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Identification of quantitative trait loci for resistance to Curvularia leaf spot of maize

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Abstract

Resistance of maize to Curvularia leaf spot (CLS), a severe foliar disease in hot and humid maize growing areas, is quantitative in nature. The $F_{2:3}$ families derived from the cross between the resistant Shen137 and susceptible Huangzao4 inbred lines, were used to detect QTL conferring resistance to CLS. Four QTL were detected on chromosomes 1, 3, 8, and 10, and could totally explain 38.8% of the total phenotypic variation. Another F_2 population consisting of 63 highly resistant and 59 highly susceptible plants, selected from 822 F_2 plants, was also subjected to QTL analysis for CLS resistance. Four QTL were found on four chromosomes 1, 4, 9, and 10, which accounted for 45.7% of the total phenotypic variation. Interestingly, a major resistance QTL, qCLS10.4, on chromosomal bin10.04 was consistently detected in both populations. Apart from additive effect, dominant effect also exerted significant influence on resistance to CLS. Therefore, breeders should avoid an early generation selection of resistant individuals in breeding program.

Keywords: maize, Curvularia leaf spot, QTL, selective genotyping

Introduction

Curvularia leaf spot (CLS) caused by the fungus *Curvularia lunata* (Wakker) Boedijn (teleomorph: *Cochliobolus lunatus* Nelson & Haasis) is an important foliar disease of corn occurring in hot, humid maize-growing areas worldwide. The disease was initially reported in China in 1990s and has become an epidemic in North and Northeast China, causing yield losses up to 20~30% (Dai et al, 1996; Lui et al, 1997). The severity of the disease depends on environmental conditions and susceptibility of maize hybrids. Infected leaves develop sub-circular white spots with dark brown peripheral rings and yellow halos that often coalesce to form larger infected areas, and then affected leaves turn yellow and dry up, resulting in a decreased photosynthetic ability.

Control measures to prevent CLS include multiple cultural disease management, such as fall tillage to bury crop residue, crop rotation, and fungicide application. The pathogens overwintered on diseased crop residues left in the field. The effectiveness of cultural practices depended on growers in a region who adopted these controls. For this reason, cultural practice is not a viable control option. Spray of fungicides was rarely economical for grain production, besides this practice would result in environmental hazards. Attempts were made to develop more safe control measures. Bacillus species were found to have chitinolytic activity of fungal mycelia. Bacillus BC121 isolate showed high antagonistic activity against *C. lunata* (Basha and Ulaganathan, 2002), while others were botanicals which have inhibitory effects on the growth of *C. lunata* (Akinbode, 2010). However, more studies were underway to validate the feasibility of using bio-control agent and botanicals against *C. lunata*. Thus, deployment of resistant variety is expected to be most effective way to control CLS. Currently, resistance to CLS is an important breeding objective in most maize improvement programs in North and Northeast China.

Many studies were conducted with the objective to understand the inheritance of CLS resistance. Using generation mean analysis, Zhao et al (2002) reported that resistance to CLS was inherited quantitatively and associated with additive and dominant genetic effects, which account for 70% of the total phenotypic variation across generations. The 113 $F_{2:3}$ families, deriving from the cross between the resistant inbred Shen135 and susceptible inbred Dan340, were evaluated for CLS resistance (Li et al, 2002). Nine QTL were detected and their resistance alleles were derived from both parents. One QTL on chromosome 10, flanked by the marker P1962430, was found to be significantly associated with resistance to CLS. Dominant and over-dominant gene actions were the major sources of genetic variance, while additive and epistatic effects were also observed. It seems very difficult to dissect the inheritance pattern of CLS resistance, since the disease severity depends on the mixture effects of genetic and environmental factors, such as additive and dominant gene action, epistasis, influence of climate changes, synergistic effects

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of co-infection with other foliar diseases, and etc.

