Significant associations between single nucleotide polymorphisms and photosynthetic parameters and grain yield in maize

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ABSTRACT

Photosynthesis is the basis of maize (*Zea mays* L.) grain formation. To further understand the genetic basis of maize photosynthetic parameters and clarify the relationship between maize photosynthetic parameters and grain yield (GY), identifying quantitative trait loci (QTLs) underlying photosynthetic parameters and GY plays an important role in improving maize yield. In this study, a set of 260 maize accessions from different geographic origins were evaluated across three developmental stages in 2 yr to identify QTLs for photosynthetic parameters and grain yield using 2824 single-nucleotide polymorphisms (SNPs) via genome-wide association analysis. The analysis revealed that maize photosynthetic parameters are substantially correlated with GY at different developmental stages. Fourteen SNPs associated with photosynthetic parameters and GY were detected at the threshold of $P \leq 0.001$ in 2 yr as well as over years. Moreover, PZE-102116144 and SYN35048 associated with stomatal conductance (g_s), and PZE-101152541 associated with photosynthetic rate (P_N), were identified at different developmental stages. Four loci were co-associated with two or more traits, such as PZE-101152541 was significantly co-associated with GY and P_N (at 25 and 35 d after pollination [DAP]), PZE-102116144 with GY, g_s (at 15 and 25 DAP), and P_N (at 25 DAP), PZE-109061997 with intercellular CO_2 concentration (C_i) (at 25 DAP) and transpiration rate (T_r) (at 25 DAP), PZE-110019199 with g_s (at 25 DAP) and GY. Based on functional annotations, two genes were considered as potential candidate genes for the identified SNPs (PZE-101152541 and PZE-109016787). The SNPs and candidate genes identified in this study might provide instrumental information for understanding the genetic mechanism of maize photosynthetic parameters and yield.

Key words: Photosynthetic parameters, SNPs, yield, *Zea mays*.

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INTRODUCTION

Maize (*Zea mays* L.) is one of the world's most important crops, serving as an essential source of food, feed, and fuel. With rapidly increasing global demands for food, bio-fuel and livestock feed, the demand for maize is increasing (Cassman and Liska, 2007; Grassini et al., 2011). Improving maize yield is the major concern of plant breeders. Photosynthesis is the primary process for crops to form grain yield. Grain yield increase in modern maize lines mainly depended on the improved chloroplast structure and more light energy catched for the photochemical reaction, thus having stronger photosynthetic capacity (Li et al., 2015). Many breeders believed that the yield increase of maize hybrids is largely attributable to the improvement of photosynthetic physiological characteristics (Chen et al., 2013). The maize yield increase largely depends on the improvement of photosynthetic characteristics in parental inbred lines (Li et al., 2013). Because photosynthesis is the basis of maize grain yield, boosting leaf photosynthesis is an important strategy to increase yield potential and biomass production (Chen et al., 2013; Li et al., 2013; Ding et al., 2014).

Many studies focused on the improvement of various crop species have revealed that photosynthetic rate (P_N) influences grain yield (GY) (Hubbart et al., 2007; Zheng et al., 2011; Peng et al., 2012). Measurements of photosynthetic gas-exchange parameters, such as P_N, stomatal conductance (g_s), transpiration rate (T_r), and intercellular CO_2 concentration (C_i), are useful to assess photosynthetic capacity and gain insight into the behavior of photosynthetic machinery (Feng et al., 2009; Khan et al., 2010; Yin et al., 2010; Song et al., 2012; Yu et al., 2015; Zhong et al., 2015; Hao et al., 2017). Significant differences in photosynthetic gas-exchange parameters have been uncovered among maize varieties. A significantly positive correlation between P_N and the yields per plant has been observed in adzuki bean cultivars from the jointing to the maturing stages (Song et al., 2012), which suggests that P_N is a potential selection marker to assess their cultivar performances. Photosynthetic parameters, such g_s and T_r, have an effect on crop GY under stressed and non-stressed conditions (Khazaei et al., 2010).

Genetic variations in maize photosynthesis parameters have been reported, but little is known about the genetic basis behind the traits. The development of genomics has provided alternative tools to improve selection efficiency in crop breeding programs. Molecular markers could be used to improve the efficiency and precision of crop breeding via marker-assisted selection

(MAS) (Collard and Mackill, 2008). Several quantitative trait loci (QTLs) associated with maize photosynthetic parameters have been reported (Yu et al., 2015). However, no consistent QTL was detected in the two populations and the contribution rate of any given single QTL was low (Yu et al., 2015). This is because most QTLs were population-specific, and the genetic background of the studied population has a strong influence (Wang et al., 2008; Xu and Crouch, 2008). Because of the existence of large confidence intervals associated with uncovered QTLs and the rarity of recombination events in the biparental populations, very few QTLs have been used in maize breeding programs (Van Inghelandt et al., 2012; Riedelsheimer et al., 2012).

With the increased availability of genomic polymorphism data, genome-wide association studies (GWASs) based on linkage disequilibrium have become a powerful approach for dissecting quantitative traits in crops (Stich and Melchinger, 2010; Yan et al., 2011; Segura et al., 2012). A GWAS is used to exploit all recombination events that occur during the evolutionary history of a natural population (Zhu et al., 2008). Marker-trait association enables researchers to exploit natural diversity and locate valuable genes in the genome (Rafalski, 2010). The main constraints on the use of the GWAS approach are genetic relatedness and population structure, which can cause spurious marker-trait associations (Chan et al., 2011; Hao et al., 2012b). Several statistical methods have been used to account for the population structure and relatedness. The unified mixed model approach (MLM) is a powerful strategy, and it can account for multiple levels of relatedness simultaneously and improve control of both type I and type II error rates (Hao et al., 2012b). In maize, GWASs have already proved successful for studying a series of morphological or metabolic traits, such as shoot apical meristem size (Leiboff et al., 2015), male inflorescence size (Wu et al., 2016), herbivore-induced volatiles (Richter et al., 2016), plant height (Li et al., 2016), etc.

