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Keywords
Zea mays L., plant height, phenotypic-selected introgression families, donor chromosomal segments, genetic variation

Disciplines
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Genetic architecture of plant height in maize phenotype-selected introgression families

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Abstract

This study aimed at developing, characterizing and evaluating two maize phenotypic-selected introgression libraries for a collection of dominant plant height (PHT)-increasing alleles by introgressing donor chromosome segments (DCS) from Germplasm Enhancement of Maize (GEM) accessions into elite inbred lines: PHB47 and PHZ51. Different backcross generations (BC_1-BC_4) were developed and the tallest 23 phenotype-selected introgression families (PIFs) from each introgression library (PHB47 or PHZ51) were selected for single nucleotide polymorphism genotyping to localize DCS underlying PHT. The result shows that most PIFs carrying DCS were significantly ($\alpha = 0.01$) taller than the respective recurrent parent. In addition, they contained larger donor genome proportions than expected in the absence of selection or random mating across all BC generations. The DCS were distributed over the whole genome, indicating a complex genetic nature underlying PHT. We conclude that our PIFs are enriched for favourable PHT-increasing alleles. These two libraries offer opportunities for future PHT gene isolation and allele characterization and for breeding purposes, such as novel cultivars for biofuel production.

Key words: *Zea mays* L. — plant height — phenotypic-selected introgression families — donor chromosomal segments — genetic variation

Plant height (PHT) is an important agronomic trait that is associated with grain yield. PHT exhibits a strong correlation with relative yield (grain production; $r^2 = 0.67$) and moderate, but highly significant, correlations with ear height and the number of leaves per plant ($r^2 = 0.88$) (Sibov et al. 2003, Aastveit and Aastveit 1993, Lima et al. 2006), illustrating the importance of PHT as fitness trait in the agricultural context. Currently, the focus is on high grain yield due to increasing plant population densities of moderate-sized, lodging-tolerant cultivars. However, an increased demand for lignocellulosic biomass for biofuels has led to a paradigm shift in plant architecture (Fernandez et al. 2009, Schwietzke et al. 2009, Hu and Lübberstedt 2015). As tall plants can produce more vegetative tissue, there is a positive and strong correlation between PHT and biomass in maize ($r^2 > 0.73$) (Lübberstedt et al. 1997). Moreover, for dual-purpose crops for which grain is still of high value, increased stover biomass is an added value. In the Midwestern and Central Plains regions, 100 million metric tons of maize stover can be produced for biofuel production (Graham et al. 2007). Moreover, selection for taller plant stature increases the yield capacity under water-limiting conditions, as PHT shows a positive and high correlation with yield under drought (Edmeades et al. 1999, He et al. 2010, Sayed 2011). Although tall plants are more susceptible to lodging, selection for traits that enhance the plant stability (Fernandez et al. 2009) can avoid the lodging problem.

However, continued selection of moderate PHT within elite germplasm may cause the reduction in genetic diversity (Hawkes 1977, Goodman 1999). Introgression library may act as a potential method to add extra genetic diversity – as introgression of unadapted exotic germplasm into elite breeding materials can reduce genetic vulnerability and broaden the variability available for selection (Tanksley and Nelson 1996). Despite poor agronomic performance, exotic germplasm has repeatedly shown to contain favourable genes (Frey et al. 1984, Elgin and Miller 1989, DeVicente and Tanksley 1993), which provides novel opportunities to maximize the genetic diversity and produce new cultivars.

Previously, introgression libraries were proposed as powerful tool to disrupt elite genetic background as little as possible by introducing a limited number of exotic segments by backcrossing (Eshed et al. 1992). Introgression involves the repeated backcrossing with the elite line as recurrent parent (RP), thereby minimizing the negative side effects attributable to genetic interactions between exotic and elite genomes. Because Eshed and Zamir (1994) constructed the first introgression line library in tomato carrying single wild tomato (*Lycopersicon pennellii*) chromosomal segments in the genetic background of cultivated tomato (*Lycopersicon esculentum*), introgression libraries were successfully used in other crop species. Beneficial effects of alleles from exotic germplasm have been illustrated for PHT (Miedaner et al. 2011), various agronomic traits (Huang et al. 2003, Pillen et al. 2003, Septiningsih et al. 2003, Tian et al. 2006, Tan et al. 2007, Von Korff et al. 2008), quality-related traits (Matus et al. 2003, Pillen et al. 2003, Kunert et al. 2007) and biotic and abiotic stress tolerance (Von Korff et al. 2005, Siangliw et al. 2007, Gu et al. 2012).

Introgression libraries ideally consist of a set of homozygous, near-isogenic lines, each carrying a single marker-defined donor chromosome segment (DCS) in the genetic background of an elite line (Zamir 2001), which facilitates the characterization and utilization of exotic segments by plant breeders (Eshed et al. 1996). These DCS are introduced by marker-assisted backcrossing (MABC), and all DCS in the introgression library jointly represent the total donor genome. So far, introgression libraries were developed for maize (Ribaut and Ragot 2007, Szalma et al.

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2007) and other economically important crop species including tomato (Eshed et al. 1992, Eshed and Zamir 1994), *Brassica napus* (Howell et al. 1996, Ramsay et al. 1996), rice (Lin et al. 1998), barley (Matus et al. 2003, Von Korff et al. 2004, 2008), soybean (Concibido et al. 2003), lettuce (Jeuken and Lindhout 2004), melon (Eduardo et al. 2005), wheat (Liu et al. 2006) and rye (Miedaner et al. 2011).

However, traditional establishment of introgression libraries using MABC is time-consuming, laborious (Zamir and Eshed 1998) and requires extensive genotyping process, although significant improvements have been made such as how to more efficiently use the high-throughput marker selection system and different crossing schemes to shorten backcross (BC) generations (Herzog et al. 2014). Moreover, for traditional introgression libraries, only two parents will be involved to construct introgression libraries, which may limit their genetic diversity. In this manuscript, we address the idea of phenotypic-selected introgression library to accumulate PHT-increasing genetic variation. Phenotype-selected introgression families (PIFs) still need several generations of backcrossing, but without marker selection or genotyping, this method is more cost-effective. Moreover, it maximizes the genetic diversity for the target trait by using more donor accessions. In summary, it has three major differences compared to traditional introgression line libraries: (i) PIFs for PHT do not necessarily cover all regions of the genome, but accumulate DCS affecting PHT within an elite/adapted genetic background; (ii) DCS originate from multiple donors; and (iii) only phenotypic selection was employed. PIF libraries enable broader and more targeted (trait-specific) use of exotic germplasm. Multiple exotic donors have the advantage over a single donor to provide a greater variety of chromosomal segments in any genomic location (Holland 2004, Zeng et al. 2007, Ladizinsky 2012). In each generation, only tall plants were selected and backcrossed, which leads to an enrichment of chromosome segments and genes with impact on increasing the PHT in isogenic background.

