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Invited Expert Review

A Sequential Quantitative Trait Locus Fine-Mapping Strategy Using Recombinant-Derived Progeny

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Abstract

A thorough understanding of the quantitative trait loci (QTLs) that underlie agronomically important traits in crops would greatly increase agricultural productivity. Although advances have been made in QTL cloning, the majority of QTLs remain unknown because of their low heritability and minor contributions to phenotypic performance. Here we summarize the key advantages and disadvantages of current QTL fine-mapping methodologies, and then introduce a sequential QTL fine-mapping strategy based on both genotypes and phenotypes of progeny derived from recombinants. With this mapping strategy, experimental errors could be dramatically diminished so as to reveal the authentic genetic effect of target QTLs. The number of progeny required to detect QTLs at various R^2 values was calculated, and the backcross generation suitable to start QTL fine-mapping was also estimated. This mapping strategy has proved to be very powerful in narrowing down QTL regions, particularly minor-effect QTLs, as revealed by fine-mapping of various resistance QTLs in maize. Application of this sequential QTL mapping strategy should accelerate cloning of agronomically important QTLs, which is currently a substantial challenge in crops.

Keywords: Fine-mapping strategy; progeny; quantitative trait locus; recombinant.

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Introduction

Cereals such as rice, maize, wheat, barley, and sorghum are the world's most important staple foods, as well as resources for livestock production, and provide >60% of the calories and protein in our daily diet. The world population is expected to reach 9 billion by 2050, which will require raising overall food production by at least 70% (FAO 2009). In addition, new demands for biofuel production and other industrial purposes also depend on an increase in crop production. Conversely, arable land and water resources for agriculture continue to dwindle, and climate change causes adverse effects on crop productivity (Chakraborty 2005; Evans et al. 2008; Butterworth et al. 2010; Ceccarelli et al. 2010). Nowadays, our challenge is to maximize crop productivity per unit area to secure a global food supply. Fortunately, genetic improvement in crops by allele mining and the application of elite alleles can achieve breakthroughs in crop productivity (Tester and Langridge 2010).

Most agronomically important traits such as yield, grain qualities, and resistance/tolerance to biotic and/or abiotic stresses are complex quantitative traits in nature. These traits are

usually controlled by multiple quantitative trait loci (QTLs) and thus are severely affected by genetic backgrounds, environmental conditions, and gene-by-environment (G×E) interactions (Holland 2007). Gaining a better understanding of the QTLs that underlie these complex traits would significantly contribute to crop productivity by pyramiding multiple favorable alleles. QTL analysis is a well-established and widely-used tool for dissecting the genetic basis of complex traits in plants (Salvi and Tuberosa 2005; Cooper et al. 2009; Yamamoto et al. 2009). Many QTLs that influence important agronomic traits have been mapped in cereals (Xu et al. 2009; Xing and Zhang 2010). Because QTL analysis typically produces a large confidence interval spanning 10–30 cM with several hundred genes, it is usually uncertain whether a QTL corresponds to one or multiple linked genes (Salvi and Tuberosa 2005; Balasubramanian et al. 2009). Moreover, major-effect QTLs are rare, and most QTLs account for a relatively small part of the total phenotypic variation (Holland 2007; Mackay et al. 2009). To date, only a small proportion of causal genes underlying major-effect QTLs have been identified and cloned in cereals (Mackay et al. 2009). Positional cloning of minor-effect QTLs is almost impossible because of their low heritability. Minor-effect QTLs do, however, play an important role in crop improvement as well, and stacking of multiple minor-effect QTLs via marker-assisted selection (MAS) would result in significant genetic gain for agronomic traits.

The most challenging step in cloning minor-effect QTLs is the fine-mapping process, which depends on precise phenotypic evaluation. Here we summarize the recent methodologies in QTL fine mapping in cereals and then introduce a comprehensive fine-mapping strategy based on both genotypes and phenotypes of recombinant-derived progeny. This proven mapping strategy is very powerful in narrowing down QTL positions to accelerate QTL cloning and application (Yang et al. 2010; Zhang et al. 2012). The relevant results are not included here, as QTL analysis has been well reviewed elsewhere (Salvi and Tuberosa 2005; Holland 2007; Balasubramanian et al. 2009; Mackay et al. 2009; Poland et al. 2009).

