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Genetic architecture of lodging resistance revealed by genome-wide association study in maize (*Zea mays* L)

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Abstract

Lodging is one of key factors influencing biomass yield, restricting planting density and reducing mechanical harvesting productivity in maize. Targeted cultivating lodging resistance varieties with screened lines is an economical and effective approach to improve ability of maize lodging resistance. To accomplish this objective, we performed phenotypic assessment of seven lodging-related traits in a diverse maize population consisting of 290 inbred lines and conducted a genome-wide association study with 201 SSR markers to detected marker-trait associations. Seven lodging-related traits all showed broad phenotypic variations. Through evaluation of stalk pushing resistance in the field for two years, a number of 32 inbred lines featured with strong lodging resistance were selected out. Correlation analysis indicated that stalk pushing resistance had a significantly positive correlation with third internode diameter and fourth internode diameter and a significantly negative correlation with ear height. Furthermore, a total of 27 and 13 significant associations for lodging-related traits were identified in year 2012 and 2013, respectively. Interestingly, three associations on chromosome 4, 5, and 6 were discovered in both years. Thus, this study provides useful information for understanding genetic architecture of lodging resistance in maize and will benefit maize marker-assistant breeding program with improving lodging resistance.

Keywords: maize, lodging resistance, genome-wide association study

Introduction

Maize is one of the most important cereal crops worldwide. It not only provides food and fodder in animal husbandry, but also plays a major role in deep processing industry. However, stalk lodging leads to maize yield losses evaluated in the range of 5-20% annually worldwide (Flint-Garcia et al, 2003a; Hu et al, 2013). Additionally, maize lodging adds the obstacle to mechanical harvesting and restricted planting density (Gou et al, 2010; Hu et al, 2012). Therefore, improving stalk-lodging resistance has become a crucial target for maize breeding projects.

Lodging is a complicated phenomenon that is mainly influenced by genetic basis (Li et al, 2014). It has been shown that lodging resistance was significantly correlated with the stalk mechanical strength, chemical composition, anatomical structures and some morphological characteristics (Kashiwagi et al, 2008; Li et al, 2014; Ookawa et al, 2010; Zhu et al, 2008). In case of stem mechanical strength, studies mainly concentrate on the stalk breaking, pushing, crushing, bending, and penetration (Berzonsky et al, 1986; Li et al, 2014; Martin et al, 1984; Sibale et al, 1992). Particularly, stalk pushing resistance (SPR) has been used primarily as an indicator to evaluate stem bending or root lodging in several crops and the results demonstrated a high positive correlation between pushing resistance and lodging in paddy fields crops (Kashiwagi et al, 2008; Terashima et al, 1992; Won et al, 1998). In case of morphological characteristics, a highly negative correlation was observed between the basal third internode diameter (TID) and the ration of maize lodging (Martin et al, 1984). In addition, studies show that the basal internode diameter of rice had a significantly positive correlation with lodging resistance and the basal internode length had a significantly negative correlation with lodging resistance (Kashiwagi et al, 2008; Zhang et al, 2005).

Despite the importance of stalk lodging in breeding, studies on genetic basis of stalk lodging is relatively lack. A few QTL mapping were conducted for stalk lodging resistance and corresponding locus associated with ear height (EH) and stem diameter were successfully identified (Flint-Garcia et al, 2003b; Hu et al, 2012; Hu et al, 2013; Li et al, 2014; Verma et al, 2005). For stalk bending strength in maize, Hu et al (2013) used a linkage map with 129 SSR markers and detected two, three, and two QTL for the maximum load exerted to breaking, the breaking moment, and critical stress, respectively. In the study of mapping SPR of stem bottom and stem diameter for rice, Kashiwagi et al (2004) detected 5 QTLs controlling SPR of stem bottom, among which, *prl5* was a major QTL that was beneficial for the increase of dry weight of stem bottom, density and carbohydrate.

