Theor Appl Genet (2004) 108:706–711
DOI 10.1007/s00122-003-1466-y

ORIGINAL PAPER

D. E. Yang · C. L. Zhang · D. S. Zhang · D. M. Jin · M. L. Weng · S.J. Chen · H. Nguyen · B. Wang

Genetic analysis and molecular mapping of maize (*Zea mays* L.) stalk rot resistant gene *Rfg*1

Received: 19 May 2003 / Accepted: 20 August 2003 / Published online: 27 November 2003

Abstract One single pathogen *Fusarium graminearum* Schw. was inoculated to maize inbred lines 1,145 (Resistant) and Y331 (Susceptive), and their progenies of F_1, F_2 and BC_1F_1 populations. Field statistical data revealed that all of the F_1 individuals were resistant to the disease and that the ratio of resistant plants to susceptive plants was 3:1 in the F_2 population, and 1:1 in the BC_1F_1 population. The results revealed that a single dominant gene controls the resistance to *F. graminearum* Schw.. The resistant gene to *F. graminearum* Schw. was denominated as *Rfg*1 according to the standard principle of the nomenclature of the plant disease resistant genes. RAPD (randomly amplified polymorphic DNA) combined with BSA (bulked segregant analysis) analysis was carried out in the developed F_2 and BC_1F_1 populations, respectively. Three RAPD products screened from the RAPD analysis with 820 Operon 10-mer primers showed the linkage relation with the resistant gene *Rfg*1. The three RAPD amplification products (OPD-20_{1000}, OPA-04_{1100} and OPY-04_{900}) were cloned and their copy numbers were determined. The results indicated that only OPY-04_{900} was a single-copy sequence. Then, OPY-04_{900} was used as a probe to map the *Rfg*1 gene with a RIL F_7 mapping population provided by Henry Nguyen, which was developed from the cross "S3×Mo17". *Rfg*1 was primarily mapped on chromosome 6 between the two linked markers OPY-04_{900} and umc21 (Bin 6.04–6.05). In order to confirm the primary mapping result, 25 SSR (simple sequence repeat) markers and six RFLP (restriction fragment length polymorphism) markers in the *Rfg*1 gene-encompassing region were selected, and their linkage relation with *Rfg*1 was analyzed in our F_2 population. Results indicated that SSR marker mmc0241 and RFLP marker bnl3.03 are flanking the *Rfg*1 gene with a genetic distance of 3.0 cM and 2.0 cM, respectively. This is the first time to name and to map a single resistant gene of maize stalk rot through a single pathogen inoculation and molecular marker analysis.

Communicated by H.F. Linskens

D. E. Yang · D. M. Jin · M. L. Weng · B. Wang (✉)
Institute of Genetics and Developmental Biology,
Chinese Academy of Sciences,
100101 Beijing, China
e-mail: bwang@genetics.ac.cn
Tel.: +86-10-64889378
Fax: +86-10-64873428

C. L. Zhang · S. Chen
China Agricultural University,
100094 Beijing, China

D. S. Zhang · H. Nguyen
Texas Tech University,
Lubbock, TX 79401, USA

Introduction

Stalk rot is a serious and widespread disease in maize that reduces both yield and quality (Chiang and Wilcoxson 1961; Sobek and Munkvold 1999; Stack 1999; Yang et al. 2002). In practical production, the disease is caused by several species of pathogens that occur singly or as part of a complex (Smeltzer 1958; Stack 1999; Yang et al. 2002). Different species of pathogens were found in different regions/countries (Chiang and Wilcoxson 1961; Munkvold et al. 1997; Wang et al. 1997; Dodd 1980; Stack 1999; Yang et al. 2002). In the past, it was generally considered that the resistance to stalk rot was controlled by multi-genes or QTLs (quantitative trait loci) (Pe et al. 1993; Stack 1999). A lot of papers about maize stalk rot have been published during the last 60 years; however, only few reports are concerned with genetics and molecular analysis of this disease (Pe et al. 1993).