Key Elements Toward QTL Fine Mapping

Quantitative trait locus fine mapping depends on three key elements: marker density, crossover density, and accurate recombinant phenotypes. Advances in cereal genome sequencing, comparative genomic analysis, and high-throughput single-nucleotide polymorphism (SNP) technology have enabled the acquisition of high-density markers along targeted regions (Holland 2007; Daetwyler et al. 2010). Genome re-sequencing in many cereal crops has created a huge number of SNPs for marker development. In maize, about 1.6 million SNPs have recently been identified in the maize HapMap project (Gore et al. 2009). In rice, Huang et al. (2010) recently detected 3.6 million non-redundant SNPs distributed across the genome, with an average of 9.32 SNPs/kb. Accordingly, a number of high-throughput SNP genotyping technologies have been developed for commercial use, and they provide an optimal opportunity to accelerate high-resolution QTL mapping and the association mapping process (Gupta et al. 2008; Yan et al. 2009; Fukuoka et al. 2010).

Sufficient recombination events within the target QTL region are necessary for successful QTL fine mapping. This mainly depends on the frequency of crossover events in the QTL region in a specific mapping population. Recombination frequency varies considerably along chromosomes (Fengler et al. 2007), creating hot-spot and cold-spot chromosomal regions. When a QTL is located in a hot-spot region, it is easier to identify numerous recombinants from segregating populations. QTLs mapped to a cold-spot region with significantly fewer crossover events require more effort to obtain new recombinants by either enlarging the segregating population or screening multiple generations across intercross/backcross populations.

Quantitative trait locus fine mapping is achievable when high-density molecular markers are used to resolve recombinants in the QTL region. In a bi-parental population, the more sequence variations that exist between parental lines, the more markers that can be developed for genotyping. Therefore, two parental lines having high sequence divergence in the QTL region are preferred, but only if such divergence does not negatively affect fertility and recombination frequency. In practice, the distal and proximal flanking markers are first used to genotype all individuals to identify new recombinants within the QTL region, followed by development of additional markers within the QTL region to resolve all recombination breakpoints. This process is repeated across multiple generations until the QTL region is restricted to a small interval. Alternatively, association mapping may also be possible to fine-map QTLs by using natural populations with quick linkage disequilibrium decay in the QTL region.

The ultimate determining factor for successful QTL fine mapping is the ability to obtain an accurate recombinant phenotype (Collins et al. 2008; Poland et al. 2009). Compared with the fine mapping of a major gene, marker development and recombinant screening are exactly the same in QTL fine mapping. In contrast, obtaining an accurate phenotype for a recombinant is much more laborious and time-consuming in QTL fine mapping. Because each individual QTL typically contributes a relatively minor effect to the target trait, it is challenging to precisely infer the QTL allele based on the phenotype. As such, various statistical methods have been explored to obtain an unbiased phenotype. Alternatively,

different populations have been adopted to increase the reliability of phenotypic evaluation.

Current Methodologies in QTL Fine Mapping

Once the QTL for an important agronomic trait is detected, the QTL region needs further delimitation to isolate the underlying genes. A widely adopted strategy is to develop a set of nearly isogenic lines (NILs) for the target QTL, which differ only in the size of the genomic segments that harbor the QTL within an otherwise uniform genetic background of the recurrent parent (QTL-NILs) (Ashikari et al. 2005; Salvi and Tuberosa 2005; Wang et al. 2005; Fan et al. 2006; Salvi et al. 2007; Shomura et al. 2008; Xue et al. 2008; Zheng et al. 2008; Li et al. 2011; Yan et al. 2011). Substitution mapping can then be used to refine the QTL interval using the QTL-NILs that carry different overlapping segments. Such an idealized population eliminates the influence of 'noisy' genetic backgrounds on phenotypic performance. Thus, the target QTL can be simplified to a Mendelian factor in the NILs. Recently, chromosome segment substitution lines (CSSLs, also called introgression lines), which are a derivation of NILs, have also been used for QTL fine mapping (Wissuwa et al. 2002; Wang et al. 2007; Tan et al. 2008; Weng et al. 2008; Zhou et al. 2009; Wei et al. 2010; Schmalenbach et al. 2011). CSSLs can be easily used to develop a segregating population for QTL fine mapping if the introgression segment harbors the QTL. In the backcrossed or selfed populations, crossovers occurring in the QTL region will produce recombinants that are identical in their genetic backgrounds but that differ in their introgression segments. Therefore, such recombinants are ideal for QTL fine mapping. The CSSLs and NILs are similar mapping populations, and both can be directly used in breeding programs once a favorable QTL has been confirmed in an elite recipient line (Takeuchi et al. 2006; Fukuoka et al. 2010). It is, however, time-consuming to develop a population consisting of idealized NILs or CSSLs. In most cases, each CSSL or NIL carries more than one segment from the donor parent, which may spoil an association between a molecular marker and a trait. Usually, only large-effect QTLs can be definitely identified using CSSL- or NIL-derived populations.

