confirmed the polymorphic DNA bands in the 16 individuals of the two bulks—eight high FHB incidence lines and eight low FHB incidence lines. After genotyping the entire RIL population with these four markers, a one-way ANOVA was used to test for marker-trait associations. Three markers (*Xbarc200*, *Xwmc149*, and *Xgwm210*) were significantly associated with narrow flower opening and low FHB incidence, while *Xgwm344* was associated with low FHB incidence (Table 1). Segregation distortion was not significant ($P > 0.05$) for any of the markers as tested by Chi-square analysis.

QTL mapping

A total of four markers were grouped and ordered using MAPMAKER/EXP 3.0B. Markers *Xbarc200* and *Xgwm210* were linked at a distance of 18.3 cM between them, while the other two markers (*Xwmc149* and *Xgwm344*) were unlinked. Using Windows QTL CARTOGRAPHER and the linkage map from MAPMAKER/EXP 3.0B, one or two

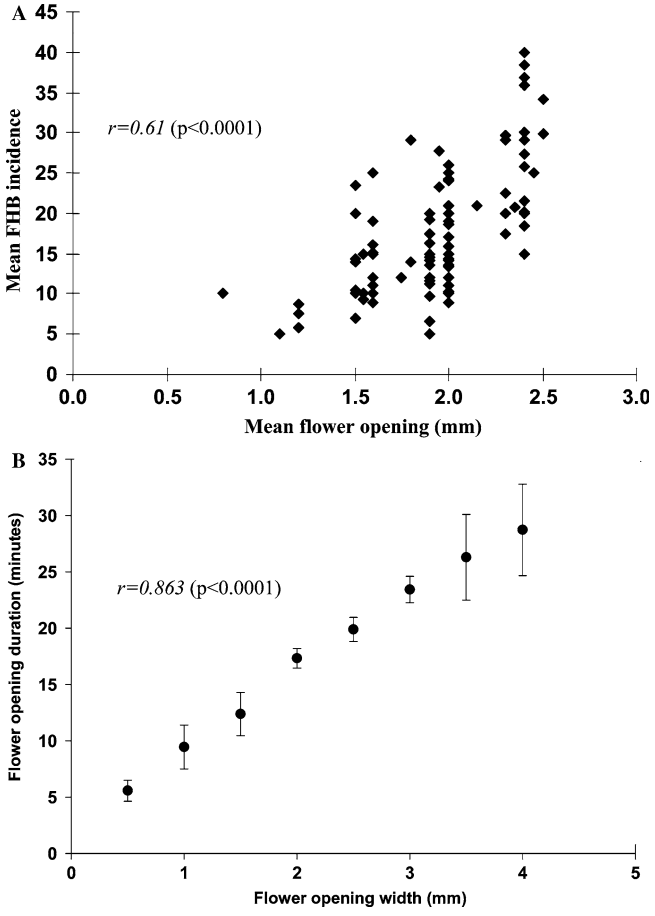

Fig. 3 **a** Mean FHB incidence (six replicated field tests in 2002 and 2003) and FOW (four replicated tests in a greenhouse and one replicated field test) of the 100 wheat RILs from a cross of Patterson × Goldfield. **b** Mean FOW rounded to the nearest 0.5 mm versus mean flower opening duration of 100 wheat RILs from a cross of Patterson × Goldfield. Significance *bars* indicate the 95% confidence interval of mean flower opening duration. Data averaged over five tests (100 individuals × 21 replications (observations) = 2,100 observations)

loosely defined QTLs for narrow flower opening and low FHB incidence were detected in the region of the markers *Xbarc200* and *Xgwm210*. *Xgwm210* has been mapped to the short arm of chromosome 2B in Chinese Spring by Röder et al. 1998, while *Xbarc200* has also been mapped to the short arm of 2B by Ward et al. 2003, using wheat Synthetic × Opata. This QTL explains 29% of the phenotypic variation for low FHB incidence (LOD score: 5.21) averaged over six environments. Averaged over five tests, a maximum LOD score of 2.1 was found for the narrow flower opening QTL, which is above the threshold of 1.1. A significant QTL for narrow flower opening was also associated with these two markers, *Xbarc200* and *Xgwm210*, in three out of the five flower opening tests. The two other markers, *Xwmc149* and *Xgwm344*, were not linked, and explained 12% and 7% of the phenotypic variation for FHB incidence, respectively.

Marker screening of other wheat lines with a low FHB incidence

The SSR markers *Xwmc149*, *Xgwm344*, *Xgwm210*, and *Xbarc200* were used to screen three Purdue wheat lines that have low FHB incidence and Goldfield in their parentage. The results shown in Table 2 and Fig. 4b suggest that these markers would be useful in screening plants with Goldfield in their parentage for low FHB.

Discussion

In this study, FHB incidence was significantly correlated to FOW: RILs with a wider flower opening tended to have a higher FHB incidence. The few RILs that displayed a high degree of flower opening and yet low incidence of FHB in the field may have partially escaped FHB infection for unknown reasons. More importantly, none of the RILs that displayed a narrow flower opening developed an incidence of FHB infection higher than the population mean. This suggests that breeding wheat for narrow flower opening may be useful in a breeding program aimed at reducing the incidence of FHB infection in wheat.