The objectives of our study were to (i) develop two maize PIF libraries using the Germplasm Enhancement of Maize (GEM) accessions as a source of DCS into the genetic background of PHB47 and PHZ51 elite maize lines, (ii) investigate the *per se* performance of introgression families resulting from BC individuals selected for PHT to study the potential utility of establishing trait-based introgression libraries using PHT as well-characterized quantitative genetic model character, and (iii) identify, localize and characterize DCS of individual PIFs to investigate the genetic architecture underlying maize PHT for future maize PHT improvement programmes using markers.

Materials and Methods

Plant materials: Two libraries of introgression families were developed. Exotic donor parents as source materials were obtained from the GEM project (Salhuana and Pollak 2006), which aims at developing adapted germplasm from unadapted sources from the Latin American Maize Project (LAMP) (Salhuana and Pollak 2006) that can be utilized in the major corn-growing areas of the United States. These exotic maize accessions were crossed with expired PVP (Plant Variety Protection) lines PHZ51 and PHB47 as RPs, resulting in two libraries based on either PHB47 or PHZ51 genetic background as PIFB47 or PIFZ51. PHB47 belongs to the Iowa stiff stalk (BSSS) gene pool, while PHZ51 is belonging to the non-stiff-stalk gene pool. As illustrated in (Fig. 1), for each donor accession, bulked pollen from donor individuals was used to cross with RP (PHB47 or PHZ51) to produce F_1 seed. Five random F_1 individuals were backcrossed with the RP plants to produce five

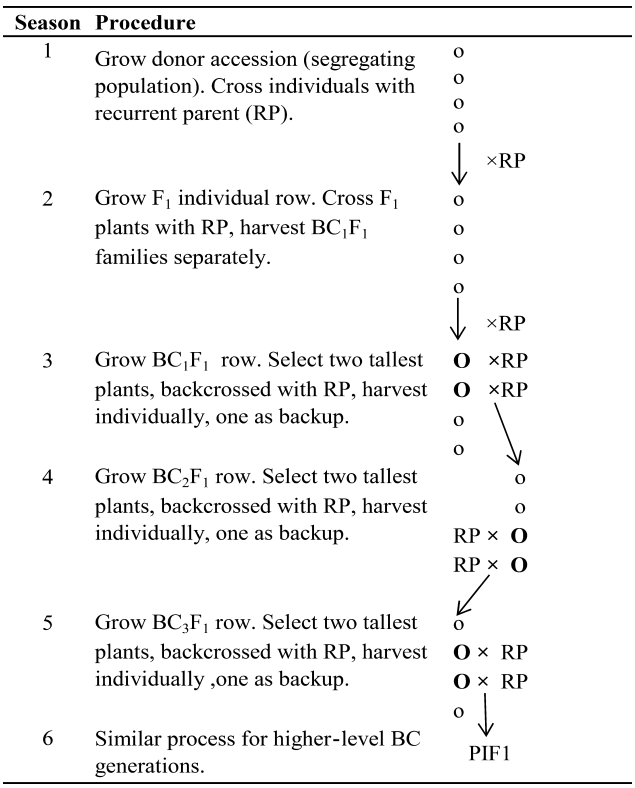

Fig. 1: Breeding scheme to generate one PIF/the donor accession is a segregating exotic source population. Bulked pollen from the donor accession was used to pollinate the recurrent parent (RP). As the F_1 is segregating, random F_1 plants were used to backcross (BC) with RP. BC_1F_1 families were harvested separately, and for selected BC_1F_1 families (similar flowering time with RP), the two tallest plants (O) were selected and backcrossed with RP, one to be planted next season and one as a backup. This process was repeated in following BC generations. The most advanced PIF per donor × background combination in terms of BC generations was used for further phenotypic and genotypic characterization in this study

BC_1F_1 families (BC_1F_1 ears were kept separate as BC_1 families). Among the five BC_1F_1 families, only the families flowering about the same time (± 5 days) as the RP were advanced. On average, one to three BC_1F_1 families were selected for each donor accession. The two tallest plants in each selected BC_1F_1 family were picked and backcrossed to the RP. Seed from the tallest plant was used to produce a $BC_{1:2}$ family (Fig. 1). $BC_{1:2}$ seed of the other selected plant was kept as a backup. This procedure was repeated to produce advanced $BC_{2:3}$ or $BC_{3:4}$ families with the goal to produce one advanced BC family per accession x background combination. In total, 58 and 62 exotic accessions were used to establish the PIFB47 and PIFZ51 libraries, respectively, including 17 accessions commonly used for both PIF libraries (Table 1). For PIFZ51, there are 2 BC_1 families, 79 BC_2 families, 42 BC_3 families and 3 BC_4 families. For PIFB47, we developed 4 BC_1 families, 39 BC_2 families, 53 BC_3 families and 4 BC_4 families.

Field evaluation: In the 2010 and 2011 growing seasons, all BC families (126 from PIFZ51, 100 from PIFB47 – some donor accessions produce more than one PIF) were planted at two locations: Agronomy farm, Iowa State University, Ames, IA (Ames), and Plant Introduction Station, IA (Ames), in 2-m plots. Twenty-five plants were planted per plot with 0.75 m between plots. BC families (226 in total) and the two parental inbred lines (PHB47 and PHZ51), included as checks, were planted in unreplicated field trials in 2010 and in a randomized complete block (RCBD) design in two replicated field trials in 2011 at two locations. Days to tasselling was recorded, when at least 50% of the plants per plot showed emerged tassels. Two weeks after tasselling, the