Pe et al. (1993) inoculated an F_2 population with a conidial suspension of *Fusarium graminearum* Schw. and analyzed them with 95 RFLP markers and ten cloned RAPD markers. Results indicated that the resistance to *F. graminearum* Schw. was a quantitative trait. Four quantitative loci were found on different chromosome regions. However, recently some researchers reported that the resistance to any specific pathogen was controlled by one dominant gene in all of the tested inbred lines (Toman and

White 1993; Chen and Song 1999; Yang et al. 2001). For example, Toman and White (1993) inoculated their F_1, F_2 and BC_1F_1 populations with a conidial suspension of *Collectotrichum graminicola,* and a single dominant gene could best explain the obtained data. Chen and Song (1999) reported that the maize resistance to stalk rot caused by *F. graminearum* Schw. was a single gene-controlling trait based on their field survey data. Our previous research suggested that the maize stalk rot resistance looked to be controlled by one dominant gene when inoculated with a single purified pathogen, but seemed to be controlled by multiple genes when inoculated with mixed pathogens. In order to clarify the genetics of maize stalk rot resistance and to have an insight into the disease at the molecular level, *F. graminearum* Schw. one of the two main pathogens, was tested regarding the genetics and chromosome location.

Materials and methods

Plant materials

Maize inbred line 1145 is highly resistant to stalk rot, while the inbred line Y331 is extremely susceptible to maize stalk rot. The above two parents and their F_1, F_2 and BC_1F_1 (F_1 was crossed back with Y331) populations were used in this study.

Pathogen

F. graminearum Schw. one of the two pathogens of maize stalk rot, was kindly provided by Professor Wang Xiao-Ming, Chinese Academy of Agricultural Sciences. The conidia were first inoculated on solid PDA (potato dextroglucose agar) medium in a culture tube. After the tube was full of mycelium, the mycelium was transferred to maize seed medium at 25°C for 4 days (Wang et al. 1997).

Inoculation and investigation

The two parental lines and their F_1, F_2 and BC_1F_1 plants were inoculated with the single pathogen *F. graminearum* Schw. in the test field of China Agricultural University and the Experimental Farm Station of the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, in the years 2000 to 2002. Fifty grams of maize seed medium inoculated with pathogen was embedded into soil around each plant when plants grew up to 12 leaves; then another 50 g of maize seed medium was embedded on another side when plants were pollinated (Wang et al. 1997; Mims and Vaillancourt 2002).

The resistant and susceptive plants of F_2 and BC_1F_1 populations were investigated four times at 1, 2, 3 and 4 weeks, after the pathogen was inoculated a second time. After the final evaluation, all of the unlodged plants were cut near the ground and the top, just below the ear, and longitudinally split the stalk to check the discoloration distortion (Wang et al. 1997; Mims and Vaillancourt 2002).

When the susceptible inbred line Y331 was planted in the test fields as a control, no stalk-rotten plant was found. These results indicated that the test fields were free of the main pathogen *F. graminearum* Schw. and they were qualified for the inoculation test.

Isolation of the original inoculated pathogen

Sterilized tissues of the first stalk node above the ground were fetched from susceptive and resistant plants at an early time and at the terminal stage of disease development. These tissues were put on the PDA plates, respectively, at 25°C for 1 day; then they were observed and examined under the microscope to test whether there was pathogen present or not (Wang et al. 1997; Stack 1999).

Statistics and genetic analysis

In the entire field tests, F_1 and the two parents were taken as controls. The numbers of susceptible plants and resistant plants were counted, and calculated in the F_2 and BC_1F_1 populations, respectively. Then, χ^2 tests were performed to determine whether the goodness of fit is a ratio of 3:1 in the F_2 generation and a ratio of 1:1 in the BC_1F_1 generation.

DNA preparation

DNA was isolated from leaves of each experimental material following the method of McCouch et al. (1988). DNA concentration was determined according to genomic DNA samples of known standard concentration.