Environmental factors can severely affect phenotypic performance, and the following experimental controls have been implemented in current fine-mapping methodologies to guarantee unbiased phenotypic evaluation: (i) selecting individuals with typical recessive phenotypes to ensure homologous alleles at the QTL; (ii) screening recombinants in NIL populations, followed by repeated phenotypic evaluations; and (iii) producing recombinant-derived progeny, followed by phenotypic evaluations. For instance, a major pleiotropic QTL, *Ghd7*, in rice was precisely mapped to a 2 284 kb region by selecting and genotyping recessive individuals from a large population for all three traits of interest (short stature, early heading, and small panicle) (Xue et al. 2008). A major-effect QTL, *tga1*, which controls the differences in fruit/ear structure between maize and teosinte, has been fine-mapped to a 1 042 bp region by comparing the phenotype to the genotype for each recombinant screened from the NIL population (Wang et al. 2005). Progeny evaluation has been used to obtain accurate phenotypes for those agronomically important QTLs with relatively low-to-moderate heritability, such as *vgt1* (Salvi et al. 2002; Salvi et al. 2007) and *tb1* (Clark et al. 2006) in maize, *Hd6* (Takahashi et al. 2001), *qSW5* (Shomura et al. 2008) and *GW2* (Song et al. 2007) in rice, and *Lr34* (Krattinger et al. 2009) in wheat. The standard analysis of variance is usually used in phenotypic evaluation to test for significant differences in mean values of the trait among different recombinants to infer whether segments in the tested recombinants carry the QTL (Belknap 2003; Clark et al. 2006).

The ideal QTL fine-mapping approach should restrict the QTL region to a single candidate gene or polymorphic site, as demonstrated in the fine mapping of a QTL for tomato fruit sugar content (Fridman et al. 2000) and for rice grain number (Ashikari et al. 2005). Although it is extremely difficult in most cases to define the QTL down to a candidate gene, QTL fine-mapping steps should delimit the QTL region to an interval with a manageable number (e.g. <10) of genes. In addition, pleiotropic QTLs are pervasive in the initial mapping, and a fine-mapping process is indispensable in ultimately uncovering whether a cluster of QTLs or a true pleiotropy is the cause of multiple phenotypic variations (Mackay et al. 2009). By comparing gene sequences and their expression levels between two parental lines, candidate gene(s) for a given QTL can be identified (Zheng et al. 2008; Hattori et al. 2009). Unbiased scans for candidate genes underlying the QTL are needed before functional validation (Mackey et al. 2009).

Association mapping provides an alternative but more risky approach for resolving the QTL of interest down to specific DNA sequences (Peleman and Van der Voort 2003; Holland 2007). It exploits linkage disequilibrium to identify relationships between phenotypic variation and genetic polymorphisms (Breseghello and Sorrells 2006; Yu and Buckler 2006). The main advantage of association mapping over linkage mapping is that it explores all historic recombination events and mutations in a given population. The disadvantage of this approach is that the mapping results are significantly affected by population structure and allelic frequency (Sorkheh et al. 2008; Mackay 2009; Yan et al. 2010). Thus, accurately estimating the population and linkage disequilibrium structures in the genomes are essential for identifying useful alleles for crop improvement. Association mapping has been extensively used in crops (Kraakman et al. 2004; Tommasini et al. 2007; Cockram et al. 2008; Huang et al.

2010; Kump et al. 2011; Poland et al. 2011; Tian et al. 2011), although only a few studies have identified reliable alleles associated with the targeted traits (Belo et al. 2008; Wisser et al. 2011). Additionally, a strategy called joint high-resolution linkage and association mapping has recently identified a handful of important alleles in maize (Harjes et al. 2008; Yan et al. 2010). It is, however, difficult to successfully detect those rare alleles or minor-effect QTLs using the association mapping approach, as high stochastic variations make the mapping results complicated.