Table 1: Exotic accessions used for establishing PIFs

No.	PIFB47	PIFZ51
1	ABANERO CUN342	Amarillo Huancabamba – PIU 17B
2	ALTIPLANO BOV903	ANCASH 291
3	Amarillo Huancabamba – PIU 38B B	ANDELA ECU699 ICA
4	ANCASHINO ANC102	Araguito – VEN 678
5	ANDAQUI CAQ307 FT	Arequipeno – ARQ 1 B
6	ANDELA ECU 699	Argentino – BOV 920
7	ARAGUITO VEN678	Arizona – LIB 16 B
8	Arequipeno – ARQ 1 B)	AvatiPichingaIhu – BR 2830
9	AvatiMorotiGuapi – PAG 139 B	Blanco Ayabaca – PIU119 B
10	Blanco Ayabaca – PIU119 B	Blanco Blandito – ECU 523 B
11	Blanco Blandito – ECU 523 B	BOFO DGO123
12	BR106:S99n99n	BR105:N99v99v
13	CABUYA SAN316 FTC	Calchaqui white flint – ARG 2420
14	CAMELIA CHI411	CANGUIL GR. ECU447
15	CANDELA ECU531	Canilla – VEN693 B
16	Canilla – VEN 693 B	Capiorosado – ARG 460
17	CatetoNortista – GIN I B	Chandelle – VEN 409
18	Chillo – ECU 458 B	Chillo – ECU 411
19	Chirimito – VEN 703 B	Chirimito – VEN 703 B
20	Comiteco – GUA 515 B	Clavito – ECU884 B
21	CON NORT ZAC161	Clavo – NAR 329
22	CON PUNT CUZ13	Comiteco – GUA 515 B
23	CONICO [PUE116]{CIMYT	CON PN CUZ13
24	Costeño – Antioquia 394 B	Coroicoblanco – BOV 406
25	COSTENO [VEN775]{ICA}	Cravoriograndense – RGS VII B
26	Cravoriograndense – RGS VII B	Cristal – SP X B
27	Cuzco gigante	Cubanodentado – BOV 585
28	DULCILLO DE NO SON57	Culli – ARG 471
29	DZ B GUA131	CUZCO CUZ217
30	EARLY CARIBBEAN MAR9	Cuzco gigante
31	ELOTES OCCIDENT NAY29	DE T CAL. GUA159
32	GORDO [CHH131]{CIMYT}	DULCILLO DEL NO SON57
33	IMA 66 Ames8554	ELOTES OCCIDENT DGO236
34	KARAPAMPA BOV978	ELOTES OCCIDENT NAY29
35	MISHCA ECU321	Huachano – LIM 43 B
36	MONTANA NAR625	Huarmaca – PIU 72
37	MORADO BOV567	Huevito – VEN 396 B
38	MOROC APC67	Jora – ANC 1 B
39	NANO # BOV1032	KCELO BOV948
40	OLITA DGO159	MISHC ECU321
41	OLOTON [GUA383]{CIMYT}	Morchon – ECU454B
42	ONAVENO SON24	Morocho – APC 77
43	PARO CUZ76	MOROCHO APC67
44	PATILLO GRANDE BOV649	MOROTI PEI
45	PERLA ANC23	Negrita de tierrafria – GUA 522
46	PISAN BOV344	NINUELO BOV1088
47	POJ CHICO BOV800	Oke – ARG 539
48	PUE116 {CIMYT}	OLLO CUN424 ICA
49	Puya – MAG 355 B	Patillo – ECU 417
50	Rabo De Zorro – ANC 325 B	Piricinco – SM 8 B
51	Racimo de Uva – ECU 517 B	PISAN BOV344
52	SABN CUN367	Rabo De Zorro – ANC 325 B
53	SAN MARCENO # [GUA724]{INIA}	Racimo de Uva – ECU 517 B
54	Semi dentadopaulista – PAG I B	Rienda – CAJ 80 B
55	TEPE CHS225	SARCO ANC184
56	Tepecintle – GUA 65 B	SG HUANCAV JUN164
57	Vandeño – GRO 96 B	Sin Clasificación – CAU454
58	YUCATAN TOL389 ICA	TuxpeñoNorteño – CHH287
59		UCHIM ECU681
60		Uchuquilla – BOV318
61		Vandeño – GRO 96 B
62		YUNGUENO BOV362

PIFs, phenotype-selected introgression families.

plant with a median PHT within each plot was selected and measured; PHT was recorded from the ground to the top of the tassel. The average of PHT across locations and replications was calculated to represent the PHT performance for each family.

DNA extraction and genotyping: Leaf samples of the selected PIFs were collected for DNA extraction after tasselling. The DNA of the BC families was extracted from the leaf materials according to the protocol described by Saghai-Maroof et al. (1984). The tallest 23 families from each introgression library (PIFB47 and PIFZ51) were selected (=subset of 'selected introgression libraries'), named as PIFB47S and PIFZ51S for genotyping with 207 mapped single nucleotide polymorphism markers (SNP) [based on ISU SNP map (Liu et al. 2010)]. The markers are distributed throughout the genome using the SequenomMassARRAY® System at the Genomic Technologies Facility (http://www.plantgenomics.iastate.edu/) at ISU. Estimation of recurrent and donor parent genome percentages for each BC family was carried out by considering alleles differing from those of the RP as donor introgressions, while marker alleles identical to the RP alleles were classified as RP alleles.

Fifty BC_1 doubled haploid (DH) lines developed from the GEM project were used for comparison as unselected library in this study (Brenner et al. 2012). Briefly, GEM-DH lines were generated by introgressing the same set of exotic maize races into the genetic background of PHZ51 and PHB47, respectively, to produce GEM-DH-B47 and GEM-DH-Z51 lines. The difference between GEM-DH lines and PIFs is that instead of repeated backcrossing with phenotypic selection, DH lines were generated from BC_1 individuals without any phenotypic selection. The 50 GEM-DH lines were genotyped with 199 SNP markers with a subset of 139 SNP markers overlapping with the 207 used for genotyping the PIFs.

Statistical analyses: Phenotypic data were subjected to analyses of variance based on a RCBD. Entry means were used in the combined analyses across locations. Only data collected in 2011 were used in the combined analyses. Location was considered as a fixed factor, whereas genotypes were considered as a random factor. The variance components for genotypes (σ_g^2), genotype × environment variance ($\sigma_{g\times e}^2$) and error variance σ_e^2 were estimated according to Hallauer and Miranda (1988). Thereafter, h_s^2 was calculated on an entry mean basis using the following formula:

$$h_s^2 = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_{g\times e}^2/n + \sigma_e^2/rn},$$

where r is the number of replications within each location and n is the number of locations.