RAPD analysis

RAPD analysis was performed following our previous report (Wang et al. 1999). The 10-base random primers were purchased from Operon Co. Amplification products were separated on a 1.0% agarose gel with ethidium-bromide staining (Wang et al. 1999).

Cloning of polymorphic DNA fragments

The target fragments were recovered and purified with a GeneClean kit (Bio-101, La Jolla, California) from the gel. The purifed products were then cloned into the pGEM-T easy vector cloning system (Promega) according to the provided instructions.

Primary mapping of the RAPD marker and the maize stalk rot resistant gene *Rfg*1

The single-copy marker was used as a probe to map the stalk rot resistant gene *Rfg*1; the RIL F_7 population consisting of 154 individuals developed from the cross of S3 and Mo17 was used for primary mapping of the cloned single-copy marker with the MAPMAKER program (Lander et al. 1987).

Fine mapping of the *Rfg*1 gene

After the *Rfg*1 gene was mapped onto a specific chromosome, a number of RFLP markers and SSR markers flanking the *Rfg*1 gene were selected and analyzed their linkage relation with *Rfg*1.

RFLP analysis was performed as described by Sambrook et al. (2001). SSR primer sequences were downloaded from http://www.maizegdb.org/ and synthesized by SBS Genetech Co., Ltd. The SSR reaction was carried out as described by http://www.maizegdb.org/. The phenotype, RAPD, RFLP and SSR data were combined for linkage analysis using the MAPMAKER Vision 3.0 program. A partial linkage map of the region surrounding the *Rfg*1 gene on chromosome 6 was constructed.

Results

Stalk rot can be caused
by the single pathogen *F. graminearum* Schw.

Stalk-rotten plants were found in F_2 and BC_1F_1 populations after inoculation with pathogen *F. graminearum* Schw. (Fig. 1). The symptoms in susceptible plants were as follow: whole plants were dried up while still green; the spike was fallen down; the side root became hazel and rotten, the brown and rotten symptom could be seen in the vertical section plane of the stalk, particularly in the first stalk above ground (Fig. 2). The symptom caused by one single pathogen *F. graminearum* Schw. was the same as that caused by the other main pathogen *Pythium inflatum* Mattews, or by a mixture of different species of pathogens. These results showed that one single pathogen, *F. graminearum* Schw. could cause maize stalk rot successfully. In addition, there was no significant symptomatic difference in the two kinds of segregation populations (F_2 and BC_1F_1).

Fig. 1 Susceptible plants in the F_2 population

Fig. 2 Vertical section plane of plants. *Left*: susceptive plant; *right*: resistant plant

Stalk rot was caused by the pathogen *F. graminearum* Schw. inoculated by us

The original pathogen *F. graminearum* Schw. that was used for inoculation was successfully isolated from stalk rot plants. The pathogen was identified as *F. graminearum* Schw. under the microscope (Fig. 3, left). In the early stage of disease development only the originally inoculated pathogen was found, but at the later and terminal stages, some other pathogens were found in susceptible plants also (Fig. 3, middle). However no pathogen was found in the resistant plants, which showed that resistant plants were not invaded by *F. graminearum* Schw. (Fig. 3, Right). The above results indicated that the stalk rot was certainly caused by *F. graminearum* Schw. inoculated by us.

Genetic analysis

The field test was performed in different places and different years. Results indicated that all of the F_1 were resistant. In the F_2 population, among the 549 F_2 plants, 408 were resistant, 139 were susceptible. Chi-square tests were performed to determine the goodness of fit to a 3:1 ratio. Results indicated that in any place and any year the ratio of resistant plants to susceptible plants was fitted to the 3:1 ratio (Table 1). These results pointed out that a dominant gene controlled the resistance to pathogen *F. graminearum* Schw. and that the resistance was from the resistant parent 1145.

