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ORIGINAL PAPER

Fine mapping of *qhir1* influencing in vivo haploid induction in maize

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Abstract Production of haploids by the in vivo haploid induction method has now become routine for generating new inbred lines in maize. In previous studies, a major quantitative trait locus (QTL) (*qhir1*) located in bin 1.04 was detected, explaining up to 66 % of the genotypic variance for haploid induction rate (HIR). Our objectives were to (1) fine-map *qhir1* and (2) identify closely linked markers useful for marker-assisted breeding of new inducers. For this purpose, we screened a mapping population of 14,375 F_2 plants produced from a cross between haploid inducer UH400 and non-inducer line 1680 to identify recombinants. Based on sequence information from the B73 reference genome, markers polymorphic between the two parents were developed to conduct fine mapping with these recombinants. A progeny test mapping strategy was applied to accurately determine the HIR of the 14 recombinants identified. Furthermore, F_3 progeny of recombinant F_2 plants were genotyped and in parallel evaluated for HIR. We corroborated earlier studies in that *qhir1* has both a significantly positive effect on HIR but also a strong selective disadvantage, as indicated by significant segregation distortion. Altogether, we were able to narrow down the *qhir1* locus to a 243 kb region flanked by markers X291 and X263.

Introduction

The development of homozygous lines is an important part of every maize breeding program. The conventional procedure to produce homozygous lines by recurrent selfing is a time-consuming and expensive process, which takes about six generations, starting from heterozygous source material (Hallauer et al. 2010). The doubled haploid (DH) technology enables reducing this time span considerably and, for this and further reasons, has been widely adopted in maize breeding during the past decade (Schmidt 2003; Seitz 2005).

One method to produce haploids is by inter-specific crosses, such as in barley between *Hordeum vulgare* L. and *Hordeum bulbosum* L., or in wheat, where maize or pearl millet is used as pollinators (Kasha and Kao 1970; Laurie and Bennett 1988). In maize, maternal haploid and paternal haploids can be generated by inducers. Paternal haploids can be produced using the *igig* genotype as female parent in crosses with the source material (Kermicle 1969; Evans 2007). In contrast, maternal haploids can be produced from

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any source material by pollination with specific male genotypes (Coe 1959). This method is commonly referred to as in vivo haploid induction.

The mechanisms for induction of maternal haploids in maize are yet not fully understood. Two major hypotheses proposed for explanation are: (1) single fertilization, in which case one of the two sperm cells is not able to fuse with the egg cell, whereas the other sperm cell fuses with the central cell; while this would lead to fully functional triploid endosperm, the egg cell is somehow triggered to develop into a haploid embryo (Sarkar and Coe 1966; Chalyk et al. 2003; Barret et al. 2008). (2) Double fertilization, in which case one sperm cell fuses with the egg cell and the second with the central cell leading to fully a functional triploid endosperm and a diploid zygote; however, thereafter the inducer chromosomes degenerate and are eliminated only in the diploid embryo but not in the endosperm, finally forming a haploid embryo and a fully functional triploid endosperm (Fischer 2004; Zhang et al. 2008; Li et al. 2009; Xu et al. 2013).

Lashermes and Beckert (1988) suggested that haploid induction rate (HIR) is a heritable trait and controlled by a small number of genes with nuclear determination. Prigge et al. (2011) reported that the inducer single cross RWS × UH400 showed both intermediate and higher HIR compared to its parent lines in combination with different testers. Thus, additive, dominance and epistatic gene action may together affect the haploid induction process. A first QTL mapping study on HIR, using Stock6 as inducer parent, detected two QTL (Deimling et al. 1997; Röber 1999): a major QTL located in bin 1.03–1.06 and a minor QTL located in bin 2.04–2.06, explaining together 17.9 % of the phenotypic variance. A major locus on maize chromosome 1 was also detected in a segregating population derived from a cross between inducer line PK6 and non-inducer line DH99, which not only caused in vivo haploid induction but also resulted in segregation distortion against the inducer gamete (Barret et al. 2008). Recently, four populations involving two inducers (CAUHOI and UH400) were produced for genome-wide QTL analysis of HIR (Prigge et al. 2012). Two major QTL, denoted as *qhir1* and *qhir8* and explaining up to 66 and 20 % of the genotypic variance in the crosses 1680 × UH400 and CAUHOI × UH400, respectively, were detected.