After excluding monomorphic markers and markers polymorphic for only one of 23 families in either PIFB47S or PIFZ51S, 71 and 84 markers were used for further analyses in PIFB47S and PIFZ51S. Graphical genotypes were obtained using the software package Graphical GenoTypes (GGT) (Van Berloo 2008). Genotyped BC families were characterized for the percentage of donor parent compared to RP alleles. Based on genotype representation with GGT, the number of DCS as well as the percentage of donor genome was determined. For the calculation of segment length and genome ratios, the half-intervals flanking a marker locus were considered to be of the same genotype as implemented by the GGT software. For a few missing marker data points, because the flanking markers are RP genotype, BC families with missing data were assumed to have the genotype of the RP for this particular marker. We define hot spots in this manuscript as meta-analyses results of previous published PHT QTL regions from Wang et al. (2006).

Results

Phenotypic characterization

Average PHT was 220.7 and 237.3 cm for the RPs PHZ51 and PHB47, respectively (Fig. 2). As expected, the means of most families from PIFB47 and PIFZ51 exceeded their respective RPs. For PIFB47 and PIFZ51, PHT ranged from 215.5 to

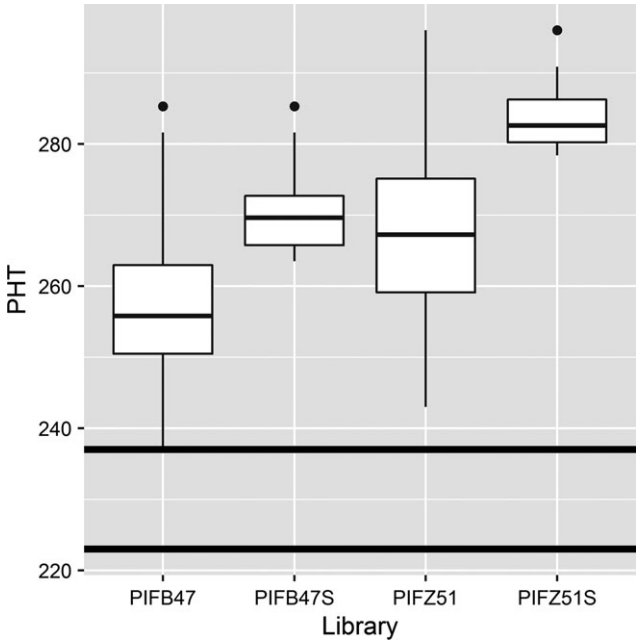

Fig. 2: Ranges of plant height of 126 (PIFZ51) and 100 (PIFB47) PIFs and the 23 selected phenotype-selected introgression families (PIFB47S and PIFZ51S)/the box defined between the first quartile (Q1) and the third quartile (Q3) represent 50% of the backcross family values. The line in the box indicates the median value. The two horizontal rows represent the means of the two recurrent parents, the upper one represents for PHB47, and the bottom one represents for PHZ51

296.0 cm and from 231.1 to 285.3 cm, respectively; 55% of PIFB47 families and 98% of PIFZ51 families were significantly taller than their corresponding RP ($\alpha = 0.01$). In contrast, only 10% and 9% of GEM-DH-B47 and GEM-DH-Z51 lines were significantly taller than their corresponding RP – most GEM-DH lines showed no difference in PHT, supporting the efficiency of phenotypic selection and indicating that the PHT-increasing donor alleles have been successfully accumulated in PIFB47 and PIFZ51 introgression libraries. DTT (Days to Tasseling) means were close to RPs in both libraries. All BC families reached tasselling within five days for PIFB47 and within seven days for PIFZ51, except for one family (PHZ51 < 2 > /(BOFO DGO123/PHZ51)/PHZ51#005)#001 > 01/Z51:1050)-B) with 10 days delayed tasselling compared to PHZ51. In contrast, most unselected GEM-DH lines show later flowering time, supporting the efficiency of phenotypic selection in preparing our PIF introgression libraries. Pearson's phenotypic correlations between PHT and DTT were low, although significant ($r = 0.19$**). When correlations were calculated separately for each library, low significant positive correlations were obtained within the PHB47 library ($r = 0.21$**). No significant correlation was found within the PHZ51 library between PHT and DTT.

Variance components for genotype generally exceeded those for genotype × environment (GxE) interactions (Table 2). Nevertheless, GxE interaction components were significant (P = 0.01). Estimates for h^2 were high: h^2 for PHT and DTT were 0.79 and 0.74, respectively.

Genetic characterization of backcross families

Generally, BC families displayed a higher percentage of donor genome than the expected 25%, 12.5%, 6.25% and 3.125% for BC_1, BC_2, BC_3 and BC_4 generations (Table 3 and Table 4). For example, there are on average 21.56% and 11.8% for BC_2 and BC_3 in the PIFZ51S (Table 3) and 16.24% and 11.47% for BC_2 and BC_3 in the PIFB47S (Table 4). The percentage of donor introgressions ranged from 8.4% to 64.6% and from 6.3% to 32.5% for PIFZ51S and PIFB47S, respectively. The number of donor segments ranged from 4 to 19 in PIFZ51S and 4 to 18 in PIFB47S.

Advanced BC generations in both selected introgression libraries (PIFB47S and PIFZ51S) showed an obvious decrease in the number of DCS and the proportion of donor genome per PIF (Table 3 and Table 4). In PIFB47S, the percentage of donor parent decreased from 24.5% (10 segments) in BC_1, 16.3% (seven segments) in BC_2, to 11.5% (six segments) in BC_3 families. In PIFZ51S, the proportion of donor parent decreased from 21.6% (nine segments) in BC_2 to 11.8% (six segments) in BC_3 families. However, one BC_4 family had a 56% donor genome proportion (19 segments), which may be due to random drift or a large linkage block inherited from its original donor. The observed donor genome proportion was greater for PIFs compared to the unselected GEM-DH lines (Table 3 and Table 4). On average, unselected GEM-DH lines had a reduced percentage of donor introgressions (21.3% and 16.5% in GEM-DH-Z51 and GEM-DH-B47 lines, respectively) compared with the expected value of 25.0% in BC_1.

Comparison between the two selected libraries (PIFB47S and PIFZ51S)

Donor chromosome segments are distributed over the whole genome for the two selected libraries (Figs 3 and 4). The maximum frequency of donor introgressions was found on Chromosome 4 in both libraries (21.4% and 33.2% in PIFB47S and PIFZ51S, respectively). Chromosomes 1 (21.2%) and 6 (17.3%) for PIFB47S and Chromosomes 2 (21.8%) and 3 (24.0%) for PIFZ51S also displayed high percentages of donor introgressions. The lowest frequency of donor allele introgressions was observed for Chromosomes 2 (9.3%), 3 (10.4%) and 7 (10.0%)

Table 2: Estimates of variance components, broad-sense heritability h^2 and 95% confidence intervals of h^2 (C.I) for phenotypically selected introgression families (PIFs)

	Variance component					
Trait	Location (σ_l^2)	Genotype (σ_g^2)	Genotype × Environment ($\sigma_{g\times e}^2$)	Error	h^2	C.I. (h^2)
PHT	450.26**	154.54**	31.81**	50.94**	0.79	0.73–0.84
DTT	10.31**	0.74**	0.25**	0.31**	0.74	0.64–0.78

PHT, plant height.
'*' and '**' show the significances at 0.05 and 0.01 of probability level, respectively.

