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Genomics tools for QTL analysis and gene discovery

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In recent years, several new genomics resources and tools have become available that will greatly assist quantitative trait locus (QTL) mapping and cloning of the corresponding genes. Genome sequences, tens of thousands of molecular markers, microarrays, and knock-out collections are being applied to QTL mapping, facilitating the use of natural accessions for gene discovery.

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Abbreviations
EST expressed sequence tag
HIF heterogeneous inbred family
LD linkage disequilibrium
NIL near isogenic line
QTL quantitative trait locus/loci
SFP single feature polymorphism
SNP single-nucleotide polymorphism

Introduction

Quantitative trait locus (QTL) mapping has been in wide use for nearly two decades during which molecular markers have become available in conjunction with interval mapping methods [1]. The goal of QTL mapping is to determine the loci that are responsible for variation in complex, quantitative traits. In some situations, determination of the number, location and the interaction of these loci is the ultimate goal; often, however, the identification of the actual genes and their functions are of interest. For example, breeding studies attempt to identify the loci that improve crop yield or quality, and then to bring the favorable alleles together into elite lines. Understanding of the response of QTL in different environments or genetic backgrounds can lead to the development of improved crop varieties through traditional breeding. If the genes underlying the QTL are known (i.e. the QTL have been 'cloned'), then transgenic approaches can also be used to directly introduce beneficial alleles across wide species boundaries. In evolutionary studies, QTL define the genetic architecture of traits that are related to fitness and that differ between recently derived species [2]; however, knowledge of the actual genes allows for studies of molecular evolution. Studies of crop varieties and their wild progenitors have been effective in identifying large-effect QTL under artificial selection during crop domestication [3].

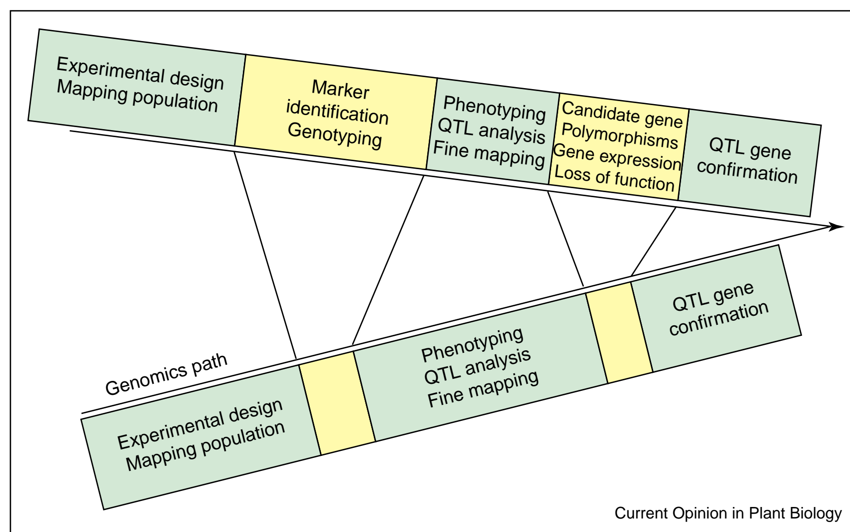
Until recently, QTL mapping was limited by the availability of molecular makers and the tediousness of their genotyping. Several high-throughput technologies that shift the burden of QTL mapping to gathering the phenotype information are now routine. In this review, we describe genomics tools and resources being used for QTL mapping (Figure 1) and cloning in *Arabidopsis thaliana*, including microarrays, which are used for both genotyping and gene expression analyses. Perhaps the most obvious genomic resource for QTL mapping is a complete genome sequence; but we also discuss what can be done with synteny and mapped expressed sequence tags (ESTs) when no complete sequence is available.

Molecular markers

Several studies have been published or are in progress that have, for all practical purposes, eliminated the need to identify new molecular markers in *Arabidopsis*. Cereon Genomics released 56 670 single-nucleotide polymorphism (SNP)/indel candidate polymorphisms from the 2–3X shotgun sequencing of the Landsberg *erecta Arabidopsi*s accession [4•]. Schmid *et al.* [5•] identified 8688 candidate SNP/indel polymorphisms from EST and sequence-tagged site (STS) reads of up to 12 accessions; in this case, the approximate allele frequencies are known for many polymorphisms. Magnus Nordborg plans to sequence up to 2000 fragments from a plate of 96 accessions for SNP discovery and linkage disequilibrium studies. At the time of writing, 15 388 polymorphisms are available from 824 fragments (M Norgborg, unpublished; http://walnut.usc.edu). The physical positions of 1267 traditional amplified length fragment polymorphisms (AFLPs) are also available [6].