Since the major QTL *qhir1* located on chromosome 1 was detected in several studies with different haploid inducers from different backgrounds, we gave *qhir1* the highest priority in fine mapping of genes underlying HIR. Our objectives were to (1) fine-map the *qhir1* locus and (2) identify closely linked markers useful for marker-assisted breeding of new inducers.

Materials and methods

Plant materials

Inbred line 1680 with no haploid induction ability (i.e., 0 % HIR) was pollinated with haploid inducer UH400, which has 8 % HIR on average (Prigge et al. 2011) and carries the dominantly inherited marker gene *R1-nj*, conferring a purple coloration expressed both in the scutellum and aleurone (Nanda and Chase 1966; Neuffer et al. 1997). The F_1 was advanced to the F_2 generation by self-pollination. F_2 plants with genetic recombination in the *qhir1* region were identified by genotyping with appropriate molecular markers (Fig. 3) and subsequently selfed to produce F_3 progeny. In order to determine the HIR of individual F_3 plants, they were pollinated onto hybrid ZD958, which served as a female tester due to its excellent agronomic performance and clear expression of the *R1-nj* marker in both the embryo and endosperm, when pollinated by inducers carrying this marker gene. In addition, two small F_2 populations of cross 1680 × UH400 were grown with an initial sample size of $N1 = 375$ in Hainan, winter 2009, and $N2 = 308$ in Beijing, summer 2010 to verify the effect of *qhir1*.

Haploid identification

Kernels in the testcross progeny with tester hybrid ZD958 displaying purple endosperm and embryo were classified as putative diploid, whereas kernels with purple endosperm and colorless embryo were classified as putative haploid following Li et al. (2009). All putative haploids were planted in the Shang Zhuang experiment station in Beijing to confirm their ploidy status by visual scoring. In comparison with diploids, haploids display shorter stature, erect and narrow leaves, and reduced growth. HIR was calculated by the following formula: $\text{HIR} = (\text{number of putative haploids/total number of } R1 - nj \text{ normal kernels}) \times (\text{number of haploids in the field/number of putative haploids}) \times 100\%$.

Development of molecular markers and genotyping

In our previous study (Prigge et al. 2012), a major QTL *(qhir1)* was detected in maize bin 1.04 with a support interval between simple sequence repeat (SSR) markers umc1917 and bnlg1811. Based on the B73 reference sequence, the physical distance between these two markers spans 7.5 Mb. The sequence between these two markers was downloaded from the maize sequence database (http://www.maizesequence.org/) to develop new markers. Primers designed by software primer 5.0 (http://www.primer-e.com/)

based on the information from the website maizesequence.org yielded three types of markers: some (100 markers) were SSR markers, and the others (200 markers) were newly designed markers based on single-/low-copy B73 reference sequences which were further developed to single nucleotide polymorphism (SNP) and insertion/deletion polymorphism (IDP) markers. First, 300 newly developed markers were tested for polymorphism between inducer UH400 and inbred 1680. Second, the map position of these markers was checked by mapping them on our original F_2 population described in detail by Prigge et al. (2012). Finally, seven SSR marker, four IDP markers and two SNP markers were developed to cover this region (Supplementary Table S1).

For DNA extraction, we used the method of Murray and Thompson (1980). Each DNA sample was genotyped for the newly designed and original markers as required by scoring PCR amplification products analyzed on 1 % agarose gels or 6 % denaturing polyacrylamide gels.

Strategy for fine mapping of *qhir1*

A progeny test mapping strategy was applied (Fig. 1) following Zhang et al. (2012). First, a large F_2 population ($N = 14{,}375 = 375 + 6{,}000 + 8{,}000$) was generated in three steps to obtain a sufficient number of recombinants that were identified by two markers flanking *qhir1* (umc1917 and bnlg1811). Second, all 14 detected recombinants (R1 to R14) were additionally genotyped by the newly developed and original markers as required (seven markers for R1, eight markers for R2 to R7, seven markers for R8 to R14) in the support interval of *qhir1* to determine the exchange boundary of each recombinant. Third, all 14 recombinant F_2 plants were advanced to generate F_3