QTL mapping for maize photosynthetic parameters has thus far been mainly based on linkage analysis. The QTLs that have currently been detected are population specific, with few QTLs consistent across various populations. A better understanding of the genetic basis of photosynthetic parameters in different maize germplasm resources would provide suitable information for marker-assisted selection (MAS) within breeding programs, especially if the identified QTLs are stable in different environments and genetic backgrounds. In the present study, a set of 260 elite maize inbred lines with extensive genetic variation was evaluated to identify QTLs associated with photosynthetic parameters and GY in multiple environments through a GWAS.

MATERIALS AND METHODS

Plant materials and plant growth conditions

An association mapping panel comprising 260 elite maize inbred lines (150 common maize and 110 waxy maize) was used for this study (Table 1). The trials were performed in 2014 (designated as environment E1) and 2015 (designated as environment E2) at the Experimental Farm of the Jiangsu Yanjiang Institute of Agricultural Sciences (31°58'48" N, 120°53'24" E), Nantong, China. To enable comparisons at similar growth stages, the 260 maize inbred lines were sown at different times in three groups according to their projected silking times (Table 1). All lines were arranged in a randomized complete block design with two replicates. Each line in a plot was planted in two 6-m long rows separated by 60 cm, with seeds in each row spaced 30 cm apart for a total of 20 plants per row. In order to control the border effect, the association mapping panel was surrounded by protective belt. To avoid potential nutrient and drought stresses, optimal nutrition and water were supplied throughout the whole life cycle.

All of the above experiments were conducted under natural irradiance. Daily relative humidity and daily mean temperatures throughout the growing season were based on data from the local meteorological station in Nantong, China. Mean daily relative humidities throughout the 2014 and 2015 growing seasons were 71.8% and 69.3%, respectively, with corresponding average daily temperatures of 24.7 and 23.5 °C.

Phenotypic data collection

Gas exchange parameters (P_N, g_s, T_r and C_i) were determined on sunny days from 09:00 to 11:30 h and 14:00 to 16:00 h with a portable photosynthesis system (LI-6400, LI-COR, Lincoln, Nebraska, USA). The air temperature of the leaf chamber was maintained at 30 °C and the photon flux density was set to 1200 μmol m^{-2} s^{-1}. The CO_2 concentration of the air in the chamber was controlled by the LI-COR CO_2 injection system, while light used for the measurements was supplied by the LI-COR LED light source. Ear leaves of four uniform plants in the middle of each plot were measured for photosynthetic parameters at 15, 25 and 35 d after pollination (DAP). Because of the large number of tested materials, two portable photosynthesis system units were used in this study.

Grain yield was estimated from 10 consistently growing plants in the middle of each plot. Ears of corresponding plants were manually harvested at maturity, dried to constant weight and threshed.

DNA extraction and single nucleotide polymorphism (SNP) genotyping

Genomic DNA from maize seedling leaves was extracted using the CTAB method (Hao et al., 2012a). All 260 maize inbred lines were genotyped for 3072 SNPs via the GoldenGate assay platform (Illumina, San Diego, California, USA) at the National Maize Improvement Center of China, China Agricultural University (Beijing, China) as described by Hao et al. (2015). These 3072 SNP markers have been applied to identify QTLs for resistance to *Aspergillus flavus* in a recombinant inbred line population in maize (Yin et al., 2014). Based on the results of a genetic

Table 1. Elite maize inbred lines (150 common maize and 110 waxy maize) used for this study.