A Sequential QTL Fine-mapping Strategy Using Recombinant-derived Progeny

Evaluation of progeny as a whole is the preferred method for obtaining accurate phenotypes for those NILs or CSSLs that share nearly identical genetic backgrounds. There are, however, several obstacles to this approach. First, many generations are needed to breed ideal NILs or CSSLs that have genetic backgrounds with very low noise. Second, fine-mapping minor-effect QTLs using NIL- or CSSL-derived progeny is insufficient because all environmental elements, residual background noise, and G × E interactions that deviate the phenotypic performance cannot be completely removed. Third, sufficient recombinants in the QTL region cannot be obtained from limited NIL or CSSL populations. For a comprehensive fine-mapping strategy, both major-effect and minor-effect QTLs should be fine-mapped using all segregating populations. The method described here uses a sequential QTL fine-mapping strategy to accomplish this goal.

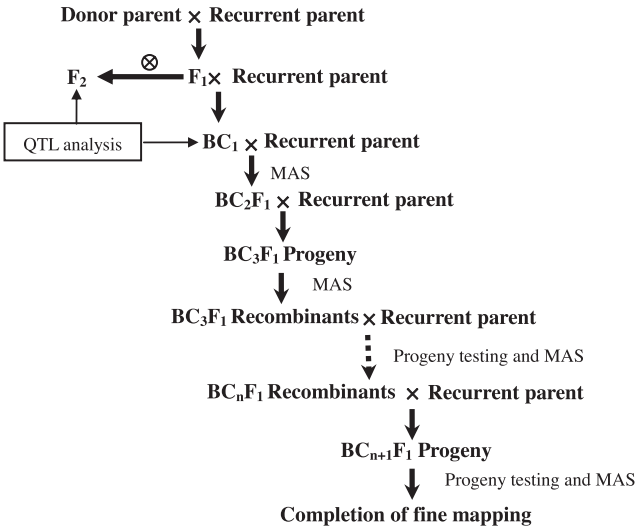


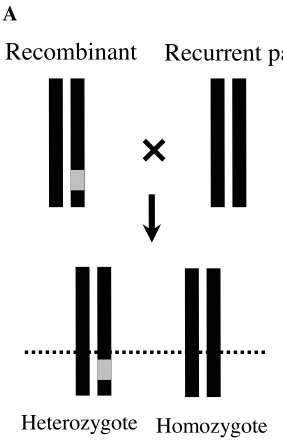
Figure 1. The sequential quantitative trait locus (QTL) fine-mapping procedure.

The donor parent and recurrent parent, which differ in the target trait, are crossed to produce the F_1 hybrid, which is either backcrossed to the recurrent parent to produce the first backcross generation (BC_1) or self-pollinated to obtain an F_2 population. Both BC_1 and F_2 populations are used for QTL analysis. With marker-assisted selection (MAS), the BC_1 individual with the target QTL is backcrossed twice to the recurrent parent to produce a BC_3 population. The recombinants from each generation are identified with QTL-tagged markers and backcrossed to the recurrent parent, and this process is repeated from the BC_3F_1 to the advanced BC_nF_1 backcross generations. Progeny testing and MAS are conducted across backcross generations until completion of the fine mapping of the target QTL.

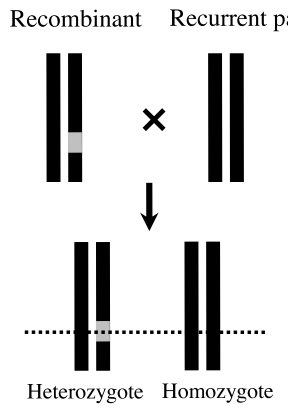
The QTL Fine-mapping Procedure

To minimize 'noisy' genetic backgrounds, we suggest initiating QTL fine mapping from the third backcrossed or selfed generation (BC_3F_1 or F_3) or even subsequent generations. With the aid of markers in the QTL region, the BC_3F_1 or F_3 individuals that underwent crossing-over within the confidence interval are selected for further backcrossing (or selfing) to produce progeny for QTL fine mapping (**Figure 1**). These backcrossed (or selfed) progeny share similar genetic backgrounds with either heterozygous or homozygous genotypes at the QTL region, hence ensuring accurate assessment of the genetic effect of the QTL.