However, previous studies on maize lodging

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mainly focus on linkage analysis, there is little known about the genome-wide association analysis (GWAS) of stalk lodging in maize. GWAS has been received unprecedented attention in virtue of its advantages, including capturing more genetic varieties and obtaining higher resolution locus in abundant natural population (Elmer et al, 2015). A large number of GWAS has been successfully conducted in many plant species, such as Arabidopsis, rice, maize, barley, tomato, oat, wheat, soybean, and sorghum (Atwell et al, 2010; Huang et al, 2010; Li et al, 2012; Morris et al, 2013; Newell et al, 2011; Rostoks et al, 2006; Stracke et al, 2009; Sauvage et al, 2014; Vuong et al, 2015). Maize was an ideal system for the application of GWAS because it has the high diversity and rapid linkage disequilibrium decay as an outcrossing species (Weng et al, 2011). With the rapid development of molecular biology and genomics, it is expected to use association analysis more widely in genetic research, germplasm enhancement and breeding in maize. In this study, considering urgent need for cultivating hybrids with high stalk lodging in Chinese maize germplasm, GWAS were performed with 201 SSR markers in a diverse maize population to determine genetic factors for stalk lodging. Our objectives in this study were to: i) determine the morphological traits significantly correlated with SPR; ii) select the inbred lines with strong lodging resistance; iii) identify the loci associated with SPR and related morphological traits.

Materials and Methods

Plants materials and field design

A set of 290 diverse maize inbred lines were used in this study. This population includes previously described 220 elite Chinese lines (the core collection and their derivatives in China) and 70 elite US lines (36 ex-PVP lines with expired plant variety protection act certificates and 34 non-PVP lines which are publically available) (Liu et al, 2014), which covers the heterosis groups of core inbred lines in China. In the summer of 2012 and 2013, all 290 inbred lines were planted at Experimental Station of Agricultural University of HeBei, Baoding, Hebei Province. A randomized complete block design was used with two replications for each inbred line. For each replication, every line was grown in a 4 m long single-row plots, and 0.60 m inter-row spacing. The planting density was 65,000 plants ha^{-1}.

Evaluation of traits

Morphological trait measurements were done according to the previous studies (Hu et al, 2012; Ma et al, 2009). Three plants with similar plant height (PH) and stem diameter (SD) in each line were chosen to test. PH and ear height (EH) were first measured with metric scale. Afterward, the third and fourth internodes above ground of maize plants were cut off using a garden shears for all lines tested. Third internode length (TIL), third internode diameter (TID), fourth internode length (FIL) and fourth internode diameter (FID) were measured with electronic micrometer. Standards for evaluating the traits referred to the book of the «Descriptors and Data Standard for maize».

Measurement of stalk pushing resistance

For each selected plant, SPR was tested with a prostrate tester (Daiki Rika Kogyou Co, Tokyo, Japan) one week after pollinated as described previously (Kashiwagi and Ishimaru, 2004). At the internode of the primary ear, we pushed the middle of the internode at a right-angle, and continued to push it until the plant inclined to 45°. SPR was determined by the mean of three tests.

Phenotypic data analysis

The mean values of traits of each inbred line were used to analyze the phenotype. One-sample Kolmogorov-Smirnov Test, descriptive statistics analysis and correlations analysis were confirmed using EXCEL and SPSS 17.0 software. Based on the change of stem pushing resistance, inbred lines in this study were divided into 5 grades. Classification criteria reference was as previous reported (Lu et al, 2005).

The broad-sense heritability (h^2B) was estimated by the following formula:

$$h^2B = \sigma^2g / (\sigma^2g + \sigma^2ge / e + \sigma^2e / re)$$

where σ^2g = the genetic variance, σ^2ge = the interaction of genotype with environment, σ^2e = the random error, e = number of environment, and r = number of replicates.

Genotypic data collection

Genomic DNA was extracted using a modified CTAB method as described (Hoisington et al, 1994). A total of 201 polymorphic SSR markers randomly distributed on 10 maize chromosomes were used to genotype the inbred lines in this study. SSR markers were obtained from the Maize GDB database (http: //www.maizegdb.org).