In order to confirm the conclusion obtained from the F_2 population, a BC_1F_1 population crossed from the

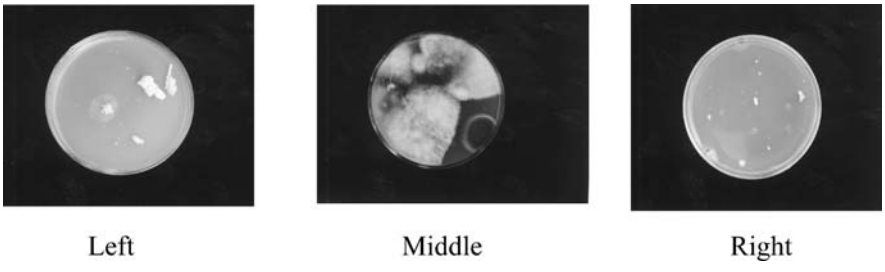

Fig. 3 Identification of the original inoculated pathogen in the susceptible and resistant plants. *Left*: only original pathogen was isolated in the early time. *Middle*: original and other pathogens were isolated in the later time. *Right*: no pathogen was isolated in the resistant plant

Table 1 The results of χ^2 test to the F_2 and BC_1F_1 population

Year		Resistant plus susceptive	Resistant	Susceptible	R/S ratio	χ^2	p
F_2	2000	252	191	61	3.13/1	0.0844	>0.05
	2001	170	127	43	2.95/1	0.0077	>0.05
	Total	422	318	104	3.06/1	0.0284	>0.05
BC_1	2001	103	53	50	1.06/1	0.0872	>0.05
	2002	87	43	44	0.98/1	0.0117	>0.05
	Total	190	96	94	1.02/1	0.0210	>0.05

Fig. 4 RAPD pattern amplified by OPA-04. M: molecular-weight marker; P_1: resistant parent 1145; P_2: susceptive parent Y331; B_R: resistant bulk; B_S: susceptible bulk; 1–30: resistant F_2 plants; 31–60: susceptive F_2 plants. * shows recombinants

F_1×Y331 was tested with this pathogen in the year of 2000 and 2001 (Table 1). There were 96 resistant plants and 94 susceptive plants in 190 BC_1F_1 individuals in these 2 years. After examining by χ^2 tests, the goodness of fit was always 1:1 in the BC_1F_1 generations in different places and different years. These results further confirmed the above idea that a dominant gene controlled the resistance to *F. graminearum* Schw. and that the resistance was from the resistant parent 1145.

Screening RAPD markers linked to the *Rfg*1 gene

From of the total F_2 plants, 120 extreme individuals (60 extreme resistant plants and 60 extreme susceptive plants) were used for RAPD analysis. In order to identify markers linked to the maize stalk resistant gene quickly, bulked segregant analysis was performed according to Michelmore et al. (1991), in which 15 individual DNAs of resistant F_2 plants were mixed with an equal amount to form a resistant bulk BR, and 15 individual DNAs of susceptible F_2 plants were mixed with the equal amount to form a susceptible bulk BS (Michelmore et al. 1991).

The RAPD analysis showed that four bands (ranging from 1 to 10) were amplified per primer on average. In total, 820 Operon 10-mer primers were screened and three polymorphic RAPD bands possibly linked to the target gene were obtained, which were designated as OPD-20_{1000}, OPA-04_{1100} and OPY-04_{900}, respectively. Then, the BC_1F_1 progeny analysis was conducted to identify the linkage relationship between the potential linked RAPD markers and the target gene *Rfg*1. The recombinant value between the *Rfg*1 gene and the three markers were about 4.1%, 5.8% and 8.3% respectively. Figure 4 shows the partial results of the progeny analysis of OPA-04_{1100}. The RAPD amplification products (OPD-20_{1000}, OPA-04_{1100} and OPY-04_{900}) were cloned and their copy numbers were determined. Results indicated that OPD-20_{1000} was a repeat sequence, OPA-04_{1100} was a multiple-copy sequence and OPY-04_{900} was a single-copy sequence. So OPY-04_{900} was used as a probe to map the *Rfg*1 gene.