Progeny derived from a single BC_3F_1 or F_3 recombinant are planted in two or three replicate plots, and the genotype and phenotype are investigated for each individual. Genotypically, progeny are divided into the heterozygous or homozygous genotype subgroups, with or without the donor segment in the QTL region, respectively. In each plot, the mean values of the trait should be calculated for each subgroup based on all of the progeny within that subgroup and are used to estimate the difference in the trait between the two subgroups. Statistically significant difference in mean values of the trait between heterozygous and homozygous genotypes among replicate plots is determined using a paired-sample *t*-test. If more than one recombinant shares the same donor segment, these recombinants are regarded as multiple samples, and the data from all recombinants are analyzed in one statistical test. No significant difference ($P \geq 0.05$) or significant difference ($P < 0.05$) between the two subgroups indicates the absence or presence of the QTL in the donor segment, respectively (**Figure 2**). Eventually, comparison of the donor regions to phenotypes for all recombinants should enable the QTL to be narrowed down. Furthermore, genotyping of all progeny enables the identification of new recombinants within the mapped QTL region, and these new recombinants are backcrossed again to the recurrent parent (or selfed) to produce the next progeny for further QTL fine mapping. This fine-mapping process will be



Replicate	Mean value of the trait		Difference	*P*-value
	Heterozygous progeny	Homozygous progeny		
1	*y1a*	*y1b*	*y1a* – *y1b*	$P \geq 0.05$
2	*y2a*	*y2b*	*y2a* – *y2b*	
3	*y3a*	*y3b*	*y3a* – *y3b*	



Replicate	Mean value of the trait		Difference	*P*-value
	Heterozygous progeny	Homozygous progeny		
1	*y1a*	*y1b*	*y1a* – *y1b*	$P < 0.05$
2	*y2a*	*y2b*	*y2a* – *y2b*	
3	*y3a*	*y3b*	*y3a* – *y3b*	

Figure 2. Progeny evaluation strategy to detect a quantitative trait locus (QTL) in the donor segment.

The recombinant screened from a previous backcross generation is backcrossed to the recurrent parent to produce its progeny. The progeny consist of the heterozygous and homozygous genotypes, with or without the donor segment at the QTL region (respectively), which segregate at a theoretical ratio of 1:1. The progeny are planted in two or three replicate plots. Differences in mean values of the trait between the two genotypes are analyzed using a *t*-test to determine whether there is no significant difference ($P \geq 0.05$; A) or a significant difference ($P < 0.05$; B) suggesting the absence or presence of the QTL in the donor segment, respectively. The donor chromosome region (gray) and QTL position (dotted line) are indicated.

sequentially carried out until the candidate genes underlying the target QTL are identified (**Figure 1**).

Minimal Progeny Numbers Required to Detect an Authentic QTL

Before a QTL can be fine-mapped, the number of progeny required to detect an authentic QTL should be estimated. In a fixed backcross generation, the heritability of a QTL determines the number of progeny required to detect the QTL (**Figure 3**). A simple simulation shows that QTLs with various coefficients of determination (R^2) require different numbers of progeny to detect the QTL at $P < 0.05$. A major-effect QTL requires few progeny, whereas minor-effect QTLs demand a large number of progeny. For example, 20 or 80 progeny are sufficient to detect a QTL ($P < 0.05$) with R^2 values of 0.30 or 0.10, respectively (**Figure 3**). With a large progeny population (e.g. 200 individuals), a minor-effect QTL with a very low R^2 value (e.g. 0.05) can also be detected (**Figure 3**). When the recombinant frequency (r) is considered as a parameter, more individuals are required to reveal a true QTL. In practice, it is thus advisable to plant more progeny than estimated to guarantee the stability of QTL fine mapping.

Through continuous backcrossing to a recurrent parent, genetic backgrounds of recombinant-derived progeny are gradually recovered to that of the recurrent parent. Accordingly, a QTL should account for more of the total phenotypic variation