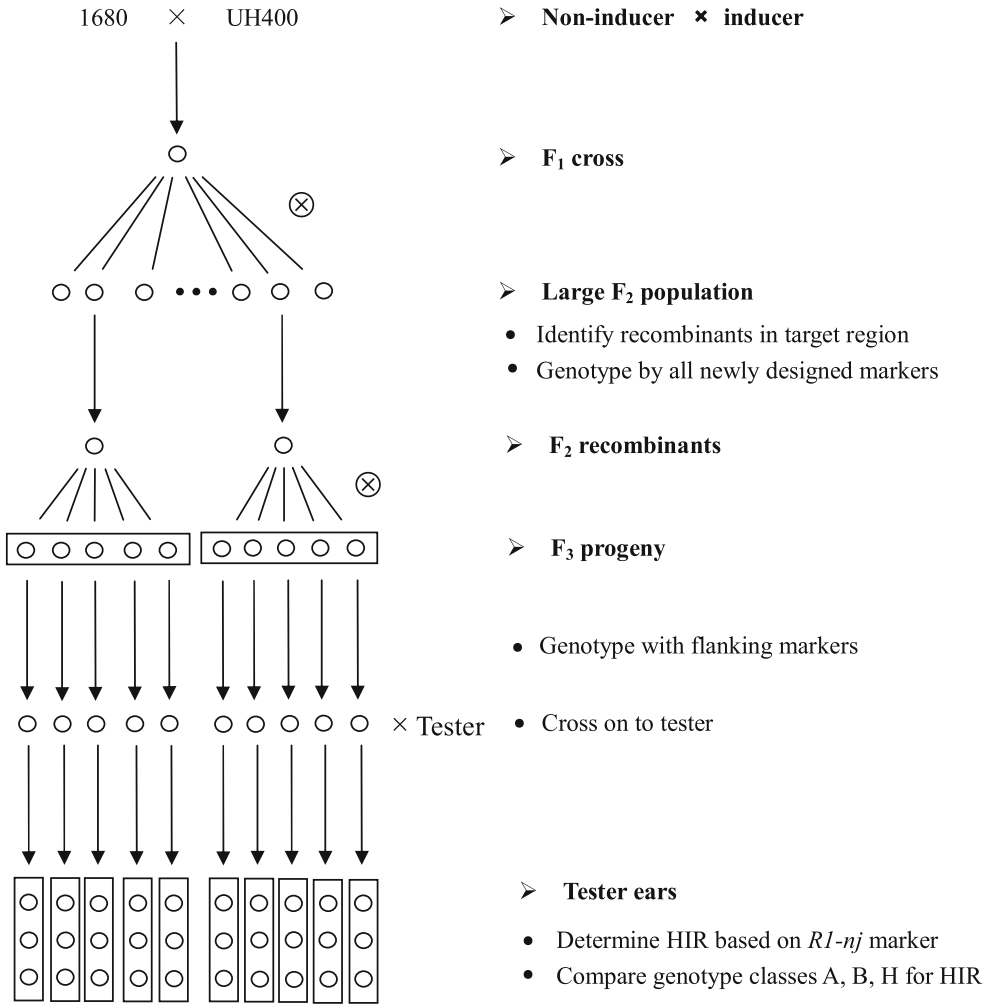


Fig. 1 Strategy for fine mapping of the *qhir1* locus. A total of 14,375 F_2 individuals from cross 1680 × UH400 was produced and genotyped by newly developed markers in the *qhir1* target region to identify a sufficient number of recombinants. These were selfed (⊗), and their F_3 progeny were (**a**) genotyped with markers from the *qhir1* region and (**b**) testcrossed onto tester hybrid ZD958 for determination of their haploid induction rate (HIR)