Primary mapping of the linked marker OPY-04_{900} and the resistant gene *Rfg*1

To determine the chromosome location of the single-copy marker OPY-04_{900}, progeny analysis was carried out in the RIL F_7 mapping population. After analysis with MAPMAKER software (version 3.0), OPY-04_{900} was mapped on chromosome 6 between two mapped markers umc21 and csu155 (or from Bin 6.04 to Bin 6.05) with the genetic distance about 10 cM and 15 cM, respectively (Fig. 5A). Because the *Rfg*1 gene is linked with OPY-04_{900}, the *Rfg*1 gene must be located on chromosome 6 also.

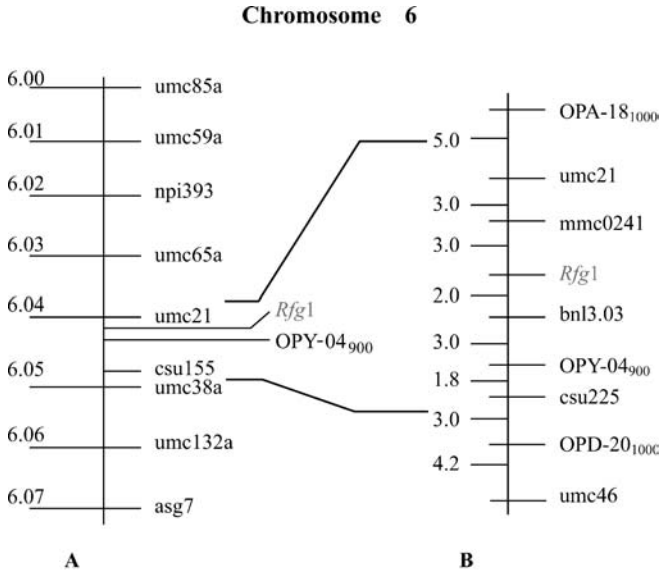

Fig. 5A, B The *Rfg*1 gene and its linked markers on chromosome 6. **B** is the enlarged area between Bin 6.04 and 6.05 in **A**

Fine mapping of the resistant gene *Rfg*1

In order to fine map the *Rfg*1 gene, six RFLP markers and 25 SSR markers surrounding the *Rfg*1 gene on chromosome 6 were analyzed with their linkage relation on *Rfg*1. Then, the phenotypic data, RAPD, RFLP and SSR were combined for linkage analysis using the MAPMAKER Vision 3.0 program. Results indicated that SSR marker mmc0241, RFLP marker bnl3.03 and the cloned RAPD marker OPY-04_{900} were the three molecular markers most closely linked to the *Rfg*1 gene. The *Rfg*1 gene was located between the SSR marker mmc0241 and the RFLP marker bnl3.03; the SSR marker mmc0241 was 3.0 cM apart from the *Rfg*1 gene on one side of *Rfg*1, while on the other side of *Rfg*1, a RFLP marker bnl3.03 and the cloned RAPD marker OPY-04_{900} were 2.0 cM and 5.0 cM apart from *Rfg*1 respectively (Fig. 5). Figure 6 shows the SSR pattern amplified by primer mmc0241, while Fig. 7 shows the RFLP pattern probed by bnl3.03. Finally, a partial linkage map of the region surrounding the *Rfg*1 gene on chromosome 6 was constructed (Fig. 5B).

Discussion

One single pathogen *F. graminearum* Schw. can cause maize stalk rot

It has been controversial for a long time that the pathogen can cause maize stalk rot. Most breeders and researchers agreed that two main pathogens, *Fusarium* and *Pythium*, together invaded the maize host to cause stalk rot (Pe et al. 1993; Wang et al. 1997; Stack 1999; Yang et al. 2002).