The current study is to attempt to answer two questions regarding CLS resistance: i) what factors influence CLS resistance; ii) can the individual QTL, responsible for CLS resistance, be mapped and if so, what is the type of gene action.

Materials and Methods

Plant Materials

The resistant inbred line Shen137 was derived from a Pioneer hybrid 6JK111 (Wang et al, 2008), and the susceptible inbred line Huangzao4 belongs to a local Chinese heterotic group, Tangsipingtou. Shen137 was crossed to Huangzao4 to produce the F_1 hybrid. The F_1 hybrid was self-pollinated to produce F_2 plants, which were self-pollinated to produce $F_{2:3}$ families. The parental lines, F_1 hybrid, and 117 $F_{2:3}$ families were field-tested at the experimental farm of Jilin Academy of Agricultural Sciences, Gongzhuling, Jilin province in 2009. Each line was planted in a single row with two replicates in a randomized complete block design. The 20 plants in the center of each row were evaluated for their resistance to CLS.

To fully understand maize resistance to CLS, the selective genotyping strategy was adopted to detect more QTL. A total of 822 F_2 plants from the same F_1 hybrid were sown in Jilin in mid-May 2010, in which 63 highly resistant and 59 highly susceptible F_2 plants were selected for QTL analysis.

Inoculations

Five isolates, collected from different growing regions, were chosen to represent the population of *C. lunata* in Northeast China and used in the field test. Sorghum grains were served as culture medium to prepare inocula. Sorghum grains were autoclaved in a wide-mouth glass canning jar, capped with kraft paper and bundled with cotton cord. Sterilized sorghum grains were inoculated with a mixture of the five *C. lunata* isolates, and then incubated at room temperature until the grains were thoroughly colonized (~3 weeks). Colonized grains were rinsed with tap water and kneaded well by hands to suspend conidia in the water. Suspension was filtered through two layers of cheese cloth, and conidium concentration was estimated using a blood-cell counting chamber. The final inoculum was diluted with tap water to ca. 5×10^3 conidia ml^{-1}, and then added Tween-20 surfactant at a rate of 2 ml $liter^{-1}$. Plants were inoculated in mid-July at the thirteen-leaf stage of development, in which inoculum was sprayed on the whorls of the plants with backpack sprayers.

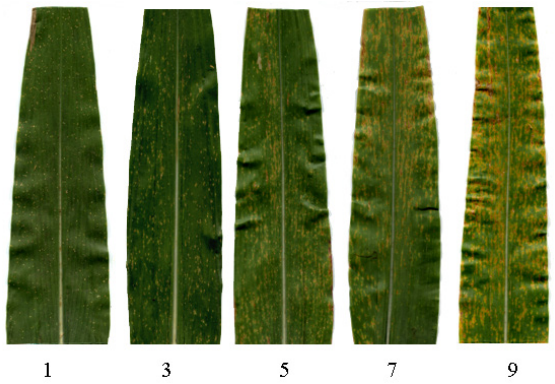

Figure 1 - visual disease rating scores of 1-9 for CLS severity.

Disease Ratings

Disease severity was initially scored 2-3 weeks after inoculation, corresponding to 7-14 days after appearance of visible symptoms, by visually estimating the percentage of infected leaf area (Figure 1). The second scoring was conducted one month after the first scoring. An odd numbered rating system was adopted with some modifications as presented in Supplementary Table 1 (Wang, 2005). The disease severity of each $F_{2:3}$ family was obtained by averaging scores of all plants within the family.

The 822 F_2 individuals were also visually evaluated with the same rating system as described above. The first and the second scorings were conducted 14 and 45 days after inoculation, respectively.