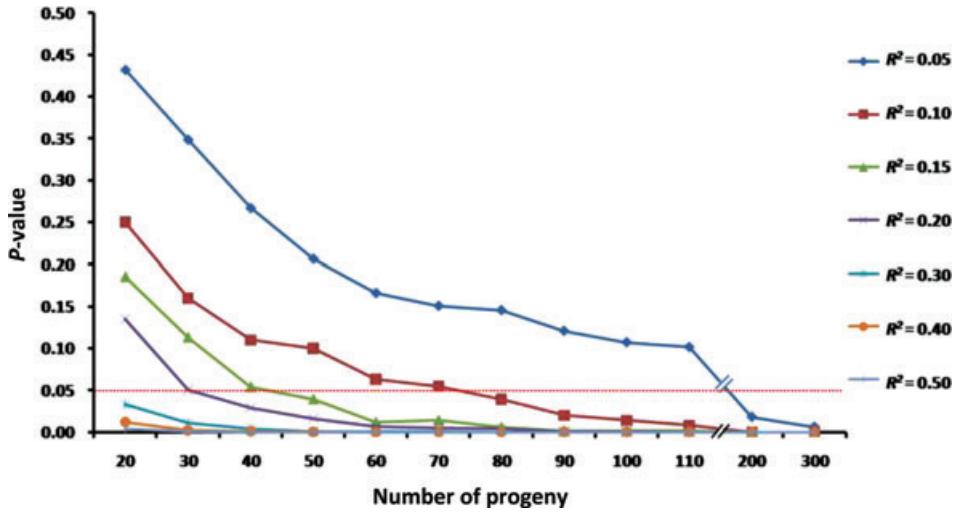


Figure 3. Number of progeny required to detect quantitative trait loci (QTLs) at various R^2 values.

The minimal number of progeny needed to detect different QTLs, ranging from 0.05 to 0.5 R^2 values, at $P < 0.05$. Here, the x-axis denotes the number of progeny, and the y-axis represents *P*-value.

in advanced generations as compared with early generations, and the R^2 value of a QTL should thus become larger through successive backcrossing. With a fixed progeny size (e.g. 30 individuals), QTLs ($P < 0.05$) with R^2 values of 0.20 or 0.10–0.15 can be detected in BC_3 or BC_4 generations, respectively. Obviously, 30 individuals are far from enough to detect minor-effect QTLs with R^2 values of <0.05 (**Figure 4**). Overall, the *P*-values sharply decrease from BC_1 to BC_4 and then become more constant in advanced backcross generations. The results imply that it would be best to start screening recombinants in BC_3 and to conduct progeny tests in BC_4 generation.

As mentioned above, both QTL heritability and genetic background uniformity are key elements in detecting the genetic effect of a QTL. Based on the R^2 value of a QTL and a fixed backcross generation, the number of progeny can be manipulated so as to reveal the true QTLs. Once the presence or absence of a QTL in the donor region can be determined for every recombinant, fine mapping of the QTL is then achievable.

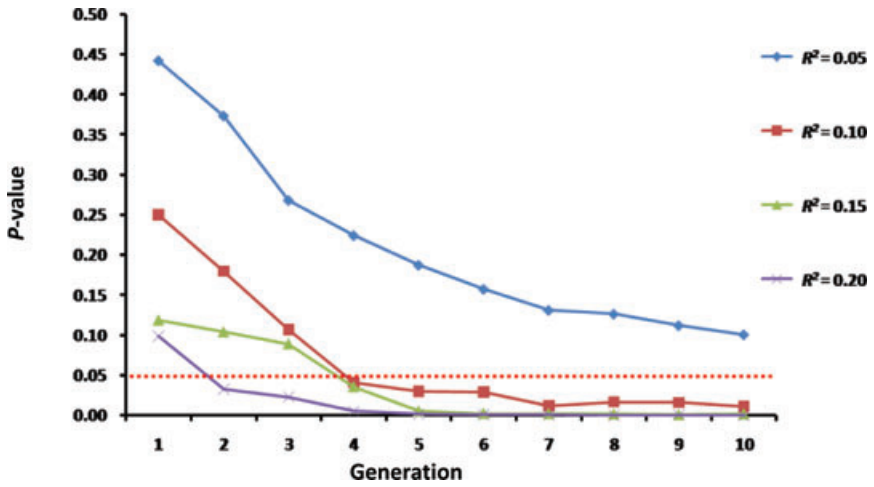


Figure 4. Backcross generation suitable to start the quantitative trait locus (QTL) fine mapping.

With a fixed number of 30 progeny, the starting generation can be estimated to detect QTLs with various R^2 values at $P < 0.05$. The x-axis denotes the number of backcross generation, and the y-axis denotes *P*-value.