progeny. Fourth, from each of these recombinant plants in F_2, that is heterozygous for at least one marker but homozygous for at least one other marker in the target region, at least 30 F_3 crossing kernels were planted in the field ear to row. Fifth, every F_3 plant was (1) genotyped by markers flanking the exchange position to determine its genotype, and (2) used to pollinate 3–4 ears of the tester hybrid ZD958 to determine its HIR. Based on the markers in the *qhir1* region, each F_3 plant was assigned to one of the following three genotype classes: (A) absence of the UH400 haplotype at *qhir1*, (B) homozygous for the UH400 haplotype at *qhir1*, (H) heterozygous for the UH400 haplotype. We were interested in testing the following three hypotheses: (1) H_0: $\mu_A = \mu_B$ vs. H_A: $\mu_A < \mu_B$; (2) H_0: $\mu_A = \mu_H$ vs. H_A: $\mu_A < \mu_H$; (3) H_0: $\mu_B = \mu_H$ vs. H_A: $\mu_H < \mu_B$. Significant differences among the three genotype classes in the F_3 progeny are expected if and only if the gene(s) underlying the *qhir1* QTL reside(s) within the chromosome segment of a given recombinant F_2 plant that is heterozygous for the target region. We applied first an *F* test to test for heterogeneity in the variances of the F_3 progeny among the three marker genotype classes of a given F_3 family. If this test was significant, we used a Wilcoxon rank-sum test instead of an ordinary *t* test to test for differences between any two genotype classes of the respective F_3 family.

Results

Haploid induction rate of the three genotype classes

The two F_2 populations of cross 1680 × UH400 grown in Hainan, winter 2009 and Beijing, summer 2010, showed a similar pattern of HIR for the three genotype classes (Fig. 2). In both environments, the F_2 plants in genotype class A (lacking the inducer haplotype from UH400 at *qhir1*) had on average significantly ($P < 0.01$) lower HIR than those in genotype class B (homozygous for the inducer haplotype at *qhir1*) and HIR of the heterozygous genotype class H was in between. Moreover, the standard deviation of HIR amongst individual F_2 plants was in both environments largest for genotype class B, intermediate for genotype class H, and smallest for genotype class A.

Fine mapping of *qhir1*

To verify the effect of *qhir1* and narrow down the region of this major QTL, the 375 F_2 plants grown in Hainan in winter 2009 (Fig. 2) were genotyped with markers umc1917 and bnlg1811. A single recombinant R1 was found between these two markers and further genotyped with five markers (X8, X18, umc2390, X41, X52),

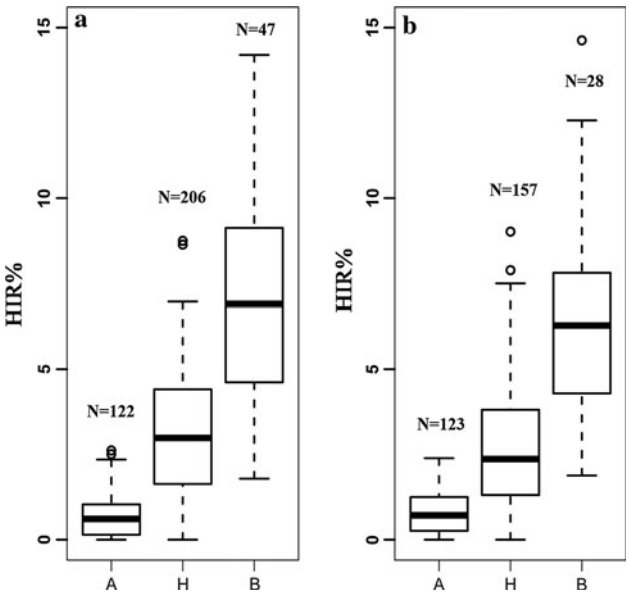


Fig. 2 Effect of the *qhir1* region on HIR in maize analyzed with an F_2 population produced from cross 1680 × UH400. Number (*N*) of F_2 plants classified as *A* homozygous for the 1680 haplotype, *B* homozygous for the UH400 haplotype or *H* heterozygous based on genotyping with two markers (umc1917 and bnlg1811) in the *qhir1* region and their corresponding average HIR. **a** F_2 plants grown in Hainan in winter 2009, **b** F_2 plants grown in Beijing in summer 2010

revealing that the recombination had occurred between markers X41 and X52 (Fig. 3). In spring 2010, 12 F_3 progeny originating from this recombinant were tested for HIR but no significant differences were found (with a significance threshold of $P < 0.01$) amongst the three genotype classes (Fig. 3). In parallel, another 6,000 F_2 plants were planted in 2010, genotyped with markers umc1917 and X52, and six new recombinants (R2–R7) were found and advanced to the F_3 generation. Six markers (X8, X18, X93, X109, umc2390 and X41) were used to resolve the exchange boundary in the newly detected recombinants (Fig. 3). Approximately 30 F_3 plants from each recombinant were genotyped and testcrossed with tester ZD958 to determine their HIR. Recombinants R2 and R4 showed that *qhir1* was located upstream of the marker X93 and recombinant R7 indicated that it was downstream of X18, so that *qhir1* was narrowed down between markers X18 and X93 with a distance of about 800 kb on the B73-based physical map (http://www.maizesequence.org).