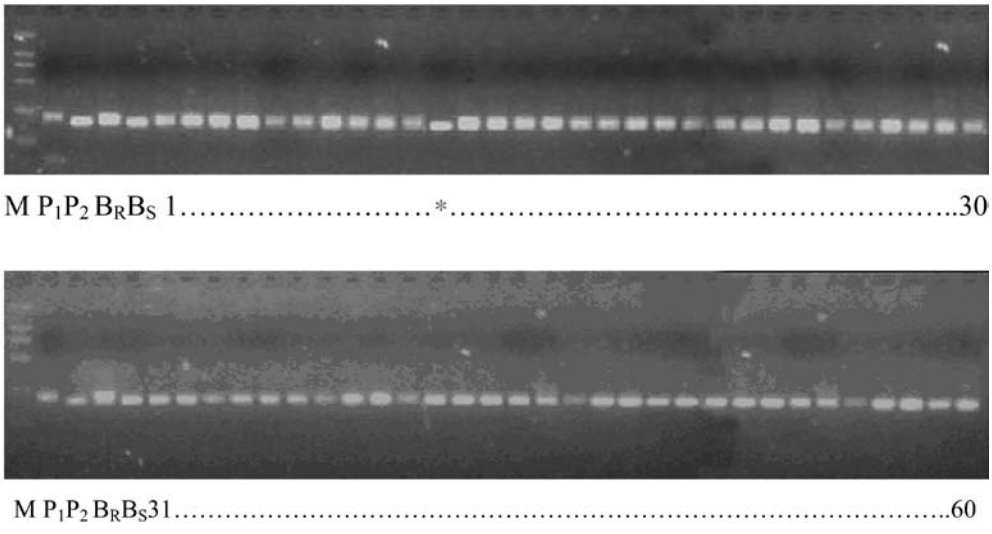

Fig. 6 SSR amplified pattern of mmc0241 to F_2 plants. M: molecular-weight marker; P_1: resistant parent 1145; P_2: susceptive parent Y331; B_R: resistant bulk; B_S: susceptible bulk; 1–30: resistant F_2 plants; 31–60: susceptive F_2 plants. * shows a recombinant

Fig. 7 RFLP analysis probed by bnl3.03. M: molecular-weight marker; P_1: resistant parent 1145; P_2: susceptive parent Y331; R: resistant F_2 plants; S: susceptible F_2 plants. DNA samples were digested with *Hin*dIII.* shows a recombinant

In our research *F. graminearum* Schw. one of the two main pathogens causing maize stalk rot was chosen to inoculate each plant of F_1, F_2 and BC_1F_1 of the cross 1145×Y331. Results demonstrated that: (1) no pathogen *F. graminearum* Schw. was found in our test field; (2) the symptom of susceptive plants inoculated with *F. graminearum* Schw. was the same as that inoculated with the other main pathogen *P. inflatum* Mattews, or mixed pathogens; (3) only the originally inoculated pathogen *F. graminearum* Schw. was isolated from susceptive plants in the early stage of the disease development. These results indicated that one single pathogen, *F. graminearum* Schw., could cause maize stalk rot.

One dominant gene controlled the resistance to *F. graminearum* Schw.

For a long time in the past, most breeders and researchers insisted that the resistance to maize stalk rot was controlled by quantitative trait loci (QTLs). Toman and White (1993) first reported that the resistance to corn rot was controlled by one dominant gene through their inoculation experiment with conidial suspension of *C. graminicola*. Chen and Song (1999) suggested that the resistance to *F. graminearum* Schw. might be a single-gene controlling trait based on their field survey data.

Our single pathogen inoculation tests carried out at different places and in different years revealed that the ratio of resistant plants to susceptive plants were always 3:1 in the F_2 population, and 1:1 in the BC_1F_1 population. These results strongly indicated that the resistance to *F. graminearum* Schw. was controlled by one dominant gene in the cross "1145×Y331", and that the resistant gene to *F. graminearum* Schw. was from the resistant parent 1145.

According to the standard principle of the nomenclature of plant disease-resistant genes, we denominate the resistant gene as *Rfg*1, because it is a dominant resistant gene and is first mapped.