The Merits of the Sequential QTL Fine-mapping Strategy

The sequential QTL fine-mapping strategy has many advantages as compared with other fine-mapping methods. First, all progeny derived from a single recombinant are randomly grown in the same plot, environmental errors are thus distributed as the normal distribution $N(\mu, \sigma^2)$, where, the parameter μ is zero and σ^2 is the variance. The 'noisy' genetic backgrounds among a single recombinant-derived progeny are also distributed as the normal distribution $N(\mu, \sigma^2)$. When the difference in the mean values between two subgroups is used to stand for phenotypic value for a given recombinant, the experimental errors resulting from environmental conditions, 'noisy' genetic backgrounds, and G $\times$ E interactions could be maximally decreased so as to reveal the authentic genetic effect of target QTLs. Theoretically, the number of progeny can be increased to reveal the true phenotypic difference between homozygous and heterozygous genotypes for any QTLs. Simulation results show the minimal number of progeny required to detect significant differences between two genotypes for different QTLs at various R^2 values (**Figure 3**). More accurate phenotype characterizations should be obtained from larger populations of progeny. Second, new recombinants within the mapped region can be obtained in each mapping generation by genotyping all progeny. The phenotypic performance of all recombinants can be compared to confirm the location of the target QTL, and the increased number of recombinants will improve the mapping precision. This effort guarantees the sequential fine mapping of the target QTL until the candidate genes that underlie the QTL are detected. In principle, this QTL fine-mapping strategy does not differ from other fine-mapping approaches. However, it provides a powerful method to minimize experimental variations so as to reveal the authentic genetic effect of a given QTL and to obtain as many recombinants as possible for QTL fine-mapping.

Application of the Sequential QTL Fine-mapping Method

In our laboratory, three QTLs have been successfully mapped to a few candidate genes with this QTL fine-mapping method. A minor resistance QTL, *qRfg2*, against *Gibberella* stalk rot in maize, could explain only 8.9% of the total phenotypic variation (Yang et al. 2010). This minor-effect QTL has been fine-mapped to a genomic interval of ~6 kb, encompassing a single candidate gene (Zhang et al. 2012; data not published). At the final mapping steps, the recombinants screened from advanced backcross generations provided unambiguous evidence for the genetic effect of *qRfg2*. Similarly, a major QTL (*qRfg1*) for resistance to *Gibberella* stalk rot and a QTL for head smut resistance (*qHSR1*) in maize have also been fine-mapped using this mapping method (Xu et al. 2010; Yang et al. 2010). Overall, the sequential QTL fine-mapping method provides a very powerful approach for fine-mapping QTLs, particularly minor-effect QTLs, which have been regarded as a major challenge in QTL cloning.

Perspectives

How to find quantitative trait nucleotides (QTNs) that underlie a QTL is a prerequisite for QTL cloning. A single QTL region may consist of multiple closely linked QTLs that often have opposite effects (Flint and Mackay 2009). A striking example was provided by a detailed dissection of the effect of a QTL on growth rate. Initially, the 210 kb region was not found to be associated with growth rate in a genome scan of QTLs in *Arabidopsis thaliana*. This genomic region was later found to contain two tightly linked QTLs with clear, albeit minor, effects on growth rate, which occur in opposite directions in the two parental strains used to construct the mapping population (Kroymann and Mitchell-Olds 2005). The dissection of a 32 kb region containing a QTL with a large effect on yeast growth ability at high temperature identified three tightly-linked QTLs with lesser effects that were the true genetic basis of this trait (Steinmetz et al. 2002). As demonstrated above, the process of defining QTNs will be accelerated by this mapping method because of its powerful minimization of experimental variations to pinpoint the QTNs underlying a QTL. Encouragingly, next-generation sequencing technologies could provide huge numbers of polymorphisms for QTL fine mapping.

When a QTL is fine-mapped, the markers within or flanking the target QTL can be used in MAS to accelerate pyramiding of QTLs that contribute to important agronomic traits. As more QTLs become fine-mapped or cloned, molecular-designed breeding becomes more realistic in crops. So far, however, only a few QTLs have been cloned, and QTL-based breeding requires further work. Instead, genome-wide selection has been proposed for genetic improvement of agronomic traits, which is based on marker-trait relationships across the whole genome. When all QTLs that underlie agronomically important traits have been detected, true molecular-designed breeding will be achieved, and agriculture will enter a new era that will allow dramatic increases in the global food supply.

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