In the spring of 2011, another 8,000 F_2 plants were genotyped and phenotyped to further narrow down to the region. These plants were screened with markers X18 and X93; seven recombinants (R8–R14) were found and genotyped with six newly designed markers (X273, X22, X224, X291, X260 and X263) (Fig. 3). In the winter 2011, on average 30 F_3 plants for each recombinant were genotyped and phenotyped for HIR. Recombinant R13 indicated that *qhir1* was located upstream of the marker X263 and

recombinant R11 indicated that it was downstream of X291. Finally, *qhir1* was mapped between markers X291 and X263, which have a physical distance of about 243 kb based on the B73 physical map. Variances for HIR were heterogenous ($P < 0.01$) among the three genotype classes in the F_3 progeny, when respective F_2 recombinant was heterozygous for the *qhir1* region and the Wilcoxon rank-sum test indicated significant ($P < 0.01$) differences among the three marker classes in the four recombinants R2, R3, R11, and R14 (Fig. 3).

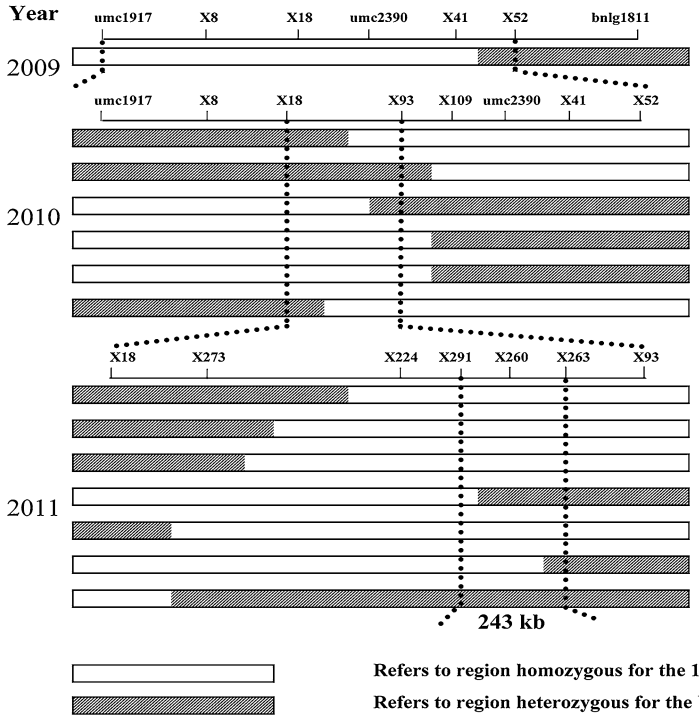
Relationship between *qhir1* and segregation distortion

Both initially tested F_2 populations showed significant ($P < 0.05$) segregation distortion for *qhir1* with strongly reduced frequencies of genotypes homozygous for the inducer haplotype (Fig. 2). For each recombinant detected in F_2, the F_3 progeny were also tested for segregation distortion of *qhir1* based on the corresponding genotypic data (Table 1). The χ^2 test revealed significant ($P < 0.05$) segregation distortion in all recombinants that putatively harbored the *qhir1* haplotype, as inferred from the result of the Wilcoxon rank-sum test for differences in HIR among the three genotype classes (Fig. 3). In all instances, the frequency of F_3 plants homozygous or heterozygous for the inducer haplotype was lower than expected based on normal Mendelian segregation (Table 1).

Discussion

Strategy for fine mapping of *qhir1*

QTL mapping represents a powerful tool to reveal the genetic architecture of complex traits but it requires reliable phenotypic data. Haploid induction ability is a complex quantitative trait with two major QTL and several minor QTL (Prigge et al. 2012), and to some degree also influenced by the environmental conditions (Kebede et al. 2011; Prigge et al. 2011). However, previous studies employing linkage mapping of QTL for HIR encountered limitations with regard to the map resolution and usually resulted in large support intervals for the detected QTL (Deimling et al.1997; Röber 1999; Prigge et al. 2012).