Table 3: Genetic characterization of PIFZ51S: Proportion of donor parent genome and its expected values and the observed number of donor chromosomal segments (DCS)

PIF number	Donor parent	BC generation	Proportion of donor parent		No. of DCS
			Expected %	Observed %	
1	Chandelle – VEN 409	BC_2	12.5	22.5	10
2	Capiorosado – ARG 460	BC_2	12.5	10.1	7
3	Negrita de tierrafria – GUA 522	BC_2	12.5	17.3	11
4	Blanco Blandito – ECU 523 B	BC_2	12.5	35.3	15
5	Rabo De Zorro – ANC 325 B	BC_2	12.5	12.7	8
6	Huevito – VEN 396 B	BC_2	12.5	21.2	10
7	Piricinco – SM 8 B	BC_2	12.5	23.4	10
8	Culli – ARG 471	BC_2	12.5	13.4	9
9	Huachano – LIM 43 B	BC_2	12.5	31.8	12
10	BOFO DGO123	BC_4	3.125	56	19
11	Racimo de Uva – ECU 517 B	BC_2	12.5	64.6	10
12	Chillo – ECU 458	BC_2	12.5	25.4	11
13	BOFO DGO123	BC_4	3.125	10.9	7
14	BOFO DGO123	BC_3	6.25	14.3	7
15	Sin Clasificación – CAU454	BC_2	12.5	18.1	9
16	Jora – ANC 1 B	BC_2	12.5	14.3	8
17	Patillo – ECU 417	BC_2	12.5	15.5	10
18	Comiteco – GUA 515 B	BC_2	12.5	20.4	11
19	BOFO DGO123	BC_3	6.25	12.4	7
20	Arizona – LIB 16 B	BC_3	6.25	8.7	4
21	Chillo – ECU 411	BC_2	12.5	19.6	10
22	Cuzco gigante	BC_2	12.5	8.4	4
23	Canilla – VEN693 B	BC_2	12.5	14.1	11
	Avg. PIFZ51S (23 ILs)		10.8	21.3	9.6
	Avg. GEM-DH-Z51 lines (20 ILs)	BC_1-DH	25	16.5	8.95

BC, backcross; DH, doubled haploid; GEM, Germplasm Enhancement of Maize; PIF, phenotype-selected introgression families.

Table 4: Genetic characterization of PIFB47S: Proportion of donor parent genome and its expected values and the observed number of donor chromosomal segments (DCS)

PIF number	Donor parent	BC generation	Proportion of donor parent		No. of DCS
			Expected%	Observed%	
1	Blanco Ayabaca – PIU119 B	BC_2	12.5	10	4
2	ELOTES OCCIDENT NAY29	BC_3	6.25	32.5	18
3	Racimo de Uva – ECU 517 B	BC_2	12.5	9	9
4	Vandeño – GRO 96 B	BC_2	12.5	20.1	13
5	Amarillo Huancabamba – PIU 38B B	BC_2	12.5	18.2	12
6	PHB47/Chillo – ECU 458	BC_2	12.5	11.6	8
7	Comiteco – GUA 515 B	BC_2	12.5	10.5	5
8	Rabo De Zorro – ANC 325 B	BC_2	12.5	18.7	10
9	CatetoNortista – GIN I B	BC_2	12.5	29.8	11
10	B47NANO # BOV1032/PHB47	BC_3	6.25	5.4	5
11	Semi dentadopaulista – PAG I B	BC_2	12.5	19.5	9
12	CON PUNT CUZ13	BC_3	6.25	6.7	4
13	GORDO [CHH131]{CIMYT})-B-B-SIB-023-001-001)#001	BC_2	12.5	8.6	5
14	CAMELIA CHI411	BC_3	6.25	10.3	8
15	Costeño – Antioquia 394 B	BC_2	12.5	22.2	11
16	B47/COSTENO [VEN775]{ICA})-B-B-SIB-018-001-001)#001	BC_3	6.25	12.2	8
17	Blanco Ayabaca – PIU119 B	BC_2	12.5	15.6	9
18	Cuzco gigante	BC_1	25	24.5	12
19	Cuzco gigante	BC_2	12.5	11.5	5
20	Blanco Ayabaca – PIU119 B	BC_3	6.25	6.9	4
21	Canilla – VEN 693 B	BC_2	12.5	20.4	7
22	TEPE CHS225	BC_3	6.25	6.3	4
23	Blanco Blandito – ECU 523 B	BC_2	12.5	18	11
	Avg. PIFB47S (23 ILs)	Mixed	11.1	15.2	8.3
	Avg. GEM-DH-B47 lines (20 ILs)	BC_1-DH	25	15.5	6.8

BC, backcross; DH, doubled haploid; GEM, Germplasm Enhancement of Maize; PIF, phenotype-selected introgression families.

in PIFB47S and for Chromosomes 6 (8.8%) and 7 (12.4%) in PIFZ51S.

In PIFZ51S, seven genomic regions displayed the highest donor genome percentages, which suggests that in these regions the RP alleles tend to be replaced by donor segments during the phenotypic selection process. The highest genome contribution (45%) was detected within the intervals ranging from 16.0 to 29.7 cM on Chromosome 4, followed by nine regions with 35%