Statistical Analysis

Heritability was estimated from replicated evaluation of the $F_{2:3}$ families in 2009. Analyses of variance were made using the GLM procedure of SAS 8.02 (SAS Institute, 1999). Variance components were estimated from mean squares (Fehr, 1987). Broad sense heritability (H^2) was calculated based on components of variance as follows, $H^2=\sigma^2_G/(\sigma^2_G+\sigma^2_{GY}/R+\sigma^2_e/YR)$ (Knapp et al, 1985). Of the components of variance, the genotype-year interaction (σ^2_{GY}) was zero in our study, and the formula, $H^2=\sigma^2_G/(\sigma^2_G+\sigma^2_e/YR)$ was applied to estimate heritability.

Genotyping and Linkage Analysis

Procedures for DNA extraction were as described by Dellaporta et al (1983) with some modifications. SSR markers that distributed across the maize genome were obtained from the Maize Genetic and Genomics Database (http://www.maizegdb.org/). Primer oligonucleotides were synthesized by Invitrogen (Beijing, China). Polymerase chain reaction was performed using Easy Taq with the recommended protocol (Trans Co, Beijing, China). Thermocycling was performed in a 96-well, thin-walled plate with the following steps: denaturation at 94°C 3 min, 35 cycles of 94°C 30 sec, 58°C 30 sec, 72°C 30 sec, followed by an extension at 72°C 10 min. The amplified products were separated on 6% denaturing urea polyacrylamide gels (Creste et al, 2001), and visualized after modified silver staining. Overall, 150 and 145 SSR markers, chosen to cover the maize genome at intervals of approximately 10-20 cM, were respectively used to construct linkage maps of the $F_{2:3}$ populations and the selected F_2 individuals, which served as the basis for QTL analysis.

In both the $F_{2:3}$ and selected F_2 populations, chi-square (χ^2) analysis was used to test the goodness-of-fit for marker segregation. F_2-derived maps were constructed with the software package MAPMAKER V3.0 using the Kosambi map unit function (Lincoln et al, 1993). Additional markers were added to linkage groups when recombination frequency was >0.4 between two markers identified as being linked on the ISU Integrated IBM 2009 genetic map (http://www.maizegdb.org/).

QTL Analysis

All necessary computations for QTL mapping and estimation of their effects were performed with the software package Windows QTL Cartographer V2.5 (Zeng, 1994; Wang et al, 2011). A LOD threshold of 2.5 was chosen to declare a putative QTL as significant using 1000 permutations runs (Doerge and Churchill, 1996). We used Model 6 of the Zmapqtl module of QTL Cartographer, for scanning intervals of 2 cM between markers and putative QTL with a window size of 10 cM. QTL positions were determined at local maxima of the LOD-curve plot in the region under consideration. QTL were declared to be common across populations if their confidence intervals overlapped, the resistant allele was contributed by the same parent, and the QTL mapped to within a 20 cM distance.

The degree of dominance (DR=|*d*/*a*|) was used to determine the type of gene action (Bohn et al, 1996). Gene action was described as additive for DR < 0.2, partially dominant for 0.2 ≤ DR < 0.8, dominance for 0.8 ≤ DR < 1.2, and overdominance for DR ≥ 1.2.

Results

Phenotypic evaluation

There were considerable variations in CLS severity within the $F_{2:3}$ population. In 2009, most of the $F_{2:3}$ families were found to show intermediate CLS resistance, in which as many as 56 $F_{2:3}$ families were rated as 3 and 45 $F_{2:3}$ families were rated as 5. The extreme resistant/susceptible individuals were observed at low frequencies in the $F_{2:3}$ and F_2 populations (Figures 2A, B).

Heritability estimation

The statistical analysis indicated that significant difference in maize resistance to CLS was present among genotypes (Table 1). The broad-sense heritability for CLS resistance was estimated to be 0.62, which was a bit higher than that (0.52) reported by Li et al (2002). The broad-sense heritability based on one location for one year would be over-estimated, because the underlying G×E variance could not be detected (Dudley and Moll, 1969).

Table 1 - Analyses of variance for CLS resistance.