Nr	Inbred line	Origin/Pedigree	Group
1	4377	(75×YJ7)×YJ7	I
2	437	75×YJ7	I
3	T458	Derived from Ye478	I
4	T75	8112×YJ7	I
5	4S	Derived from Huangzaosi	I
6	T877	(YJ7×E28)×YJ7	I
7	S951	(8711×8709)×90SEY	I
8	C72	(V59×Huangzaosi)×S901	I
9	ND1145	Recycled line from hybrid 78599	I
10	Qi319	Recycled line from hybrid 78599	I
11	T249	5U×P6C1	I
12	CA375	Derived from 13QPMCO	I
13	Dan598	Derived from Mixed Population of Dan340 and P78599	I
14	HL04-40	Derived from synthetic variety	I
15	Shen137	Derived from 6JK111	I
16	4AYC	4S×YC	I
17	Su195	2163×Luyuan92	I
18	Wu314	(302×HBL)×Huangzaosi	I
19	U8112	Derived from U8	I
20	F2	Derived from Ye478	I
21	X19M	Derived from mutant of Ye478	I
22	Zheng58	Derived from Ye478	I
23	H991	Ye478×5003	I
24	N18	Maize inbred line from 78599	I
25	N19	Maize inbred line of N18 from USA	I
26	N3	Maize inbred line of N3 from USA	I
27	N21	Maize inbred line of N21 from USA	I
28	Dan340	Baizhulu-9×wild maize	I
29	K22	K11×478	I
30	T803	8112×5003	I
31	C8605	7922×5003	I
32	Ye478	8112×5003	I
33	SMLYC	SML×YC	I
34	7922	Recycled line from hybrid 3382	I
35	DH02	Derived from Mixed Population of Lv28 and Lv9	I
36	Mo17	187-2×103	I
37	Zi330	OH43×Keli67	I
38	DH65232	6237×5003	I
39	8723	Unknown	I
40	Xun92-6	C7-2×J7H	I
41	9801	502×H21	I
42	Y85	515×P78599	I
43	YJ7	Recycled line from hybrid 78599	I
44	Zong3	Derived from Zi330 Synthetic	I
45	Zong31	Derived from Zi330 Synthetic	I
46	PHC	(HX162×330)×Tuxpeno	I
47	P178	Recycled line from hybrid 78599	I
48	Zhong128	2118×Zhongzi7490	I
49	P138	Recycled line from hybrid 78599	I
50	Q1261	Q12×61	I
51	K12	Huangzaosi×Weicunhuang	I
52	ZGF	Derived from Synthetic Population	I
53	Z25F	Z2×5F	I
54	Huangzaosi	Tangsipingtou	I
55	Luyuan92	Yuanqi123×1137	I
56	Ye107	Derived from XL80	I
57	DH1M	7922×478	I
58	JHM	Derived from Jinhai muben	I
59	JHF	Derived from Jinhai fuben	I
60	Z25M	Z2×5M	I
61	J-2	Recycled line from hybrid 78599	I
62	'66'	8112×78599	I
63	568G	8112×78599	I
64	L162	8112×75	I
65	Zheng37	(138×Zheng22)× Ye52106	I
66	DD60	Derived from Liaoning	I
67	J853	Huangzaosi×Zi330	I
68	T1003	S4×4S	I
69	T1002	Recycled line from Xianyu	I
70	T1004	4S×Zheng58	I
71	T1005	Qi319×X7	I
72	T1006	Qi319×78599	I
73	T1007	Shen137×4S	I
74	T2001	Recycled line from hybrid Xianyu335	I
75	T1008	E28×CML×Dan340	I
76	T1009	E28×CML×4S	I
77	T1010	K12×MDR×D340	I
78	T1011	K12×MDR×4S	I
79	T1012	(5U×P6C1)×CML	I
80	T1013	568G×S3×568G	I
81	T1014	Zong3×T877	I
82	T249·7	T249×7	I
83	T43.7·C72	T43×7×C7-2	I
84	T1015	T249×78599	I
85	T1016	(75×78599)×C7-2	I
86	T1017	Unavailable	I
87	T1018	Unavailable	I
88	T1019	Hai9-21	I
89	T1020	Unavailable	I
90	T1021	D805-4-4	I
91	R1	Derived from tropical population S4	I
92	R2	Derived from tropical population S187	I
93	R3	Derived from tropical population S2	I
94	R4	Derived from tropical population S5	I
95	R5	Derived from tropical population S6	I
96	R6	Derived from tropical population S7	I
97	R7	Derived from tropical population S8	I
98	R9	Derived from tropical population Pob501	I
99	R10	Derived from tropical population Pob502	I
100	R11	Derived from tropical population Suwan	I
101	N1	Inbred line of N1 from USA	I
102	N9	Inbred line of N9 from USA	I
103	N10	Inbred line of N10 from USA	I
104	N11	Inbred line of N11 from USA	I
105	N16	Inbred line of N16 from USA	I
106	N23	Inbred line of N23 from USA	I
107	N24	Inbred line of N24 from USA	I
108	N25	Inbred line of N25 from USA	I
109	N26	Inbred line of N26 from USA	I
110	A489	Derived from Nanjing	I
111	LS335M	Recycled line from hybrid Xianyu335	I
112	YC2	Y85·C72	I
113	N42	T75×T178	I
114	E77	E7×7×9045	I
115	N27	Zong31×S51M	I
116	SC	4S×C72	II
117	SD4	4S×Dan598	II
118	DH4866	7922×Ye478	II
119	Ji53	Jiqun2×Co-2	II
120	DH9M	Derived from Shandong	II
121	ZGF	Derived from Suqian	II
122	N1012	T366×T2×T2	II
123	Dan231	Derived from Liaoning	II
124	T10015	Recycled line from hybrid 78599	II
125	N51	XD×20M	II
126	JX	Derived from tropical population	II
127	T1000	Derived from tropical population	II
128	QX7	Qi319×X7	II
129	N120	Derived from USA	II
130	N121	Derived from USA	II
131	BSC	BSSS(R)×C7	II
132	JH78-2	Recycled line from hybrid 78599	II
133	Zheng39	(Ji533×Zheng32)×Ji533	II
134	P420	PA91×LH98A	II
135	AL1	A619×L120	II
136	OhL	Oh43×L120	II
137	K24	LH19×LH39	II
138	778	78×YJ7	II
139	JH3372	Shen5003×Zi330	II
140	B37644	(B37×644)×B37	II
141	BH739	B73(2)×H93	II
142	VPA	Va85×Pa91	II
143	AL9	A619×L120	II
144	K27	Mo17 backcross 5 recovery	II
145	K29	Mo17(3)×W153R type	II
146	MH	(Mo17×H99)×LH53	II

Continuation Table 1.