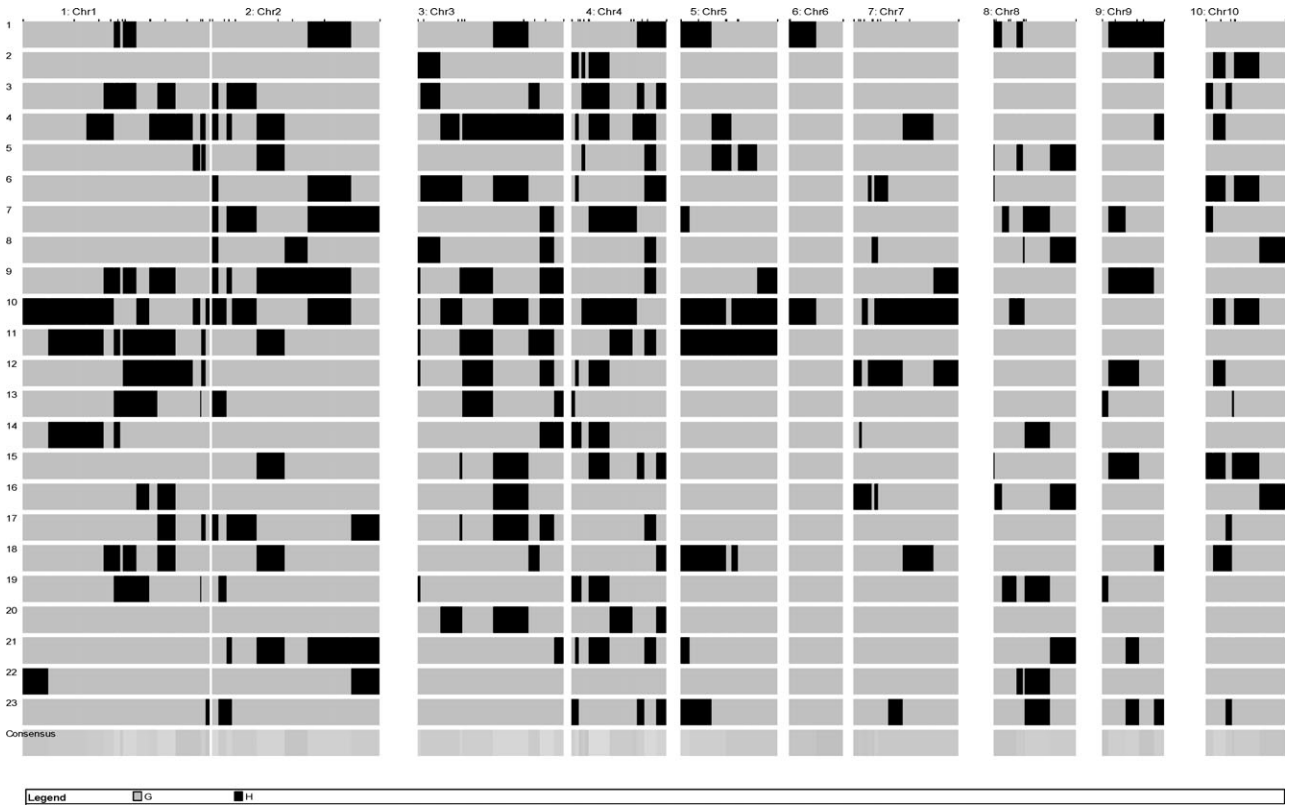

Fig. 3: Genotypic characterization of the top 23 phenotype-selected introgression families within PIFB47S/the marker position (vertical bars) is presented above the figure, black represents heterozygous state of the donors, and grey represents for recurrent parents

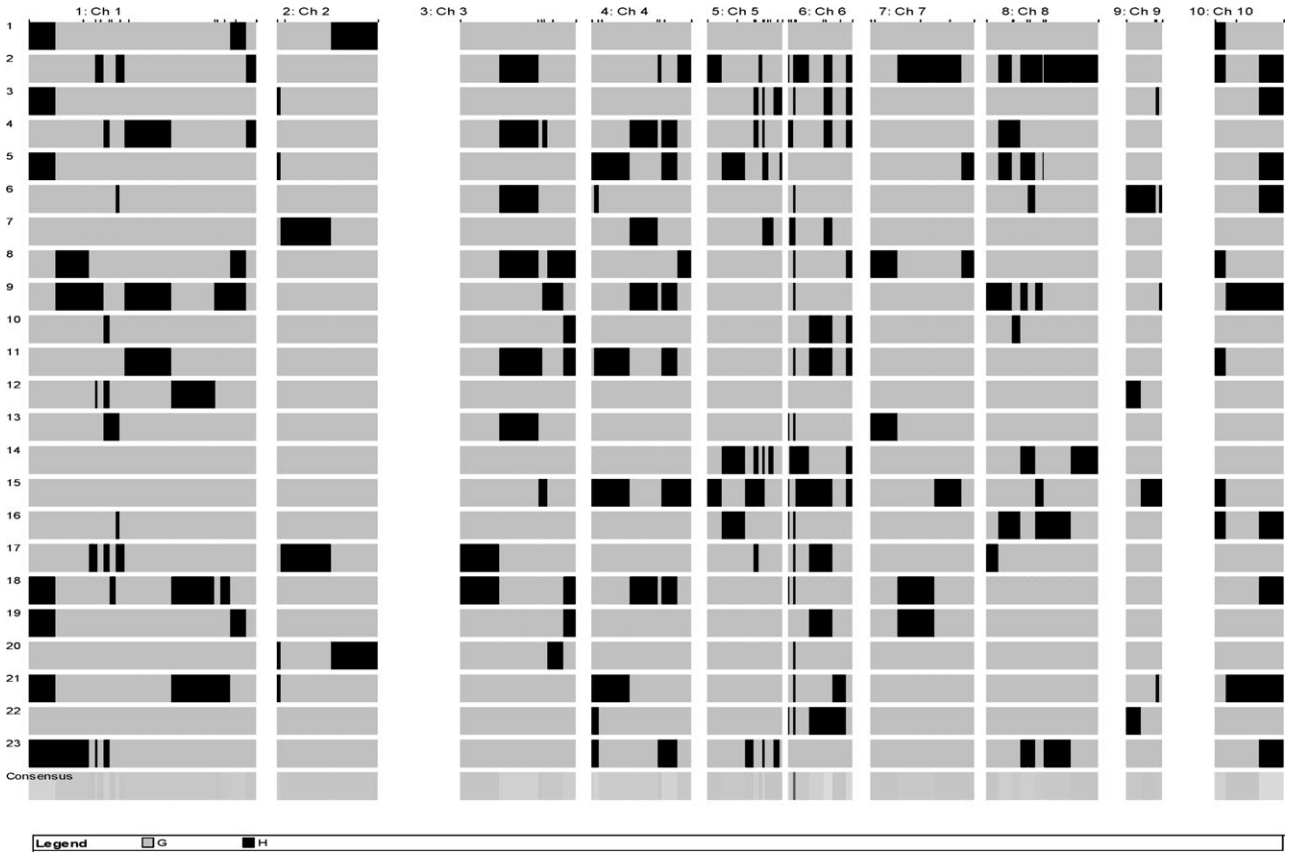

Fig. 4: Genotypic characterization of the top 23 phenotype-selected introgression families within PIFZ51S/the marker position (vertical bars) is presented above the figure, black represents heterozygous state of the donors, and grey represents for recurrent parents