Variance source	Df	SS	MS	F value	Pr (>$F_{0.001}$)	Significance
Genotype (G)	116	470.64	4.057	2.6408	1.52E^{-07}	***
Replication (R)	1	34.45	34.446	22.4202	6.25E^{-06}	***
Residual error (σ)	116	178.22	1.536			

*** Significance at P < 0.001; Df - degree of freedom; SS - sum of squares; MS - mean squares; Pr - probability.

SSR segregation and map construction

The SSR linkage map of the $F_{2:3}$ population covered 1,824.5 cM (122.5-250.5 cM per chromosome) with an average marker distance of 12.2 cM. For the selected F_2 population, the linkage map covered 1,545.8 cM (107.2-222.7 cM per chromosome) and the average distance between markers was 10.7 cM. All markers were anchored onto the chromosomes according to the marker orders provided in the ISU Integrated IBM 2009 genetic map (http://www.maizegdb.org/), so the linkage maps were constructed for two populations and combined to create an integrated linkage map (Figure 3).

The majority of markers analyzed fit the expected 1:2:1 ratio for the homozygous Shen137, heterozygous, and homozygous Huangzao4 genotypes. Significant deviation from the expected segregation was observed only for one marker in the $F_{2:3}$ population and nine markers in the selected F_2 population. Of these 9 markers, six linked markers (between bnlg1583 and bnlg127), spanning 27.2 cM on chromosome 9, showed significant deviation toward the resistant parent Shen137. However, no evidence was observed that the distorted segregation influenced the order of markers.

QTL affecting CLS resistance

CLS resistance was subjected to QTL mapping using SSR markers mapped in the $F_{2:3}$ population. Four QTL were detected on chromosomal bins 1.11, 3.04, 8.06 and 10.04, designated as *qCLS1.11*,

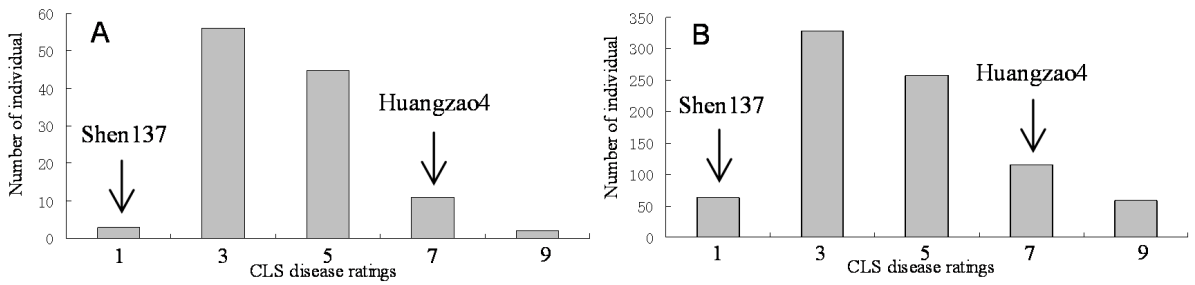

Figure 2 - Distribution of CLS disease severities (A - $F_{2:3}$ families, B - F_2 population).