Nr	Inbred line	Origin/Pedigree	Group
147	K25	LH55×LH47	II
148	MA	(Mo17×ASA)×Mo17(2)	II
149	N32	PH814×PH207	II
150	N33	PH814×PH848	II
151	Hu-2	T5×5U	II
152	H	Derived from Hengbai522	II
153	ZFF	Bainuo	II
154	XH361	Rudonghongnuo×361×361	II
155	T5T5	Derived from Tongxi5	II
156	06X-6	366×YJ7	II
157	W6	361×Shengnongnu	II
158	RF	Jingchenghuanuo	II
159	T5	Derived from synthetic population from Qihaichong	II
160	R-8	Landrace with unknown pedigree	II
161	T2-1	Derived from T2 of Thailand	II
162	T2-2	Derived from T2 of Thailand	II
163	NFT5T5	(Zhiyunuo×T5)×T5	II
164	HMZW	Landrace from Haimen	II
165	NFHH	(Zhiyunuo×HB522)×HB522	II
166	YHuF	Derived from Yunan	II
167	YHeF	Derived from Yunan	II
168	T4-2	Derived from T4 of Thailand	II
169	T361	T5×WA4	II
170	2214	Nuo22×Nuo24	II
171	Hu-1	HB522×U8112	II
172	H13	366×Luyunuo1	II
173	NF	Derived from landrace of Zhiyunuo	II
174	H9	Derived from Hengbai522	III
175	Ybw1	Derived from HB522	III
176	HDWF	Landrace from Hainan	III
177	HLZ	Derived from Hainanzhinuo	III
178	L8	Jingheinuo	III
179	ZJM	Derived from Nanjing	III
180	T354	T5×GW112	III
181	JNHWF	JNH×WF	III
182	Huang1	Recycled line from Luyunuo1	III
183	Zhenxiongnuo	Landrace from Yunnan	III
184	Bainian	Derived from synthetic landrace	III
185	Shennongnuo	Derived from Hubei landrace	III
186	N1001	Recycled line from hybrid Zhongnuo2	III
187	N1002	HB522×T4	III
188	HT4	H366×T4	III
189	N1005	T361×T2	III
190	N1006	T2×HB522	III
191	N1007	T2×HB366	III
192	N1008	T354×T2	III
193	N1009	Luyunuo1×T2	III
194	Thu	T2×Hu-2	III
195	N1016	T5×N27	III
196	HTT	H366×T2×T2	III
197	RX6	RF×Xiang618	III
198	RH-5	RF×H9	III
199	HRF	Hu-2×RF	III
200	HNW	Landrace from Hainan	III
201	WH8	Derived from USA	III
202	T5V	Derived from T5	III
203	N1035	W5×T2	III
204	EH6	E77×H366	III
205	N1025	Zhengheinuo1×T361	III
206	N1026	((NF×354)×354)×354	III
207	HWT	HW×T2	III
208	T1001	(75×78599)×(5U×P6C1)	III
209	HD	H×Dan340	III
210	N1029	Hu-1×RF	III
211	N2001	Recycled line from hybrid Xiangnuo618	III
212	SNN	Landrace of Shengnonnuo	III
213	J2ML	Derived from J2M	III
214	N1011	Recycled line from hybrid Jingkenuo2000	III
215	N1014	RF×HU-1	III
216	N1021	T5×T2-1	III
217	SW1	Derived from wxsu	III
218	L1M	Derived from Guangxi	III
219	N1003	(Rudonghongnuo×T5)×T5	III
220	N1015	T5×RF	III
221	N1010	T2×Hu-2	III
222	N1004	HB522×T4	III
223	W25	Derived from Zhinuo	III
224	N1023	U8112×T5	III
225	W23	Derived from Bainuo	III
226	N1028	HB522×Dan340	III
227	N1019	(T1×Shengnongnuo)×T1	III
228	HB522	Landrace of Hengbaiduoshui V	III
229	N1017	M020×HB522×T1	III
230	RDHW	Derived from Jiangsurudong	III
231	N1036	Huanuo	III
232	BN2	Recycled line from hybrid Zhinuo3	III
233	11HW-2	Derived from Sichuan	III
234	N1024	T877×T366	III
235	LSHW	Landrace from Hainan	III
236	H408	Derived from Huaiyingzhinuo	III
237	N2002	Recycled line from hybrid WH8	III
238	RH-2	Landrace of Ronghong	III
239	K23	L150	III
240	T4-1	Derived from T4 of Thailand	III
241	SW4	Derived from wxsu	III
242	MHH-2	Landrace of Minghehong	III
243	L1F	Derived from T4 of Guangxi	III
244	N1018	T366×T1	III
245	YSN	Derived from Sichuan	III
246	N1027	Huyunuo1×T2	III
247	ZX①	Derived from Yunnanzhenxiong1	III
248	ZX②	Derived from Yunnanzhenxiong2	III
249	ZX③	Derived from Yunnanzhenxiong3	III
250	N1031	Derived from T	III
251	N1030	TZ×228TZ	III
252	N1020	Recycled line from hybrid Wannian1	III
253	N1013	RF×Xiangnuo618	III
254	N1034	W314×HZH	III
255	N1033	W314×228	III
256	N1032	W313×3WX	III
257	N1022	T5×Bainian	III
258	W24	Derived from Bainuo	III
259	J2FL	Derived from J2F	III
260	J2FP	Derived from J2F	III

Note: The 260 inbred lines were sown in three groups according to their projected silking times. Group III, Group II, and Group I were sown at 28 March, 1 April, and 5 April in 2014, and at 26 March, 30 March, and 3 April in 2015, respectively.

diversity analysis, 2824 SNPs with minor allele frequencies ≥ 5% and missing data ≤ 20% in the present population were used for subsequent analysis.

Population structure and kinship analysis

Population structure based on the 2824 SNPs was inferred using the software program STRUCTURE 2.3 (Hubisz et al., 2009). Five independent Markov chain Monte Carlo runs of 100 000 iterations (after discarding the burn-in) were performed for each hypothetical number of subpopulations (k) ranging from 1 to 10. To estimate the most likely number of subpopulations present, the number of subgroups (Δk) was maximized according to Hao et al. (2015). STRUCTURE HARVESTER (http://taylor0.biology.ucla.edu/structureHarvester/) (Earl and von Holdt, 2012) was used to visualize the STRUCTURE output and implement the Evanno method. A membership value (Q value) > 0.5 was used as a criterion to assign each maize inbred line

into a subpopulation. A population structure matrix (Q) was generated for further analysis.

A relative kinship matrix (K) comparing all pairs of the 260 maize accessions was calculated using the software program SPAGeDi (Hao et al., 2015), with the negative value of kinship set as zero.