Genotype-phenotype association mapping

The association analysis between SSR markers and phenotypic traits was carried out using TASSEL5.0 software package. Briefly, based on the population structure matrix (Q) calculated by STRUCTURE and the kinship matrix (K) obtained from TASSEL5.0, both of which act as covariate, together with genotypic data and phenotypic data, TASSEL5.0 was employed to detect marker-trait association at Log10(p) > 2.5.

Results

Evaluation of phenotypic variations

Descriptive statistical results of lodging resistance related traits of two years were listed in Table 1. From the Table 1, we observed that the absolute values of skewness and kurtosis of all 7 traits were less than 1 indicating that these traits were in conformity with normal distribution. Furthermore, the variation range of the whole traits presented wide phenotypic diver-

Table 1 - Statistic summary of phenotypic diversity in 290 inbred lines for 7 traits scored over two years.

Trait	Year	Average	Range	SD[a]	Skewness	Kurtosis	h^2B[b]
SPR /N	2012	8.82	1.63~20.97	16.80	0.61	-0.13	0.62
	2013	7.26	0.74~17.50	12.28	0.79	0.44	
TIL /mm	2012	89.88	59.50~128.17	172.58	0.40	-0.12	0.64
	2013	78.23	39.2~120.13	221.66	0.04	0.38	
TID /mm	2012	16.28	9.65~23.59	4.30	0.09	0.26	0.70
	2013	16.35	10.37~21.62	4.28	-0.03	-0.13	
FIL /mm	2012	105.35	74.00~147.67	224.66	0.45	0.02	0.82
	2013	96.07	49.47~143.00	273.37	0.01	0.60	
FID /mm	2012	16.08	9.42~23.19	4.08	0.01	0.47	0.77
	2013	16.07	11.90~21.95	3.65	0.22	-0.19	
PH /cm	2012	184.56	125.00~238.33	560.15	-0.11	-0.19	0.71
	2013	190.52	126.48~252.25	645.02	-0.20	-0.41	
EH /cm	2012	63.02	35.88~101.67	195.54	0.27	-0.35	0.78
	2013	71.33	33.13~111.93	232.96	0.24	-0.34	

[a] SD standard deviation.
[b] h^2B broad-sense heritability.

sity. SPR in 2013 showed the broadest variation with its range from 0.74 N to 17.50 N, followed by SPR in 2012 with its range from 1.63 N to 20.97 N. The following corresponding order of variation range was EH in 2013, TIL in 2013, FIL in 2013, EH in 2012, FID in 2012, TID in 2012, TIL in 2012, TID in 2013, FIL in 2012, PH in 2013, PH in 2012, and FID in 2013. All the traits measured had rather higher heritability, among which, SPR showed the lowest heritability of 0.62, while FIL had the highest heritability of 0.82. One-sample Kolmogorov-Smirnov Test was also employed to estimate whether the seven traits conformed to normal distribution or not, and the results presented that $P > 0.05$ for all traits, which stating that they conformed to normal distribution. Phenotype correlated with lodging resistance.

Phenotypic correlation analysis of lodging resistance related traits

Phenotypic correlation analysis of averages of two years for all traits was shown in Table 2. It was shown that SPR had a significantly positive correlation with TID and positive correlation with FID, while it was negatively correlated with EH. Moreover, obviously, EH and PH were both significantly positively correlated with other traits except for SPR.

SPR clustering analysis

Whereas the significant difference of lodging resistance resulting from genotype variation, the inbred lines in this population were divided into 5 grades (Table 3). Different grades were classified according to the trait of SPR which gradually decreased from the first grade to the fifth one. The first grade covering 15.9% of all inbred lines had the strongest lodging resistance, and the representatives were Wu 109, Ye 8112, PH6WC and so on. Lines belonging to the second strongest grade accounted for 12.4% of all lines, and the representatives were Mo17, Zong 3, 78599 and so on. The third strongest grade showed the largest proportion of 40.8% and had representatives of B73, Chang 7-2, Huangchang b and so on. The forth strongest grade and the weakest grade respectively had a proportion of 14.4% and 16.4% of all lines, and the representatives were correspondingly shown in Table 3.