The continuous distribution of FHB incidence and FOW in the recombinant inbred population suggests that two or more genes control these traits. This was supported by the identification of three SSR markers (*Xbarc200*, *Xgwm210*, and *Xwmc149*) located in at least two different regions of the genome having significant association ($P < 0.05$) with both low FHB incidence and narrow flower opening. This observation suggests that flower opening may be responsible, at least in part, for the low FHB incidence in this population. One of the markers, *Xgwm344*, had significant marker-trait association with low FHB incidence, but not with narrow flower opening. It is likely that this marker represents another factor for low FHB incidence besides narrow flower opening (possibly glume toughness or physiological resistance). Two of the markers (*Xgwm210* and *Xbarc200*), which were associated with a major QTL likely located on the short arm of 2B, accounted for 29% of the phenotypic variance for FHB incidence averaged over six environments, while the other two markers (*Xwmc149* and *Xgwm344*) explained 12% and 7% of the phenotypic variation, respectively. The effect of the QTL for low FHB incidence located between markers *Xgwm210* and *Xbarc200* was significant in all four tests in 2003 and in two of three tests in 2002.

The detection of a significant QTL for flower opening in the same region as one for low FHB incidence between the markers *Xbarc200* and *Xgwm210* provides further evidence that narrow flower opening causes a low incidence of FHB. The fact that the LOD score for FOW was lower than for low FHB incidence is partially due to the large amount of variation in the phenotypic data which did not allow the QTL for flower opening to

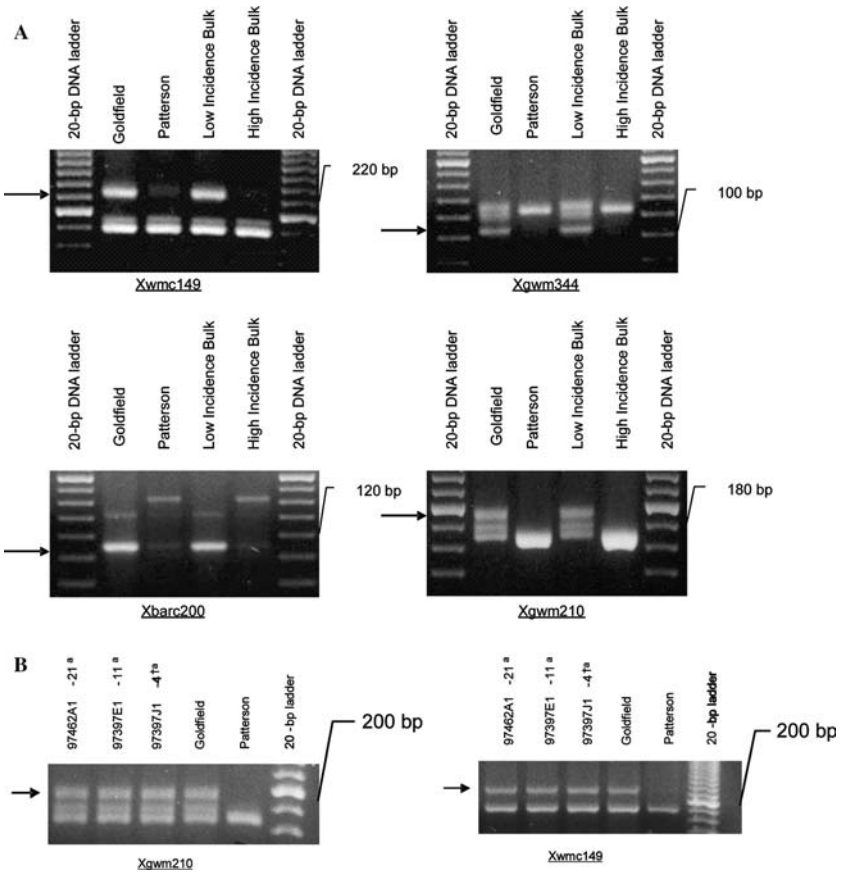

Fig. 4 a Ethidium bromide-stained agarose gel electrophoresis showing polymorphisms between the wheat parents Patterson and Goldfield and the low/high incidence bulks of a recombinant inbred population from a cross between these parents. **b** Polymorphisms between the wheat parents Patterson and Goldfield and the low-FHB-incidence Purdue wheat lines 97462A1-21, 97397E1-11, and 97397J1-14 that have Goldfield in their parentage. The critical bands are indicated by *arrows*. The superscript "a" indicates Goldfield in the pedigree

be as significant as for low FHB incidence. Part of the experimental error can reasonably be attributed to sunlight intensity differences throughout the experiment (cloudy days versus sunny days). Other factors, such as humidity, wind movement, and water stress differences, which may affect flower opening should not have accounted for error due to randomization of the experimental units. Although we have observed no evidence of resistance in either of the two parent lines in tests in this study or in previous observations, it is certainly possible that resistance to FHB due to epistasis is present in some of the RILs.