Statistical and association analyses

Statistical analyses were performed using SAS 9.1 (SAS Institute, Cary, North Carolina, USA). ANOVA was performed using SAS PROC GLM. Mean values of phenotypic traits were calculated using SAS PROC MEANS. Regression coefficients and Pearson phenotypic correlations among traits were calculated using SAS PROC REG and PROC CORR, respectively. To minimize the effects of environmental factors in subsequent analyses, the best linear unbiased predictions (BLUPs) for the tested traits over years in each line were estimated using PROC MIXED (Hao et al., 2015).

To identify SNPs associated with the studied traits, an association analysis was performed using a Q+K mixed linear model (MLM) in TASSEL 4.0. Based on a threshold of -log $P \geq 3.00$ ($P \leq 0.001$), SNPs significantly associated with phenotypic traits were identified.

To identify the best alleles of significantly associated SNP markers, average phenotypic values of the corresponding alleles were measured based on the BLUPs of the phenotypic values of each trait in the two years. The trait value for each tested maize line was the mean BLUP value calculated from observations of each line in the two years.

RESULTS

Phenotypic variations and heritability of photosynthetic parameters and GY

BLUPs, relevant statistical parameters, and broad-sense heritabilities for all five studied traits are shown in Table 2. Extensive phenotypic variations were observed among different environments (Table 2). ANOVA revealed that variances of genotypes, environments, and interactions between genotypes and environments (G×E) were highly significant at the $P \leq 0.01$ level for all five traits (Table 2). Values of photosynthetic parameters at 25 DAP were significantly higher than at 15 DAP and 35 DAP during 2 yr. The heritability of photosynthetic parameters and GY varied across different growth stages. Except for C_i, the heritabilities of photosynthetic parameters at 25 DAP were higher than those at 15 DAP and 35 DAP.

Phenotypic correlation analysis

Correlation coefficients based on the BLUP model analysis are summarized in Table 3. In all cases, correlation coefficients were significant or highly significant in different stages. GY was highly positively correlated with P_N, C_i, g_s and T_r at 15, 25 and 35 DAP, which suggests that photosynthetic capacity influences GY during the grain-filling process. P_N, g_s and GY were highly positively correlated during the three developmental stages, with correlation coefficients ranging from 0.535 (between GY and g_s at 15 DAP) to 0.817 (between P_N and g_s at 25 DAP). In most cases, correlation coefficients at 25 DAP were higher than those at 15 and 35 DAP.

Population structure analysis and GWAS

A Bayesian model-based method as implemented in STRUCTURE was used to infer population structure and assign individuals to subpopulations. As k increased from 1 to 10, the ln P(D) value corresponding to each hypothetical k increased, with no peaks evident (data not shown). As shown in Figure 1, the likelihood of Δk was much higher at $k = 2$ than at $k = 3$-10 (Figure 1), which suggests that the population could be clustered into two major subpopulations. The corresponding Q-matrix (at $k = 2$) was generated for the subsequent GWAS.

SNP markers associated with the five studied traits were identified using a MLM (Q+K) in TASSEL 4.0. A total of 34 marker-trait associations involving 23 SNPs were associated with maize photosynthetic parameters and yield in different years and over years at a threshold level of $P \leq 0.001$ (-log $P \geq 3$) (Figure 2). Among the significant marker-trait associations, 23 marker-trait associations representing 14 SNPs remained significant in 2 yr and over years at the threshold of $P \leq 0.001$, these SNPs were distributed among 8 of chromosomes, excluding chromosome 3 and 8 (Table 4). Of these 14 SNPs, 4 were associated with P_N, 4 with C_i, 5 with g_s, 3 with T_r and 3 with GY. PZE-101152541 associated with P_N was identified at 25 and 35 DAP. PZE-102116144 associated with g_s was identified at 15 and 25 DAP, and SYN35048 associated with g_s was identified at 15, 25 and 35 DAP.

Four *loci* were co-associated with two or three traits. PZE-101152541 on chromosome 1 was co-associated with P_N (25 and 35 DAP) and GY. PZE-102116144 on chromosome 2 was co-associated with P_N (25 DAP), g_s (15 and 25 DAP) and GY. PZE-109061997 on chromosome 9 was co-associated with T_r (25 DAP) and Ci (25 DAP), and PZE-110019199 on chromosome 10 was co-associated with g_s (25 DAP) and GY.

Identification of candidate genes

Among the 34 significant associations (-log $P \geq 3$) we identified, a total of 14 SNPs were associated with 9 genes (Table 3). According to the maize gene annotation database at MaizeGDB (http://www.maizegdb.org), the putative genes, where the associated SNPs located in, indicated that genes of GRMZM2G317770 on chromosome 1 and GRMZM2G002227 on chromosome 9 were the most likely candidate genes for PZE-101152541 (associated with P_N at 25 and 35 DAP and GY) and PZE-109016787 (associated with g_s at 15), which are candidate genes encoding protein kinase (Table 4). In *Arabidopsis thaliana*, the protein kinase in chloroplasts is known to regulate photosynthesis (Schliebner et al., 2008).

Table 2. Descriptive statistics, results of ANOVA, and broad-sense heritabilities of grain yield and photosynthetic parameters at different growth stages among 260 maize inbred lines across 2 years.