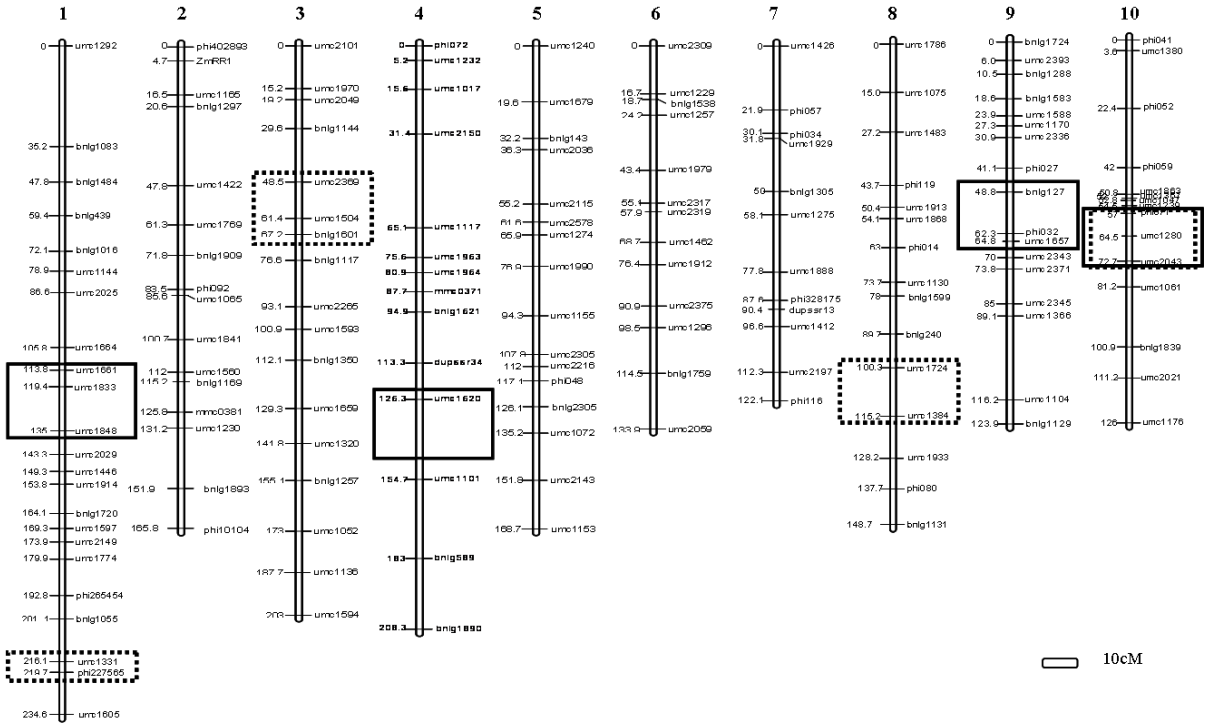

Figure 3 - Genetic linkage map of maize derived from Shen137×Huangzao4.
Dashed boxes indicate the QTL intervals detected in the $F_{2:3}$ population, while solid line boxes correspond to the QTL intervals detected in the selected F_2 population.

qCLS3.4, *qCLS8.6*, and *qCLS10.4*, respectively (Figure 3, Table 2). The percentage of phenotypic variance explained by each QTL ranged from 0.4% (*qCLS1.11*) to 28.9% (*qCLS10.4*). The combined action of all these QTL accounted for 38.8% of the total phenotypic variation.

The selected F_2 population was also subjected to QTL analysis for CLS resistance. Initially, five QTL were detected. Among them, two distinct peaks in the LOD curve appeared on chromosome 1. Using 1-LOD drop-off method (Lander and Botstein, 1989), the LOD score dropped less than 1 unit for two peaks, suggesting the two peaks could be considered as a single QTL. Thus, four QTL were located on chromosomal bins 1.07, 4.07, 9.04, and 10.04, and named as *qCLS1.7*, *qCLS4.7*, *qCLS9.4*, and *qCLS10.4*, respectively (Figure 3, Table 2). The percentage of phenotypic variance explained by each QTL ranged from 5.2% (qCLS1.7) to 19.5% (qCLS10.4). All these QTL combined could attribute to 45.7% of the total phenotypic variation.

Gene action of QTL

The DR values were estimated for all QTL detected in the current study, ranging from additive (DR < 0.2) to over-dominance (DR > 1.2) (Figure 4). For the $F_{2:3}$ population, two QTL, *qCLS1.11* and *qCLS8.6*, showed significant over-dominance, and the remaining two QTL, *qCLS3.4* and *qCLS10.4*, exhibited typical additive gene action. For the selected F_2 population, the four QTL showed either partial dominant (*qCLS1.7*, *qCLS9.4*, and *qCLS10.4*) or additive (*qCLS4.7*) genetic effects.