	N_{F3} [a]	N_T [b]	HIR% [c, d] of F_3 progeny			Inference [e]
			A	H	B	
R1	12	36	0.19a	0.28a	0.61a	N
R2	49	111	0.49a	2.37b	3.65c	Y
R3	30	113	0.24a	1.46b	2.42c	Y
R4	52	105	0.13a	0.21a	0.29a	N
R5	44	98	0.13a	0.17a	0.15a	N
R6	52	154	0.64a	0.46a	0.86a	N
R7	9	33	0.51a	0.60a	0.36a	N
R8	96	272	0.42a	0.44a	0.32a	N
R9	37	94	0.43a	0.51a	0.34a	N
R10	33	110	0.36a	0.40a	0.50a	N
R11	31	106	0.30a	2.35b	2.99b	Y
R12	35	94	0.14a	0.09a	0.14a	N
R13	31	85	0.41a	0.47a	0.23a	N
R14	22	54	0.17a	2.13b	n.a. [f]	Y

Fig. 3 Fine mapping of the *qhir1* region. *Left side* the physical position of markers mapping to bin 1.04 is shown on *top* of the bars. The designation of the F_2 recombinants (R1–R14) in 2009, 2010, 2011 is given on the *right side* of each bar. *Right side* mean haploid induction rate (HIR %) of F_3 progeny: [a]N_{F3} number of plants in the F_3 progeny, [b]N_T number of tester ears per F_3 family, [c]numbers in a line followed by the same letter are not significantly different from each other based on a Wilcoxon rank-sum test at the 1 % probability level, [d]inference on haploid induction ability based on result of the Wilcoxon rank-sum tests, [e]*n.a.* not available. *A* homozygous for the 1680 haplotype, *B* homozygous for the UH400 haplotype or *H* heterozygous, *Y* F_2 recombinant harbors the qhir1 QTL allele in heterozygous state, *N* F_2 recombinant does not harbor the *qhir1* allele

Table 1 Genotype distribution of F_3 progeny produced from recombinant plants in the F_2 population

ID of recombinant	No. of F_3 progeny[a]				χ^2 test[b]	
	A	B	H	Total	A:B:H = 1:2:1	A:B = 1:1
R1	3	2	7	12	0.5	0.17
R2	19	3	27	49	10.96*	10.54*
R3	13	2	15	30	8.07*	8.13*
R4	11	15	26	52	0.62	0.65
R5	14	8	22	44	1.64	1.68
R6	11	16	25	52	1.04	1.12
R7	2	3	4	9	0.33	0.72
R8	23	19	54	96	1.83	0.85
R9	8	10	19	37	0.24	0.23
R10	7	9	17	33	0.27	0.26
R11	13	3	15	31	6.31*	6.31*
R12	11	9	15	35	0.94	0.93
R13	8	6	17	31	0.55	0.27
R14	4	0	18	22	10.36*	4.73*

[a] F_3 plants were classified into three genotype classes for the *qhir1* region: *A* homozygous for the 1680 haplotype, *B* homozygous for the UH400 haplotype, *H* heterozygous

[b] χ^2 test for segregation distortion

* Significant at the 5 % probability level

Promising approaches for fine mapping of QTL are analyses of near-isogenic lines, linkage disequilibrium mapping and progeny testing. Near-isogenic lines were successfully used for fine mapping QTL in maize (Graham et al. 1997; Koester et al. 1993) with the advantage to reduce much of the "noise" caused by the genetic background. Instead of constructing near-isogenic lines, which is very laborious and time-consuming, we adopted a progeny test method to fine map the *qhir1* locus. This method was originally proposed by Lander and Botstein (1989) to obtain more accurate phenotypic data by phenotyping a large number of progenies. Averaging over the data from progenies reduces the experimental error and 'noisy' genetic background so as to reveal the authentic genetic effect of the target QTL (Yang et al. 2012). In the present study, we applied the basic idea of the progeny test to F_2 plants that were recombinant for the target region and, consequently, were heterozygous for a certain chromosome segment in this region. By planting F_3 progeny from these recombinants in the same plot and by genotyping and phenotyping testcross progeny of individual F_3 plants, we obtained reliable estimates of HIR for the three genotype classes of the target region as a prerequisite for fine mapping. Another effective method is saturation of the *qhir1* region with SNPs and looking for the SNPs with maximum segregation distortion, thereby capitalizing on the assumption that *qhir1* is also responsible for segregation distortion in bin 1.04. This approach was proposed by Barret et al. (2008) to identify markers tightly linked to *qhir1* in a single large F_2 population and to circumvent the extensive phenotyping for HIR; it could provide a key to monitor the 243 kb interval for putative candidate genes based on the maize sequence.