GWAS analysis

The study of Liu et al (2014) employing the same population and the same SSR markers had certificated that the genetic diversity of SSR used in this study was abundant enough to further carry out GWAS, and they had prepared both population structure and kinship matrix for GWAS. The mixed linear model (MLM) in TASSEL5.0 was employed to carry out GWAS for seven traits of two years, and the results were shown in table 4. A total of 40 significant trait-marker associations were detected within 26 makers distributed on Chr1, Chr2, Chr3, Chr4, Chr5, Chr6, Chr9, and Chr10. For SRP in 2012, three markers located on Bin1.05, Bin2.03, and Bin6.05 were detected with explaining 30.2% of phenotypic variation, while one marker on Bin6.05 was detected for it in 2013. There was only one locus anchored on Bin6.05 that was associated with TIL with a phenotypic explanation of 7.2%. For

Table 2 - Correlation analysis of traits related to lodging resistance.

Traits	SPR	TIL	TID	FIL	FID	PH
TIL	0.016					
TID	0.414**	0.152*				
FIL	0.016	0.914**	0.084			
FID	0.143*	0.083	0.687**	0.001		
PH	0.095	0.415**	0.427**	0.456**	0.276**	
EH	-0.157*	0.244**	0.426**	0.261**	0.316**	0.648**

* significant at P< 0.05; ** significant at P< 0.01

TID in 2012 and 2013, a total of 11 controlled locus were detected on bin1.04, bin1.05, bin2.1, bin3.1, bin4.08, bin5.05, bin6.02, bin6.06, bin9.03, and bin9.08 with phenotypic explanation from 0.2% to 15.0%. The detailed information for the rest four traits was shown in Table 4. Among all the markers detected, umc1462, bnlg589, and mmc0241 were stable ones co-detected in two years for FIL, FID, and PH. Moreover, several bins were hot spots covering more than one trait, and they were bin1.04, bin1.05, bin3.1, bin4.05, bin4.08, bin4.1, bin5.05, bin6.02, bin6.05, bin6.06, and bin9.08, Especially, bin6.05, which contained markers associated with five traits, was a bin deserving further attention.

Discussion

Maize lodging resistance is a complex trait that is affected by several factors. Thus, developing an effective way to assess stalk-lodging resistance has important significance for the identification of maize germplasm and lodging resistance breeding. The most direct method to evaluate lodging resistance is to count the lodged plants at harvest. Although the way was accurate, it was seriously dependent on the environmental factors and the heritability is lower (Thompson et al, 1963). In recent years, numerous methods have been used to predict lodging resistance potential, which include chemical methods and mechanical methods. Moreover, stalk mechanical strength had shown a highly relationship with stalk lodging resistance through studies on correlation between the rind penetrometer resistance (RPR) and stalk lodging (Flint-Garcia et al, 2003a; Hu et al, 2012, 2013). Zuber and Grogan et al (1961) had reported the effect of stalk crushing strength on lodging. Compared with those two traits, SPR can more directly reflect the stalk lodging resistance in the field. Although previous studies on SPR in crops have been reported, little is known on comprehensive evaluation of SPR about the germplasm resources of diversity. In present study, 290 inbred lines derived from China and America were used to assess the SPR of maize. The results indicated a significant difference between the inbred lines and presented the continuous variation and a wider range. Meanwhile, all traits had higher broad-sense heritability, so these characteristics explained that the SPR could effectively reflect the stalk lodging resistance. Based on the classification results of SPR, the germplasm resources of Reid heterosis group should be valued, because of 31.2% strongly lodging resistance inbred lines within our investigation belonged to the Reid subgroup (Liu et al, 2014). In contrast, the 55.5% weakly lodging resistance inbred lines originated from the TSPT subgroup (Liu et al, 2014). The result was consistent with pervious study (Fu et al, 2011).