donor genome contribution distributed over five chromosomes. The second highest donor genome contributions were located on Chromosomes 1, 2, 3 and 4. Regions with low frequency of RP allele substitution by donor segments were clustered in 10 regions on seven chromosomes: Chromosome 1 (0 and 201.4–230.1 cM), Chromosome 2 (99.3 cM), Chromosome 4 (17.7–29.0 cM), Chromosome 6 (12.9 cM), Chromosome 7 (11.5–34.3 cM), Chromosome 9 (30.3 and 81.2 cM) and Chromosome 10 (83.6 and 146.7 cM). For PIFB47S, 10 regions with a high donor replacement frequency were distributed over six chromosomes. The highest donor genome percentages were detected on Chromosome 6 at 12.1 cM (65%) and 47.7 cM (45%), followed by the telomeric regions of Chromosomes 1, 4, 6 and 10 with 35% donor allele contributions. Sixteen regions with a low replacement frequency were identified for PIFB47S distributed over the whole genome with 15% of donor genome.

In several cases, introgressions coming from different donor parents were shared by selected BC families in both libraries. The most frequent donor regions detected in both selected libraries were at 93.5 and 117.1 cM on Chromosome 1, 21.4 cM on Chromosome 2, 201.9 cM on Chromosome 3, 16.0 and 101.0 cM on Chromosome 4, 12.1 cM on Chromosome 6, 41.3 and 77.5 cM on Chromosome 8 and 72.4 cM on Chromosome 9.

Discussion

Our study aimed to establish two phenotypic-selected introgression libraries to accumulate dominant maize PHT-increasing alleles from various donors into the same genetic background. Tall plants were selected that flowered about the same time as the RP, to reduce the confounding effect of flowering time and PHT. Various unrelated PHT QTL from different donor parents may facilitate the discovery of novel PHT-related genes, and they may be useful for breeding varieties aiming at maximizing biomass yield (Fernandez et al. 2009).

The two introgression libraries used in this study consist of BC families carrying DCS, which were introgressed into a common elite genetic background of either PHB47 or PHZ51. The selected and genotyped tallest 46 BC families (23 in each RP background) with significantly ($\alpha = 0.01$) taller PHT than their RPs contained 4–19 DCS with donor genome percentages ranging from 6.3% to 64.6%. Compared to high-throughput genotyping technologies that produce thousands of markers for each individual, the number of markers used in this study was limited. However, because most of the individuals are only BC_2-BC_3, the number of SNP markers used in this study is sufficient to capture large DCS (Figs 3 and 4) – in PIFZ51S, the average donor genome proportion is 21.3%, and there are on average 9.6 donor segments, so the average length is 21.3%/9.6 = 2.22% of the genome. We used 84 markers across the genome and the average length between each two markers is 100%/84 = 1.19% <2.22%; in PIFB47S, the average donor segment length is 1.83% and the average length between each two markers is 1.4 < 1.83 – the number of markers are able to capture the large donor segments. Distribution of DCS across the whole genome in these selected PIFs is consistent with quantitative inheritance of PHT. In the present study, most PIFs were generated after two to three generations of backcrossing. Without selection, the proportion of donor genome should decrease by 50% after each generation of backcrossing. A lower-than-expected donor genome proportion was observed ($BC_1DH = 16 < 25\%$) for the unselected GEM-DH lines. This can be explained by monomorphic markers between the RP and donors, which leads to an underestimated donor genome proportion. In contrast, PIFs had a higher level (on average 8%) of donor genome proportion ($BC_2 = 19.1\%$, $BC_3 = 11.6\%$, etc.) than expected without selection. Because some markers are monomorphic between RP and donor parent and the number of SNP markers is relatively small in this study, the true donor genome proportion in PIFs is expected to be even higher. This indicates that the selection process was successful and that donor alleles related to PHT were accumulated in our PIF libraries. Some alleles with small effects related to PHT may be lost during the selection process. However, PIFs are still able to capture more DCS relating to tallness compared with traditional introgression methods. Substantial hitchhiking and linkage drag around the target locus can be expected (Stam and Zeven 1981), resulting on average in 36-cM-long donor regions in BC_3 for a chromosome of 200 cM. Our phenotypic selection process likely selected multiple loci related to PHT in each PIF, explaining the much higher donor genome proportion found in our study. A rough calculation could be that if only one locus affecting PHT was selected, then additional donor genome proportion is expected to be 2% [36 cM/1800 cM – ISU SNP map (Liu et al. 2010)]. If two independent loci are selected, then it should be 4%, etc. Based on the BC_1DH information, when the expected donor genome proportion is 25%, on average there is 9% (25–16%) lower donor genome proportion likely due to monomorphic markers, which means that when the observed donor proportion is 16%, there will be 9% underestimated. So on average, when the observed extra donor genome proportion is 8%, there will be 8%*9%/16% = 4.5%. The extra donor genome proportion for the PIFs should then be (8% + 8%*(9%/16%) = 12.5%), which means that there were around seven (12.5%/2%) loci selected for each PIF on average. For the PIF with the largest donor genome proportion, the number of loci would be 41 (one BC_2F_1 – (64.6–12.5)*(25/16)/2).

To differentiate genome regions with the effect on PHT from regions carrying DCS and present due to random drift, we determined hot spots for PHT in the genome. A hot spot in our study was defined as genomic region, where the RP allele is replaced by DCS in more than seven PIFs across both PIFB47S and PIFZ51S. A DCS is expected to occur at 50% in BC_1F_1, 25% in BC_2F_1 and 12.5% in BC_3F_1 due to the random drift. Because there were 1 BC_1, 33 BC_2, 10 BC_3 and 2 BC_4 families within our PIF library, we calculated an approximate weighted probability for a DCS to occur by random drift among our selected 46 PIFs as P = 0.25*(1/46) + 0.125*(33/46) + 0.0625*(10/46) + 0.03125*(2/46) = 0.1. Based on a binomial distribution mass function (Frisch et al. 1999) of F (7, 46, 0.1) = 0.92, the probability of more than seven individuals carrying a donor allele SNP by random drift rather than by selection pressure is smaller than 0.08. The more often a DCS substitutes a particular region, the more likely it becomes that this substitution is not only by chance but indeed due to selection. Our hypothesis is that hot spots are more likely to carry PHT-related QTL. This is similar to introgression mapping process proposed by Thurber et al. (2013). Therein, for all BC_4-derived lines, the theoretical expectation of finding a donor segment without selection was 3% and a cut-off of 4% was used to define the regions retained due to selection. As a result, 28 hot spots met our criterion, spread across the whole genome (Table 5). The number of hot spots in our study was comparable with the number of QTL identified in previous studies of maize using large mapping populations. In a study based on a population size of N = 976 in maize, a total of 30 QTL were detected for PHT (Schön et al.