The resistance gene *Rfg*1 was mapped on chromosome 6

Three RAPD markers linked with the *Rfg*1 gene were identified by RAPD analysis combined with BSA analysis. The *Rfg*1 gene was mapped on chromosome 6 between the SSR marker mmc0241 and the RFLP marker bnl3.03. This was confirmed, at the molecular level, that the resistance to *F. graminearum* Schw. was controlled by one dominant gene.

This paper first reported that the resistance to stalk rot caused by *F. graminearum* Schw. was controlled by one dominant gene named *Rfg*1. The new resistant gene was tagged and mapped using the molecular-marker technique combined with BSA analysis.

Acknowledgements This work was supported by the Natural Science Foundation Project of China (39893350) and the State High-Tech Program of China (2001AA211111). The maize RFLP probes were kindly provided by Professor Ed Coe, University of Missouri, USA. The strain of *F. graminearum* Schw. was kindly given by Professor Wang Xiao-Ming, The Chinese Academy of Agricultural Sciences. All maize seeds were gifted by Professor Song Tong-Ming, China Agricultural University.

References

Chen SJ, Song TM (1999) Disease resistance of maize stalk rot. Simple genetics controlled by a single gene. Acta China Agric University 4:56

Chiang HC, Wilcoxson RD (1961) Interactions of the European corn borer and stalk rot in corn. J Econ Entomol 54:850-852

Dodd JL (1980) The role of plant stresses in development of corn stalk rots. Plant Dis 64:533–537

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Etoh T (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

McCouch SR, Kochert G, Yu ZH, Wang ZY, Khush GS, Coffman WR, Tanksley SD (1988) Molecular mapping of rice chromosomes. Theor Appl Genet 76:815–829

Michelmore RW, Paran I, Kesseli V (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to detect markers in a specific genomic region by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Mims CW, Vaillancourt LJ (2002) Ultrastructural characterization of infection and colonization of maize leaves by *Colletotrichum graminicola*, and by a *C. graminicola* pathogenicity mutant. Phytopathology 92:803–812

Munkvold GP, Hellmich RL, Showers WB (1997) Reduced *Fusarium* ear rot and symptomless infection in kernels of maize genetically engineered for European corn borer resistance. Phytopathology 87:1071–1077

Pe ME, Gianfranceshi L, Taramino G, Tarchini R, Angelini P, Dani M, Binelli G (1993) Mapping quantitative trait loci (QTLs) for resistance to *Gibberella zeae* infection in maize. Mol Gen Genet 241:11–16

Sambrook J, Fritsch EF, Maniatis T (2001) Molecular cloning: a laboratory manual, Chapter 6, 3rd edn. Cold Spring Harbor Laboratory Press, New York, 2001

Smeltzer DG (1958) Relationship between *Fusarium* ear rot and corn earworm infestation. Agron J 50:53–55

Sobek EA, Munkvold GP (1999) European corn-borer (Lepidoptera: Pyralidae) larvae as vectors of *Fusarium moniliforme*, causing kernel rot and symptomless infection of maize kernels. J Econ Entomol 92: 503–509

Stack J (1999) Common stalk rot disease of corn. Plant Dis 6: 1–8

Toman J, White DG (1993) Inheritance of resistance to *anthracnise* stalk rot of corn. Phytopathology 83: 981–986

Wang B, Zhang CL, Weng ML (1999) Obtaining of the specific SCAR marker in maize inbred line 8112. High Tech Commun Lett 9:45–47

Wang XM, Zhu XY, Lin HX (1997) Study on the technique of isolation and pathogenicity of the maize stalk rot pathogens. Acta Phytopathol Sinica 27:29–35

Yang DE, Chen SJ, Wang YG (2001) Genetic analysis of resistance to stalk rot (caused by *Pythium inflatum* Mattews) in maize. China Acta Phytopathol 28:392–396

Yang DE, Zhang CL, Wang YG (2002) Review of maize stalk rot in China. J Maize Sci 1:4