The QTL-*qCLS10.4* closed to the marker umc1280 on bin 10.04 showed the largest genetic effect on CLS resistance, and was constantly detected in both the $F_{2:3}$ and selected F_2 populations. In the $F_{2:3}$ population, *qCLS10.4* showed the positive additive effect; while, in the selected F_2 population, *qCLS10.4* exhibited the positive additive and partial dominance effects. These findings indicated that the resistance allele at this *qCLS10.4* locus was derived from the susceptible parent Huangzao4, rather than the resistant parent Shen137.

To further confirm the allelic effect of QTL-*qCLS10.4*, three closely-linked markers, *umc1280*, *phi071* and *umc2043*, were selected to determine the genotypes at the *qCLS10.4* locus for the 117 $F_{2:3}$ families. The average CLS severity incidence and its distribution were calculated for each genotype within the whole $F_{2:3}$ families. The homozygous genotype with the Shen137 allele at all three markers showed the highest CLS severity, while the homozygous genotypes with the Huangzao4 alleles displayed the lowest CLS severity, and the heterozygous genotypes showed an intermediate CLS severity (Supplementary Figure 1). This corroborates the fact that the resistance allele at the *qCLS10.4* locus does derived from the susceptible Huangzao4, rather than the resistant Shen137 parent.

Table 2 - Putative QTLs for CLS resistance detected in the $F_{2:3}$ population and the selected F_2 population.

Population	Chr	Bin	Name	Position (cM)	NML	LOD	Additive	Dominant	R^2 (%)	Total R^2 (%)
$F_{2:3}$	1	1.11	*qCLS1.11*	183.8	phi227562	2.64	0.38	-0.60	0.4	38.8
	3	3.04	*qCLS3.4*	59.2	umc1504	2.81	-0.46	0.03	6.2	
	8	8.06	*qCLS8.6*	140.5	umc1724	4.29	-0.30	-0.59	3.3	
	10	10.04	*qCLS10.4*	53.3	umc1280	7.76	0.97	-0.10	28.9	
Selective F_2	1	1.07	*qCLS1.7*	108.0	umc1661	3.86	-1.34	-1.15	5.2	45.7
	4	4.07	*qCLS4.7*	162.0	umc1620	2.90	2.58	-0.23	15.5	
	9	9.04	*qCLS9.4*	72.0	phi032	2.51	1.36	-0.75	5.5	
	10	10.04	*qCLS10.4*	56.1	umc1280	12.7	2.33	1.72	19.5	

NML, the nearest marker locus; R^2, percentage of the phenotypic variance explained by a QTL

Discussion

If a number of QTL detected had allelic effects opposite to those predicted by the parents, an individual with a combination of complementary positive QTL alleles would exceed their parents in trait performance, so-called transgressive segregation. Besides, nonadditive gene action (overdominance) might also contribute to transgressive segregation (DeVicente and Tanksley, 1993). In the current study, some QTL detected did have allelic effects opposite to their parents in both the $F_{2:3}$ and selected F_2 populations. Theoretically, CLS resistance might exhibit vast transgressive segregation; however, this did not happen (Figure 2). We assumed that: i) lots of minor QTL undetectable in the current study; ii) complex gene action at various QTL; iii) environmental influence on CLS development, all contributed to the complexity of maize resistance to CLS and distorted the transgressive segregation.