Table 5: Positions and frequencies of selected hot spots

Chr.	Position (cM)	Donor loci frequency (%)
1	0	19.55
1	93.5	21.75
1	107.2	15.2
1	117.1	23.9
1	125.3	17.39
1	174.5	17.39
1	221	15.2
2	1.1	19.57
2	21.4	23.9
3	174.5	17.39
3	201.9	26.05
4	16	28.25
4	29.7	21.74
4	94.1	15.2
4	101	34.75
5	16	19.55
5	70.8	15.2
5	71.9	17.35
6	12.1	41.3
6	47.7	21.74
6	79.7	17.39
8	41.3	19.55
8	59	15.2
8	62.2	15.2
8	77.5	17.4
8	80.8	17.35
9	72.4	19.55
10	140.1	19.57

2004). Melchinger et al. (1998) detected a total of 31 QTL for PHT among 344 maize F_3 families derived from a biparental cross. Wang et al. (2006) found 40 'real' PHT QTL using meta-analyses by collecting all previous PHT QTL results distributed over the whole maize genome. We found that 12 hot spots in our study overlapped with respective 'real' QTL, which means that these 12 hot spots overlapped with repeatedly reported QTL in previous mapping studies for PHT QTL. For example, the hot spot on Chromosome 4 with the maximum frequency of donor introgressions overlapped with previously reported PHT QTL (Wang et al. 2006). This number of overlapping with previous published PHT hot spots is higher than expected by chance: the probability of finding 12 hot spots by chance based on 28 regions detected in our study is below 0.1. Around 31% of the total markers overlap with the 'real' QTL. Based on a binomial distribution mass function (Frisch et al. 1999) of F (11, 28, 0.31) ~0.9, the probability of finding 12 or more overlap regions with the 'real' QTL just by chance is around 0.1. These 12 overlap regions may be the main clusters of PHT-related genes segregating within elite maize materials.

For the 126 PIFs from the PIFZ51 and 100 PIFs from the PIFB47, we only genotyped 23 PIFs from each library and only the regions without an effect on flowering time were selected. This may be the reason that we did not capture more 'real' QTL. We also detected 16 hot spots different from the 40 'real' QTL. As most of the previous PHT QTL mapping studies were based on elite germplasm, it is no surprise to find novel PHT-related regions in our study, because we utilized offspring of exotic x elite crosses. Across the 28 regions, the region around 12.1 cM on Chromosome 6 and 101 cM on Chromosome 4 [based on the ISU SNP map (Liu et al. 2010)] had the highest DCS substitution rates: 18 of 46 PIFs carried DCS in these two regions.

Our approach of utilizing a small population size while tapping into extensive genetic diversity by using multiple donor accessions is different from biparental QTL mapping and introgression mapping populations. Small population sizes influence the power to detect QTL and the accuracy of estimated QTL effects (Melchinger et al. 1998). In a study performed by Beavis (1994) using 400 maize F_3 families derived from the B73 × Mo17 cross to determine the effect of population size on the number of QTL detected, only four QTL were detected in the combined mapping population of size N = 400, further reduced to 1–3 in each of the subsets of N = 100 families. In another study performed by Schön et al. (2004), a total of 30 QTL for PHT were detected, but with a very large mapping population of N = 976 maize $F_{2:5}$ families derived from a biparental cross. When multiple sets of smaller population sizes N = 488, 244 and 122 $F_{2:5}$ from the same biparental cross were used by sampling without replacement, the number of QTL detected decreased to 17.6, 12.0 and 9.1, respectively. Therefore, our PIF library method provides a promising opportunity to efficiently use the genetic potential of exotic germplasm and targeting genomic regions underlying quantitative traits based on a small number of selected families.

The method to use introgression libraries to discover PHT QTL was successful in previous studies (Pillen et al. 2003, Septiningsih et al. 2003, Liu et al. 2006, Von Korff et al. 2008) and for other agronomically important traits (Huang et al. 2003, Pillen et al. 2003, Septiningsih et al. 2003, Tian et al. 2006, Tan et al. 2007, Von Korff et al. 2008). Even for introgression lines with extra donor segments, new statistical methods have been developed for QTL detection (Falke et al. 2014). A preselection for target genes underlying a quantitative trait or phenotype in previous BC generations was shown to be helpful for accelerating the recovery of the elite RP genome (Frisch 2005, Falke et al. 2008). Our PIF library method is comparable to other traditional introgression libraries, with the difference being that only phenotypic selection was executed, multiple donor parents were used and we only focused on one trait. Compared with the traditional introgression libraries, the use of PIFs has advantages: (i) low cost phenotypic selection, (ii) multiple donors and (iii) focus on only one trait to accumulate respective genomic regions. However, this approach of using PIFs may only be useful for highly heritable traits and a limited use for only one trait – PIFs may not be suitable for complex traits that are more difficult to phenotype with lower heritability. However, the fast-developing novel high-throughput phenotyping techniques may provide more opportunities for PIF method. Without high-throughput marker-assisted selection, there may be linkage drag problems associated with PIF method; however, PIF is still a very efficient way to maximize the genetic variation and to establish prebreeding materials.

Flowering time and PHT are closely correlated. We intentionally and successfully enriched for DCS affecting PHT, but not flowering time as documented by much lower correlations between both traits ($r^2 = 0.19$) compared to other studies, for example $r^2 = 0.34$ in the NAM (Nested Association Mapping) population and $r^2 = 0.78$ in NCRPIS panel (Peiffer et al. 2014). In this way, the PHT-related genomic regions will not be confounded with flowering time. This is valuable to develop silage and dual-purpose maize varieties to increase the biomass yield via PHT without affecting the maturity.

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Author Contribution

AAG and SH contributed equally to this work. AAG, YC, EAB and BK established the experimental population (PIFs) and collected data. SH and AAG analysed the data and wrote the manuscript. MB and TL designed the experiment and involved in the revision of the manuscript. All authors read and approved the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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