Traits	DAP	Year	Mean	SD	Min	Max	G	E	G×E	h^2(%)
					μmol m^{-2} s^{-1}					
P_N	15	2014	25.85	5.47	19.30	34.69	**	**	**	56.45
		2015	22.03	3.65	19.63	33.74				
		BLUPs	24.07	3.06	19.13	34.68				
	25	2014	26.64	6.52	15.00	43.62	**	**	**	63.21
		2015	30.02	5.95	18.39	43.20				
		BLUPs	28.38	2.25	17.67	41.64				
	35	2014	24.16	5.45	10.05	43.04	**	**	**	59.43
		2015	25.72	6.78	10.95	42.54				
		BLUPs	25.12	2.56	11.95	39.72				
					μmol mol^{-1}					
C_i	15	2014	213	12	156	278	**	**	**	51.76
		2015	218	18	162	289				
		BLUPs	216	19	159	283				
	25	2014	238	17	164	287	**	**	**	53.81
		2015	231	20	171	291				
		BLUPs	234	19	169	289				
	35	2014	218	21	157	271	**	**	**	54.82
		2015	216	16	159	278				
		BLUPs	217	15	158	275				
					mol m^{-2} s^{-1}					
g_s	15	2014	0.354	0.143	0.13	0.81	**	**	**	47.21
		2015	0.387	0.102	0.14	0.79				
		BLUPs	0.371	0.118	0.14	0.80				
	25	2014	0.386	0.109	0.15	0.88	**	**	**	49.23
		2015	0.392	0.121	0.16	0.86				
		BLUPs	0.389	0.110	0.15	0.87				
	35	2014	0.376	0.152	0.14	0.76	**	**	**	46.42
		2015	0.367	0.173	0.15	0.72				
		BLUPs	0.369	0.147	0.14	0.74				
					mmol H_2O m^{-2} s^{-1}					
T_r	15	2014	5.364	1.237	1.98	10.76	**	**	**	51.24
		2015	5.765	1.329	1.87	10.87				
		BLUPs	5.543	1.179	1.91	10.82				
	25	2014	6.321	1.987	2.34	11.42	**	**	**	52.12
		2015	6.453	1.869	2.01	11.52				
		BLUPs	6.389	1.141	2.21	11.49				
	35	2014	5.879	2.012	2.12	10.56	**	**	**	50.26
		2015	6.213	1.897	2.17	10.87				
		BLUPs	6.102	1.768	2.15	10.76				
					g					
GY		2014	320.73	57.25	158.75	490.90	**	**	**	64.89
		2015	301.71	72.10	147.30	502.40				
		BLUPs	347.72	91.77	151.35	491.23				

P_N: Photosynthetic rate; C_i: intercellular CO_2 concentration; g_s: stomatal conductance; T_r: transpiration rate; GY: grain yield; DAP: days after pollination; G: genotype; E: environment; G×E: Genotype × Environment; h^2: broad-sense heritability; BLUPs: best linear unbiased predictions.
**Significant at the 1% probability level.

DISCUSSION

Understanding the genetic mechanism of maize photosynthetic parameters and GY may provide a new approach for maize improvement. Identification of QTLs for photosynthetic parameters is very important for elucidation of their genetic basis and facilitation of MAS in maize genetic improvement programs. In the present study, the photosynthetic parameters were diverse in the studied population, and the heritability values of P_N at 15, 25 and 35 DAP were relatively low (Table 2), consistent with results reported for soybean (Yin et al., 2010). This result indicates that photosynthetic parameters are susceptible to environmental factors, thereby hindering the improvement of these traits using conventional breeding programs. Therefore, further studies should be conducted to dissect the precise cause of controlling photosynthetic parameters for maize in our study.

Population stratification in mapping panels can cause spurious marker-trait associations (Hao et al., 2015). To account for population structure in association analysis, MLM-based (Q+K) association mapping has been found

Table 3. Correlation coefficients among photosynthetic parameters and grain yield at 15 (T1), 25 (T2) and 35 d (T3) after pollination (DAP).

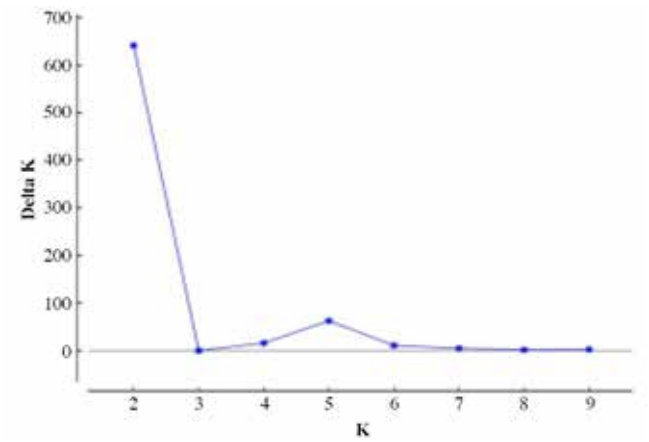
T1	C_i	g_s	T_r	GY
P_N	0.212*	0.699**	0.463**	0.589**
C_i		0.392**	0.545**	0.512**
g_s			0.574**	0.535**
T_r				0.324**
T2	C_i	g_s	T_r	GY
P_N	0.201*	0.817**	0.523**	0.646**
C_i		0.478**	0.556**	0.478**
g_s			0.523**	0.642**
T_r				0.339**
T3	C_i	g_s	T_r	GY
P_N	0.125*	0.705**	0.452**	0.567**
C_i		0.401**	0.499**	0.445**
g_s			0.512**	0.548**
T_r				0.341**

P_N: Photosynthetic rate; C_i: intercellular CO_2 concentration; g_s: stomatal conductance; T_r: transpiration rate; GY: grain yield; DAP: days after pollination.
*, **Significant at the 0.05 and 0.01 probability levels, respectively.

to be well suited in our study, which greatly reduced false positives (Hao et al., 2015). In this study, 260 maize inbred lines were classified into subpopulations that were generally consistent with their known pedigrees. The MLM model applied in our study has been successfully used in other studies to account for population structure (Zhao et al., 2007; Hao et al., 2015).