There are many factors influencing an identification of QTL in a segregating population (Asíns, 2002). It is well known that accurate assessment of disease severity is essential for estimating the positions and effects of QTL and for elucidating inheritance of resistance. To assess foliar disease severity, the major components which represent an infection rate of the pathogen, like infection frequency, latent period, and spore production, are normally taken into account (Parlevliet, 1979), and all these parameters are reflected in the final stage as a percentage or proportion of the total affected area (James, 1974). The mature CLS spots appear as minute and 0.5-2 mm long lesions, so it is difficult to measure spot size in ways like area, diameter, and length. In addition, lesion number in the infected area is also an undesirable measure of CLS severity, since the number of lesions is directly proportional to the concentration of inocula. Therefore, the disease-severity scoring was conducted twice at the thirteen leaf stage which is assumed to be the most appropriate time to reveal maize resistance to CLS.

In the current study, two methods were used for QTL detection. One method is to adopt an average value of F_3 progeny to represent the phenotypic value of a single F_2 plant, so called the $F_{2:3}$ design (Fisch et al, 1996). Theoretically, half of the F_3 progeny derived from a heterozygous F_2 plant will be fixed to either paternal or maternal homozygous genotype. Overall, F_3 individuals will have more extreme genotypes than their heterozygous F_2 plant, resulting in more powerful phenotyping by using $F_{2:3}$ progeny than a single F_2 plant (Zhang and Xu, 2004). The other method is to adopt a selective genotyping strategy to guarantee phenotypic evaluation by using individuals with extreme phenotypes. It is speculated that the individuals with extreme phenotypes may carry large numbers of either positive or negative QTL alleles and thus provide more linkage information than other individuals (Lander and Botstein, 1989). The power to detect QTL by selective genotyping depends on several factors, including the population size, a fraction of the selected plants for genotyping, the QTL effects, and marker-QTL distance (Navabi et al, 2009). In a study performed by Ayoub and Mather (2002), genotyping 10% of the population was sufficient to permit detection of all major QTL. But, Lee (2005) used genetic simulation to reveal that selection can reduce an accuracy of QTL detection and bias an estimation of QTL effect. In the present study, the use of a rather large population (N = 822), an intensive selection (P = 14.8%), and a relatively good marker coverage (10.7 cM) have provided sufficient power to detect the major QTL. The most important QTL *qCLS10.4*, detected

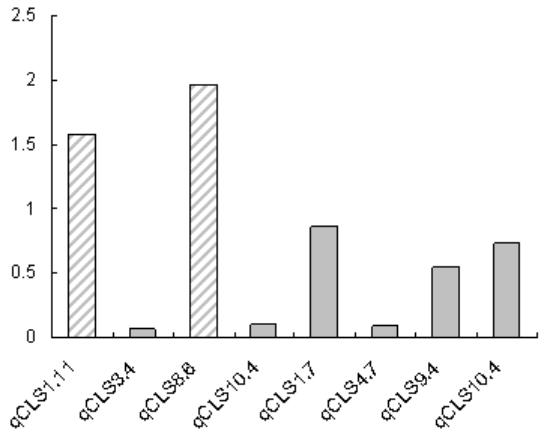

Figure 4 - The histogram of the DR values for all detected QTL.
DR were calculated for each QTL detected to determine its gene action model. Striped bars correspond to those QTL with significant overdominance.

in the $F_{2:3}$ population, could also be detected by the selective genotyping, indicating that the strategy is powerful enough to reveal authentic QTL as reported by other studies (Foolad and Jones, 1993; Foolad et al, 1997; Foolad et al, 2001; Zhang et al, 2003; Micic et al, 2005; Linde et al, 2006). It is therefore concluded that selective genotyping could be efficiently used for analysis of resistance QTL in an independent mapping population.