qhir1 plays an important role in haploid induction

While HIR is a typical quantitative trait controlled by several loci, the *qhir1* region explained in most studies the largest proportion of the genetic variance for HIR amongst the identified QTL (Prigge et al. 2012). It was repeatedly detected in crosses with inducers Stock6 (Deimling et al. 1997; Röber 1999), PK6 (Barret et al. 2008), and UH400 (Prigge et al. 2012). Since Stock6 is an ancestor of many inducers, there is strong evidence that *qhir1* is essential in the process of in vivo haploid induction. Our study corroborated these findings by demonstrating that plants lacking the inducer haplotype at the *qhir1* locus have low HIR, whereas those carrying the inducer haplotype in homozygous or heterozygous state have significantly higher HIR. Since Stock6 has only a HIR of 2.3 % (Coe 1959), whereas modern inducers can have a HIR of more than 8 % (Röber et al. 2005; Prigge et al. 2011; Prigge and Melchinger 2012), we conclude that besides *qhir1* other QTL, too, affect HIR. In support of this hypothesis, we found in the F_2 population a large variance for HIR amongst F_2 plants homozygous for the inducer haplotype at *qhir1*, which could be exploited by selection (Fig. 2). This is in agreement with a hypothesis proposed by Prigge et al. (2012), who argued that *qhir1* is required for haploid induction ability, but several other QTL may act as enhancers for the function of *qhir1* and, if accumulated in one genotype, these loci together may improve the HIR.

Associated effects of the *qhir1* region

Our mapping approach corroborated the intimate relationship between HIR and segregation distortion reported in the study of Barret et al. (2008) and Prigge et al. (2012). This association may be due to one gene or closely linked genes. The mechanism of segregation distortion may involve true meiotic drive, which acts during meiosis, or gamete abortion or inactivation, which acts after meiosis (Phadnis and Orr 2009). A detailed analysis of segregation distortion in the cross 1680 × UH400 and its underlying causes is presented in a companion paper (Xu et al. 2013).

Implications for breeding of haploid inducers

The *qhir1* region has a considerable selection disadvantage in maize populations for two reasons: (1) it is subjected to strong segregation distortion and (2) the resulting haploids show poor fitness. Consequently, there is strong selection

pressure in nature to eliminate the favorable HIR-enhancing haplotype and this complicates also de novo and maintenance breeding of haploid inducers. Marker-assisted selection (MAS) could be used to overcome this problem by screening segregating material for presence of the inducer haplotype at the *qhir1* locus. The efficiency of MAS depends strongly on the degree of linkage between the flanking marker(s) used for diagnosing the presence of the target haplotype (Frisch et al. 1999). In the present study, we were able to identify markers X291 and X263, thereby defining a physical interval of less than 243 kb for the *qhir1* locus. The recombination frequency between these two markers was less than 5×10^{-4}, because we detected in a total of 14,375 F_2 individuals only 14 recombinants in the target region. Thus, these two markers have nearly the same predictive power as a functional marker and are expected to be highly reliable for monitoring the presence of the inducer haplotype at the *qhir1* locus in segregating material. Since the variance for HIR among F_2 individuals homozygous for the *qhir1* haplotype was large and indeed considerably larger than for the other two genotype classes, which may be caused by epistasis (Fig. 2), there should be ample opportunities for further improvement of HIR by selection. Thus, one could preselect segregating populations for presence of inducer haplotype at *qhir1*, and then select for high HIR either by marker-assisted selection for other QTL influencing HIR or whole genome selection (Technow and Melchinger 2013) or phenotypic selection within this genotype class.

Our study also provides an excellent starting point for map-based cloning of the gene(s) underlying the *qhir1* locus. Both, reliable diagnostic markers for the *qhir1* haplotype and knowing the function of the gene(s) underlying haploid induction will benefit the development of new inducers with higher HIR and improved agronomic characteristics.

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Conflict of interest None.

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