Genotyping technologies, which allow these markers to be processed quickly, have also come on line rapidly. Several approaches require that fragments spanning an SNP be amplified, after which extension reactions interrogate the polymorphic base [7–9]. Several approaches differ mainly in the way that the alternative alleles are detected. Often, the SNP amplification reactions can be multiplexed; but usually, a maximum of 10 SNPs can be amplified per reaction. The individual marker price continues to decline as these methods become routine.

Figure 1



Genomics technologies have reduced several time-consuming steps (yellow boxes) in the identification of the genes that are responsible for quantitative traits. High-throughput genotyping techniques and large marker collections help at early stages, whereas gene expression and knock-out collections are important at late stages.

Array hybridization is another source of polymorphisms. Affymetrix-type high-density oligonucleotide arrays may contain millions of 25mer features. Each has the potential to identify a marker when the arrays are hybridized with labeled total genomic DNA [10••,11]. At present, data are available for more than 19 000 non-singleton single feature polymorphism (SFPs) from 14 accessions (http://naturalvariation.org/sfp). With SFPs, the actual base-pair change is not known; all that is known is that a particular 25mer is likely to have a change because of the differential hybridization of the genomic DNA from two parents. Thus, genomic DNA hybridized to expression arrays can be used as both a discovery and genotyping platform. This is an attractive method if many genotypes are required per sample. Spotted oligoarrays may be a less expensive alternative [12] but may suffer from lower reproducibility and batch-to-batch variation.

Analysis methods

Bulk segregant mapping is aptly suited to parallel genotyping methods. Array hybridization with DNA from pools of segregating lines, selected for alternative phenotypes, can quickly identify the location of large-effect mutations [10••]. Recently, we have extended this technique to quantitative traits by pooling lines that have extreme phenotypes (D Wolyn, JO Borevitz, J Chory, unpublished).

Linkage disequilibrium (LD) analysis in particular will benefit from high-resolution genotyping because several adjacent SNPs are needed to determine haplotypes. Hence, genomics has helped to realize LD as a tool for fine mapping of QTL [13]. Current studies aim to develop databases of high-resolution genotype information from a large collection of *Arabidopsis* accessions (http://walnut.usc.edu). This collection can be phenotyped for the trait of interest. With this information at hand, LD-mapping studies aim to associate quantitative phenotypes with haplotype information, a process known as '*in silico*' mapping [14]. The information provided by *in silico* maps, in conjunction with that from traditional QTL mapping studies, may provide a powerful way of quickly localizing QTL candidate genes. The extent of LD in *Arabidopsis* is estimated to be 50–250 kb [15], which limits the resolution for fine mapping but, conversely, makes it more likely that significant associations will be found in genome-wide scans. In maize, in which LD is on the scale of a few kilobases, associations can identify the underlying gene if properly controlled at other loci [16,17,18•,19].

Given plentiful markers and high-throughput genotyping technologies, QTL studies are limited by reliable phenotypic measures and multiple observations. Experimental design is therefore paramount. Every QTL experiment includes several sources of variation; for example, variation between experiments, between lines, and within lines. The importance of each source depends on the goals of the experimenter, and its impact depends on the design used. With unlimited resources, replication of the complete experiment (including generation of the mapping population) at different times and in different places surely decreases all sources of variation. Whenever resources of fixed, however, it makes sense to choose a design that minimizes the sources of variation that have most impact on the experimental goals. For example, if

one would like to conclude that QTL are repeatable across several independent experiments (perhaps across seasons or locations), then several studies must be performed. If the fine mapping of QTL is the primary goal, then number of recombinant lines that are used should be maximized and experimental variance minimized, often in a single large experiment. In this case, the experiment has been performed only once and, with the resulting data alone, one cannot conclude that the QTL are repeatable. QTL must be confirmed in near isogenic lines (NILs) or heterogeneous inbred families (HIFs) [20,21]. NILs contain a small introgressed fragment in an isogenic background, whereas HIFs are derived from a single recombinant inbred line that segregates a single QTL region in an inbred background that is a mixture of the two parents.

Once QTL are confirmed, they can be characterized further in several environmental conditions and/or genetic backgrounds. The NIL or HIF is also the starting material for the fine-mapping and cloning of the QTL. The availability of plentiful polymorphisms is a boon for fine-mapping because marker discovery is often rate-limiting at this stage. In some cases, the QTL can be mapped directly to the gene [22,23]. Usually, the selection of candidate genes can begin once QTL have been localized to a relatively narrow region (3 cM or less).