From this study, it seems likely that two or more genes control flower opening. This is in contrast to a study by Chhabra and Sethi (1991) that showed open flowering in durum wheat was controlled by one dominant gene and that cleistogamy (closed flowering) was expressed when two recessive genes were present. How-

Table 1 One-way *anova* for markers that are linked to low FHB incidence and narrow flower opening width (FOW) in a recombinant inbred wheat population derived from Patterson × Goldfield

Marker	Chromosome	Mean of Goldfield allele		Mean of Patterson allele		FHB *P*-value	FOW *P*-value
		FHB[a]	FOW[b]	FHB[a]	FOW[b]		
Xbarc200	2B	17.8	1.76	24.1	2.16	< 0.0001	0.0083
Xgwm210	2B	17.9	1.79	23.9	2.15	< 0.0001	0.0115
Xwmc149	2B	18.5	1.81	24.9	2.16	0.0045	0.0315
Xgwm344	7B	19.9	1.96	22.2	1.97	0.0015	0.8521
All four alleles[c]	–	15.7	1.54	27.6	2.25	< 0.0001	< 0.0001

[a]Mean percentage of *Fusarium*-infected spikes of al RILs containing specified allele; 20 observations per mean
[b]Mean FOW of lines containing the specified allele; 21 observations per mean
[c]Does not include marker *Xgwm344* for FOW alleles

Table 2 Presence (+) or absence (−) of the Goldfield marker allele associated with low FHB incidence

Line	FHB incidence[a, b]	FOW (mm)[c]	Marker			
			Xbarc200	*Xwmc149*	*Xgwm344*	*Xgwm210*
Patterson	48.2	2.25	−	−	−	−
Goldfield	20.6	0.88	+	+	+	+
97397J1-4[d]	22.3	0.94	+	+	+	+
97397E1-11[d]	25.0	0.91	+	+	+	+
97462A1-21[d]	22.1	1.18	+	+	+	+

[a]The FHB data were averaged over three locations in 2004
[b]Number of diseased spikes/number of total spikes per head row
[c]The FOW were averaged over three replications at one location in 2004
[d]Goldfield present in pedigree

ever, it is important to distinguish that in this study the narrow flower opening parent Goldfield was not truly cleistogamous (0° opening) in that Goldfield exhibited at least some degree of flower opening. Therefore, the trait they were studying in durum wheat was possibly different from the trait(s) being evaluated in this study. Alternatively, the genetics of FOW might be confounded with the genetic materials used in different studies.

Flowers that opened wider tended to stay open longer. Therefore, it seems likely that lines with a narrow flower opening are able to escape FHB infection by reducing the time and area in which *Fusarium* spores can enter the floret and initiate infection. It is possible that narrow flower opening is closely linked and segregates with other morphological or active types of resistance that may be causing the low incidence of FHB. For example, Percival (1921) noted that dense-eared wheat tended to have a higher retention of anthers. Since anthers contain betaine and choline, both of which stimulate the growth of FHB, dense-eared wheat may be more susceptible.

It was noted in our study that anthers would sometimes become trapped inside of the floret during anthesis. This was especially in narrow flower opening lines, presumably because there was less room and time for the anthers to be extruded. Percival (1921) also noted this phenomenon by writing ''As often is the case, extruding anthers may get trapped between the folded margins of the palea or caught between the tips of the closing glumes''. Therefore, it is possible that narrow flowering leads to a higher percentage of anthers becoming trapped within the floret. In this situation, the floret would act as a morphological barrier between the anthers and *Fusarium* spores by not allowing them to come into contact with each other, thus leading to a lower incidence of the disease. Other morphological features, such as glume compactness and toughness, for example, may be partially responsible for narrow flower opening.

The marker validation results suggest that the markers would be useful in screening plants for low FHB incidence with Goldfield in their pedigree. It seems reasonable that the cultivar Frontana has genes for low FHB incidence at different loci than those in Goldfield, since a recent study by Steiner et al. (2004) found significant QTLs for low FHB severity and incidence on chromosomes 3A and 5A of Frontana. From this study it appears that flower-opening width in wheat is a complex trait that is controlled by more than one genetic factor. Although the exact cause of low FHB incidence in this population is not entirely understood, it can be concluded that in order to increase the durability and level of FHB resistance in wheat, it may be useful to breed for both morphological traits that may lead to low FHB incidence, such as narrow flower opening, and physiological resistance, such as a type-II resistance.

Determinations of duration and degree of flower opening in wheat and associated FHB incidence are tedious and time-consuming. Thus, the identification of DNA markers associated with low FHB incidence is particularly valuable in wheat improvement for protection against this important disease. In our study two or more persons, as needed, were involved in measuring flower opening each day during flowering. Just prior to the onset of flowering, plants were placed along the edge of a bench in the greenhouse, along with note cards for each plant. Each observer was responsible for the measurements of duration and width of flower opening for up to 15 plants simultaneously.

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