Maize germplasm accessions used in this study included landraces collected in China and America, which represented a wealth of genetic variations for the application of GWAS. The diversity could be determined by identifying alleles that would be used for achieving the genetic improvement. GWAS provides a useful and powerful approach to accomplish this objective. Precise phenotypic measures were crucial for genotype-phenotype association analysis (Li et al, 2014). We analyzed various morphological and stalk strength, which included PH, EH, TIL, TID, FIL, FID, and SPR. Pushing resistance had been taken as an index to assess stem bending or root lodging in several crops (Kashiwagi et al, 2004; Terashima et al, 1992; Won et al, 1998). For example, five QTLs for pushing resistance were detected on chromosomes 4, 5, 6, 11, and 12, which accounted for 63.6% of the phenotypic variation. In addition, Kashiwagi et al (2008) revealed that pushing resistance was positively correlated with stem diameter. In this study, we also detected four locus associated with pushing re-

Table 3 - The types and classification standards according to loading resistance coefficient.

Type	Classification (N)	Proportion (%)	Inbred lines
I[a]	11.20	15.9	Wu109, DH857, Shen137, Shan89
II[b]	9.53 < 11.20	12.4	Mo17,78599, Zong3
III[c]	6.00 < 9.53	40.8	B73, Chang7-2, Ji853, Huangchang b
IV[d]	4.88 < 6.00	14.4	Huangzaosi, Chang K, 200B
V[e]	<4.88	16.4	Jun928, Zheng32, Jun971

[a]more strongly lodging resistance, [b]strongly lodging resistance, [c]middle lodging resistance, [d]weakly lodging resistance, [e]more weakly lodging resistance.

Table 4 - The marker associated with traits for each individual year and explained phenotype variation.

Trait	Bin	Locus	P-markers[a] 2012	P-markers[a] 2013	R^2(%)[b]
SRP	1.05	umc1558	9.58E-04		11.9
	6.05	umc1250		4.40E-04	6.3
	2.03	umc1776	1.60E-04		10.6
	6.05	umc1020	7.78E-04		7.7
TIL	6.05	umc1462		2.41E-03	7.2
TID	5.05	mmc0282	9.02E-05		14.9
	1.05	umc1558	1.40E-04		9.4
	4.08	umc1371	3.48E-04		5.8
	9.08	umc1505	8.53E-04		7.0
	6.02	umc1178	9.07E-04		14.1
	3.10	bnlg1098	9.14E-04		15.0
	1.04	umc2228	1.23E-03		0.2
	4.08	umc2286	1.76E-03		8.2
	6.06	umc2170		4.72E-04	8.4
	2.10	umc2184		5.34E-04	12.2
	9.03	bnlg430		1.21E-03	10.3
FIL	6.05	umc1462	1.21E-03	1.19E-03	5.0-7.2
	5.07	bnlg2305		1.92E-03	8.0
FID	5.05	mmc0282	5.93E-05		14.6
	1.05	umc1558	3.97E-04		8.1
	4.08	umc1371	4.63E-04		7.1
	6.02	umc1178	6.48E-04		13.3
	9.07	umc1936	8.43E-04		4.4
	3.10	bnlg1098	1.23E-03		15.8
	4.10	bnlg589	1.58E-03	1.33E-03	9.5-14.3
	1.04	umc2228	2.66E-03		0.1
	9.08	umc1505	2.83E-03		8.6
	6.06	umc2170		6.28E-04	8.5
	10.06	umc1993		2.64E-03	4.6
PH	6.05	mmc0241	1.14E-04	1.99E-03	13.5-10.2
	10.00	umc1380	2.88E-03		11.5
	4.05	bnlg1755	1.14E-03		12.9
EH	3.09	bnlg1257	1.78E-03		10.2
	5.03	umc1937	2.34E-03		16.4
	9.00	umc1957	2.59E-03		11.6
	10.05	umc1506		9.80E-04	3.2
	3.10	bnlg1098		1.72E-03	18.4
	4.10	bnlg589		1.86E-03	17.1
	4.05	bnlg1755		1.98E-03	15.0
	6.05	umc1462		2.81E-03	8.4