Among the QTL detected, the QTL-*qCLS10.4* closed to the marker umc1280 (AGPv2 position 128.1 Mb) on bin 10.04 was consistently detected in two mapping populations, and other QTL showed population-specific feature. The *qCLS10.4* had allelic effects opposite to that predicted by the parents. In other word, the susceptible Huangzao4 has at least the resistance allele at *qCLS10.4*, although a combination of resistance alleles at various QTL loci in Huangzao4 is not sufficient to render it resistance to CLS. The bin 10.04 region where the common QTL resides in this article was previously reported to be associated with many disease and pest resistance. The locus phi062 (AGPv2 position 111.8 Mb) between SSR markers umc1077 (AGPv2 position 102.6 Mb) and umc2350 (AGPv2 position 120.6 Mb) on bin 10.04 was one of the high-frequency markers associated with resistance to downy mildew in Asia (George et al, 2004). The locus umc64a (AGPv2 position 115.6 Mb) between SSR markers umc1077 and umc2350 on bin 10.04 was found to affect resistance against second generation European corn borer (2ECB) (Schon et al, 1993). The locus umc1246 (AGPv2 position 97.9 Mb) on bin 10.04 was closed to a QTL for resistance to the Gibberella stalk rot (Yang et al, 2010). It was not sure whether these resistance loci are a cluster of genes or a single pleiotropic locus, and thus the genetic basis of resistance to pathogens and pests has yet to be elucidated.

In our experiments, all QTL detected in 2009 or 2010 explained less than 50% of the total phenotypic variation. The remaining phenotypic variation was probably attributable to the undetected QTL and environmental factors. The numbers of QTL mapped were in all cases minimum estimates of the total number of loci that contributed to variation in the traits (Mackay, 2001a). Increasing the sample size would enable mapping of small-effect QTL and differentiation of linked QTL with the advantage of the large numbers of recombinant events. The number of QTL detected in crosses between inbred lines was also a minimum because one could only map QTL at which different alleles were fixed in the two parent strains, which are a limited sample of the existing genetic variation (Mackay, 2001b). A QTL with a large percentage of phenotypic variation may result from cosegregation of a major-effect qualitative trait gene in the same region for the same trait. The percentage of phenotypic variation was not a fixed property of a QTL but varied with population, number of individuals evaluated, and environments employed. Breeding with major-effect QTL was more straightforward than with minor-effect QTL (St Clair, 2010). Compared with previous results (Zhao et al, 2002; Li et al, 2002), it is speculated that a complex gene action may be involved in resistance against CLS. Therefore, breeders should avoid an early generation selection of CLS resistant individuals in breeding program.

Marker-assisted selection has been used in breeding programs for both gene introgression and pyramiding, particularly for disease resistance in primary crops. Previously, it was assumed that most markers associated with QTL from preliminary genetic mapping studies were directly useful in marker-assisted selection (MAS). However, it has become widely accepted that high resolution mapping of QTL and/or QTL validation might be required (Collard et al, 2005; Langridge et al, 2001). In the subsequent research, fine mapping and validation of a resistance QTL will be conducted in different genetic backgrounds to verify if the QTL was effective.

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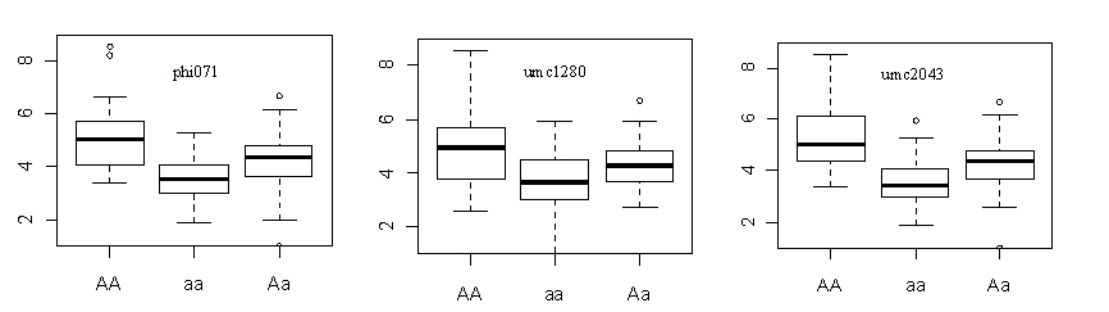
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