Photosynthetic parameters are complex traits with a dynamic character that is regulated by different physiological activities. In maize, grain filling is a critical and dynamic process that determines grain yield (Zhang et al., 2013). In maize, leaf photosynthesis is a major source of carbohydrates during grain filling (Liu et al., 2011). Consequently, an understanding of the genetic basis of photosynthetic parameters during the grain-filling process is needed. In the present study, 23 SNPs in the BLUP data set were found to be associated with studied photosynthetic parameters and yield in 2 yr (Table 4). Most SNPs associated with photosynthetic parameters were detected only at a specific developmental stage, and only three SNPs, SYN35048, PZE-102116144 and PZE-101152541, were identified at two or three developmental stages. For example, SYN35048 associated with g_s was simultaneously detected at 15, 25 and 35 DAP. These results reveal that most genes (or QTLs) controlling maize photosynthetic parameters are activated at different developmental stages, with only a handful of genes (or QTLs) stably possessing the most promising association at different developmental stages. The stable genes (or QTLs) expressed at different developmental stages can be used for marker-trait assisted selection. In the present study, the three SNPs identified at different development stages may be the most promising marker.

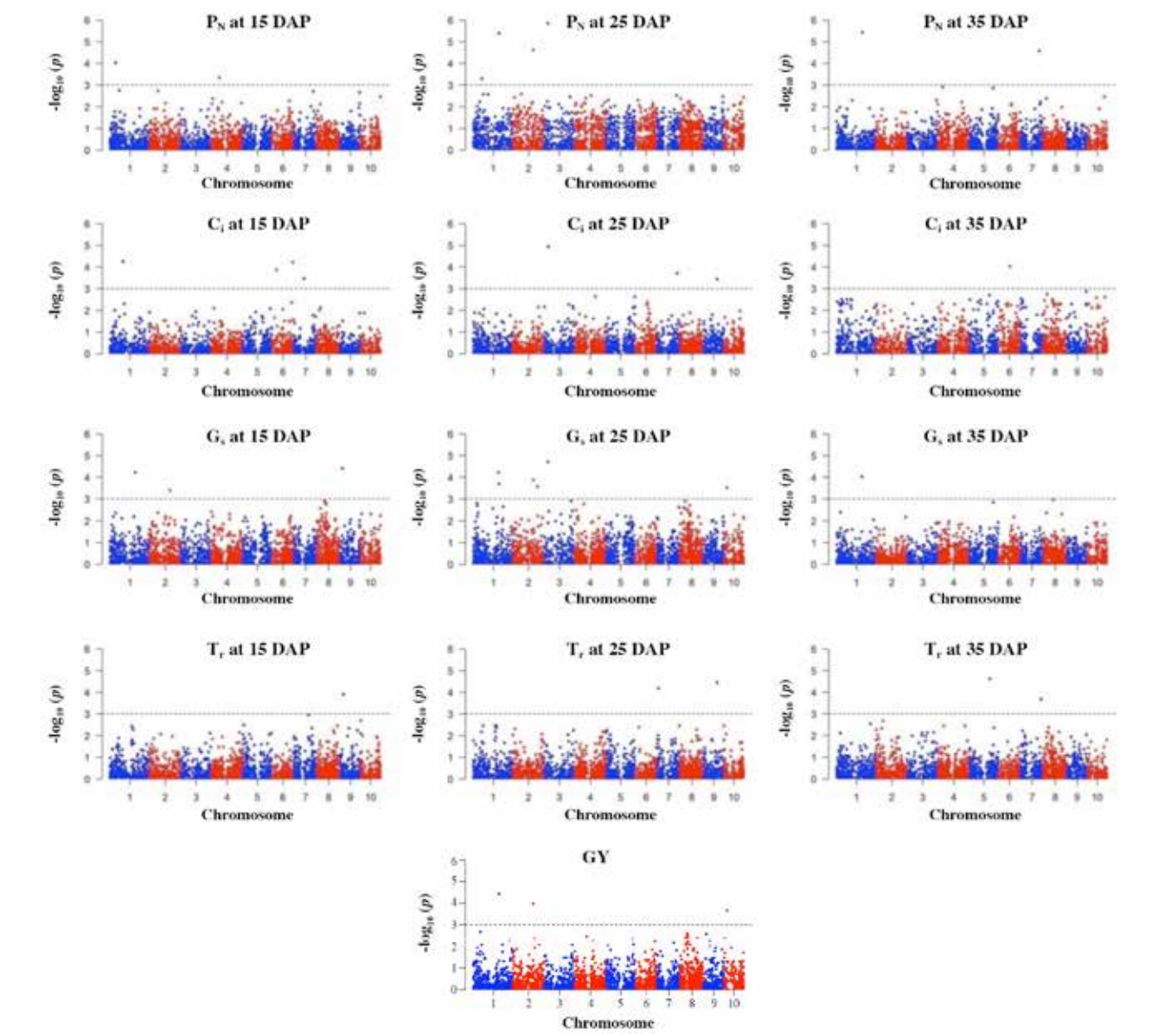
Figure 1. Number of subgroups (Δk) values calculated in a population structure analysis of 260 maize inbred lines using STRUCTURE.



Previous studies of crop plants have revealed that QTLs for closely correlated traits generally map to the same or a nearby genomic region (Yang et al., 2007; Yin et al., 2012). Similar results were observed in this study. Four SNPs were significantly associated with two or more traits, a finding also supported by the significant correlation among the studied traits (Table 4). For example, PZE-101152541 and PZE-102116144 were co-associated with P_N and g_s at 25 DAP, and there were significant correlations between P_N and g_s at 25 DAP. Some putative genes for controlling these traits might be located in or near these co-associated regions, a situation that could facilitate the pyramiding of elite alleles for different traits in maize MAS schemes (Hao et al., 2012a). PZE-101152541 and PZE-102116144 were co-associated with GY and P_N in 2 yr, indicating the possible existence of a single gene with pleiotropic effects that is tightly linked with multiple genes.