[a]P value of corresponding detected markers, [b]phenotypic contribution of corresponding detected markers. Gray line indicates associations identified in both years.

sistance, which located on chromosomes 1, 2, and 6. The QTL located on bin 6.05 was the most important one with a high frequency for SPR. Furthermore, it is worth noting that the loci had an over-lapping confidence interval with the traits for SPR, TIL, FIL, PH, and EH. These results might also offer important reference information for breeders to select an effective strategy to improve stalk strength by using SPR as an index.

Our study revealed that EH had a highly negative correlation with pushing resistance, which was consistent with previous studies. For example, Horner et al (1976) reported that EH was decreased by 9% over seven cycles of selection for lodging rate. Feng et al (2010) had suggested a significant correlation between EH, PH, and lodging resistance. Three $F_{2:3}$ populations were employed to map RPR, EH, and RPR being EH optimized, which finally obtained 27 controlled locus across Chr1, Chr 2, Chr 3, Chr 4, Chr 5, Chr 6, Chr 7, Chr 8, Chr 9, and Chr 10 (Flint-Garcia et al, 2003b). Tang et al (2005) used a population with 266 $F_{2:3}$ families from Yuyu22 to characterize EH and stem diameter and total seven QTLs of EH were detected in two-location field tests. These QTLs were distributed in four genomic regions, whose explanation ranged from 4.95% to 13.57%. Whereas, eight locus of stem diameter were located on Chr. 1, 3, 6, and 8 and we found adjacent bins of 3.09 and 3.10 in the present study. For PH, we detected three SSR markers located on bin6.05, 4.05 and 10.00. Tang et al (2013) had reported the significant association of PH located on bin6.04 - 6.06 which were consistent with our study. Moreover, our study found that the PH was not significantly correlated with the pushing

resistance. This view was consistent with the results of Kashiwagi et al (2004, 2008), but different with the results of Verma et al (2005). The causes might be the difference of experimental materials, experimental design and environmental conditions between studies.

Several studies have been shown the relationship between stalk characters and lodging resistance (Hu et al, 2012, 2013; Kashiwagi et al, 2004, 2008; Tang et al, 2005; Zhang et al, 2005; Zhu et al, 2008). For example, Martin et al (1984) had indicated that lodging rate was negatively correlated with TID. And varietal differences among ten rice cultivars showed that stem diameter was a key factor in lodging resistance, which suggested that an increase in basal internode diameter could contribute to greater pushing resistance (Kashiwagi et al, 2008).This conclusion has been confirmed in this study. Zhang et al (2005) revealed that the basal internode diameter was strongly positively correlated with lodging resistance. However, the basal internode length had a contrary effect. Besides, Zhu et al (2008) totally acquired 21 QTLs for PH, 12 QTLs for EH, 17 QTLs for basal internode, and 9 QTLs for basal elongation internode controlling lodging-resistance by employing a RIL population. Additionally, Hu et al (2013) and Tang et al (2013) had reported that QTLs of stem diameter were identified on bin5.03 and bin3.04, in the same way, we also detected significantly associated locus for FID on bin3.04 and bin5.03. These results both suggested that the associated locus detected were true and reliable. Due to the locus situated on bin6.05 were detected several times and had significant correlation with multiple traits. The marker mmc0241 located on bin6.05 represented stable association with PH. Interestingly, the QTLs of PH were identified many times on the same chromosome region, which suggested that this region deserved further investigation for finding candidates genes underlying lodging resistance. The study indicate that GWAS can be executed as an effective strategy for identifying complex traits in maize and for narrowing the GWAS-detected genomic regions, which facilitates positional cloning of the causal genes.

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