Among the 14 associated SNPs, nine were located inside genes, the others were intergenic. According to the maize gene annotation database at MaizeGDB (http://www.maizegdb.org), the putative genes of GRMZM2G317770 on chromosome 1 and GRMZM2G002227 on chromosome 9 were the most potential candidate genes. The candidate genes of GRMZM2G317770 and GRMZM2G002227 encode protein kinases, which were related to the photosynthetic light reaction in the previous studies in Arabidopsis thaliana (Schliebner et al., 2008). However, other associated SNPs were not involving some putative candidate genes; the causal genes might exist within the genomic regions in LD where associated-markers located (Hao et al., 2012b). In the further studies, choice of a larger population size with more diverse genetic background, and use of more markers (especially markers from key genes of photosynthetic metabolic networks), will directly improve the scanning power and the accuracy of detection, and capture the key loci and/or candidate genes underlying the photosynthesis and yield in maize (Yan et al., 2011; Hao et al., 2012b).

Figure 2. Manhattan plots of the results of a genome-wide association study for photosynthetic parameters and grain yield at different developmental stages over 2 years.



P_N: Photosynthetic rate; C_i: intercellular CO_2 concentration; g_s: stomatal conductance; T_r: transpiration rate; GY: grain yield; DAP: days after pollination.

CONCLUSIONS

Our results demonstrate that maize photosynthetic parameters are significantly or highly significantly correlated with grain yield (GY) at different developmental stages. Fourteen single-nucleotide polymorphisms (SNPs) associated with photosynthetic parameters and GY were detected in two years as well as over years. PZE-102116144 and SYN35048 associated with stomatal conductance (g_s), and PZE-101152541 associated with photosynthetic rate (P_N), were identified at different developmental stages. PZE-101152541 was significantly associated with GY and P_N (at 25 and 35 DAP), PZE-102116144 with GY, g_s (at 15 and 25 DAP), and P_N (at 25 DAP), PZE-109061997 with intercellular CO_2 concentration (at 25 DAP) and transpiration rate (at 25 DAP), PZE-110019199 with g_s (at 25 DAP) and GY. Based on functional annotations, two genes (GRMZM2G317770 and GRMZM2G002227) were considered as potential candidate genes for the identified

Table 4. Single-nucleotide polymorphisms (SNP) markers with significant association signals for all five traits in 2 years and best linear unbiased predictions (BLUP).

Trait	DAP	Marker	Chr.	Position	-log*P*			Candidate genes	Annotation
					E1	E2	BLUPs		
P_N	15	PZE-104035252	4	44957920	3.25	5.13	4.62	GRMZM2G097395	Major facilitator superfamily protein
	25	PZE-102116144	2	153865305	3.27	4.09	4.62	GRMZM2G070284	R-interacting factor 1
	25	PZE-101152541	1	195912125	4.12	3.89	5.43	GRMZM2G317770	Protein kinase
	25	PZE-101080365	1	65046906	3.65	4.37	3.3	Intergenic	
	35	PZE-101152541	1	195912125	3.89	3.76	5.43	GRMZM2G317770	Protein kinase
g_s	15	SYN35048	1	192405003	3.53	3.53	4.23	GRMZM2G096806	Pentatricopeptide repeat (PPR) superfamily protein
	15	PZE-109016787	9	16900615	3.22	3.53	4.41	GRMZM2G002227	Protein kinase
	15	PZE-102116144	2	153865305	3.27	4.09	3.39	GRMZM2G070284	R-Interacting factor 1
	25	SYN35048	1	192405003	3.75	3.78	4.03	GRMZM2G096806	PPR superfamily protein
	25	PZE-110019199	10	23214551	3.38	4.31	3.52	GRMZM2G036427	Universal stress protein
	25	PZE-102137972	2	186789026	3.76	4.01	3.57	GRMZM2G001977	Gibberellin receptor GID1L2
	25	PZE-102116144	2	153865305	3.21	3.57	3.88	GRMZM2G070284	R-Interacting factor 1
	35	SYN35048	1	192405003	3.75	3.78	4.03	GRMZM2G096806	PPR superfamily protein
C_i	15	PZE-107040041	7	78361592	3.45	5.23	3.47	GRMZM2G110548	Enhancer of polycomb-like transcription factor protein
	15	PZE-106015862	6	37901146	4.78	4.13	3.87	GRMZM2G069162	Ankyrin repeat domain-containing protein 28
	25	PZE-109061997	9	103853485	3.98	4.16	3.45	Intergenic	
	35	PZE-106041140	6	89686818	4.23	4.19	4.43	GRMZM2G044398	Zinc finger C-x8-C-x5-C-x3-H type family protein
T_r	25	PZE-109061997	9	103853485	5.12	8.23	4.45	Intergenic	
	35	PZE-107104221	7	157194519	4.39	3.98	3.68	Intergenic	
	35	PZE-105102393	5	154318857	4.23	4.99	4.62	Intergenic	
GY		PZE-110019199	10	23214551	4.36	3.73	3.66	GRMZM2G036427	Universal stress protein
GY		PZE-102116144	2	153865305	3.27	4.09	3.98	GRMZM2G070284	R-Interacting factor 1
GY		PZE-101152541	1	195912125	4.12	3.89	4.43	GRMZM2G317770	Protein kinase

P_N: Photosynthetic rate; C_i: intercellular CO_2 concentration; g_s: stomatal conductance; T_r: transpiration rate; GY: grain yield; DAP: days after pollination; Chr.: maize chromosome number; ns: nonsignificant. -Log *P*: Significant at -log $P \geq 3.00$ ($P \leq 0.001$); E1: Environment in 2014; E2: Environment in 2015.

SNPs (PZE-101152541 and PZE-109016787). The SNPs and candidate genes identified in this study might provide instrumental information for understanding the genetic mechanism of maize photosynthetic parameters and yield.

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