Candidate genes

When a full-genome sequence is available, perusing the annotation can often suggest genes in the QTL interval for further study. Predicted functions and gene ontologies help to guide the selection of candidate genes. The process of selecting candidate genes relies on a wealth of information gained through traditional genetics and molecular approaches. Keeping gene annotation up to date with current publications is an important task. Recently, there have been some successful examples of the use of the candidate gene approach to identify QTL genes. The gene encoding the CRYPTOCHROME2 photoreceptor was shown to be responsible for the phenotypic variation associated with a flowering-time QTL [24]. In rice, three QTL have been identified as candidate genes [25–27] whose function was known from studies of *Arabidopsis*.

Once a candidate gene is selected, the first follow-up experiment is usually to sequence the gene in the two parental lines and to look for variation that is predicted to have a functional consequence. As few QTL have been cloned, it is hard to make generalizations about what kind of changes will have phenotypic consequences, but certainly nonsense polymorphisms and deletion polymorphisms make the candidate gene more likely. Amino-acid changes [24,28], as well as expression level changes, may also be important in providing functional variation [29–31]. Several functional alleles have been identified at some QTL loci [32–36]. In such cases, the previous identification of high-density polymorphisms allows the interval to be screened for changes that might have functional consequences [37]. In this regard, genomic DNA hybridization to arrays can reveal changes and potential deletions in genes that make excellent candidates [10••]. A new flowering-time QTL has been identified by this approach in our group (J Werner *et al.*, unpublished).

Gene expression studies in which the NIL QTL is compared to that from the parental line (or an alternative QTL allele from another HIF) for differences in gene expression can also be used to identify candidate genes. Several replicate lines are used to control for biological variance and potential maternal effects. The conditions and tissue selected for the gene expression study must be chosen on the basis of the phenotype of the QTL. Experiments that look at differences in gene expression under several conditions will be more powerful. A set of conditions in which the QTL has no effect provides a control for changes that are unrelated to the phenotype; however, changes in constitutive gene expression may also suggest QTL candidate genes. Gene expression studies also characterize the downstream transcriptional response of the QTL. Thus, genes with expression-level differences that map to the QTL are candidate genes, whereas genes that map to other locations are part of the molecular phenotype caused by the QTL. An alternative experimental design involves the use of lines from the mapping population that have extreme phenotypes. Replicate pools of extreme lines can be profiled independently, so that differences in gene expression will be specific to the phenotype and genotype that separates the pools. This strategy was recently used to identify candidate genes for drought response QTL in rice (S Hazen, personal communication). Large-scale studies are underway to map QTL for gene-expression differences (eQTL) by individually profiling lines from a mapping population. Often, the eQTL map to the gene itself, indicating that *cis* changes are responsible for the different levels of expression; however, the presence of groups of genes that are coordinately regulated by a single unlinked QTL suggests that *trans*-acting factors are controlling expression [38••,39•].

QTL gene confirmation

Once candidate genes have been identified, they need to be tested functionally. A first test of gene function is to identify a null mutation. In *Arabidopsis*, thanks to several massive functional-genomics projects, we have a near-saturating collection of sequence-indexed T-DNA disruption mutants (http://signal.salk.edu; [40•,41••]). More than one null allele can often be identified for most candidate genes within the QTL interval, and the quantitative phenotypes of these alleles can subsequently be measured. The ultimate step in QTL confirmation is to reintroduce alternate alleles (using transgenic techniques)

into reciprocal QTL lines or null mutant backgrounds to show that each allele has a significantly different effect on the phenotype. To date, this has been done for at least two plant QTL [24,42]. Another elegant way to confirm a QTL gene is to use gene replacement, which has been demonstrated successfully in rice [43]. Gene replacement can be used to specifically substitute alleles at the QTL locus while maintaining the correct genomic context, as was performed recently in *Drosophila* [44•].

Conclusions

Several genomics tools are available in *Arabidopsis* that facilitate QTL mapping and cloning (Figure 1). What can be done if an organism does not have a complete genomic sequence? One approach is to use synteny with a relative that has a sequenced genome to identify candidate genes in the region of the QTL [45]. If ESTs are available, they should be mapped so that they can also serve as candidate genes should they fall within the QTL region. One approach to map ESTs quickly is to use oligonucleotide arrays designed from ESTs. Customized arrays can now be designed with no up-front costs [46•]. The hybridization of genomic DNA from parental lines will identify polymorphisms in 25mer features on the array that correspond to these ESTs. Oligonucleotide arrays can then be used to create a high-density genetic map by genotyping a mapping population. This high-density genetic map will resolve the location of many of the ESTs that can serve as candidate genes. Furthermore, these arrays have a dual purpose as they can also be used in expression studies to identify candidate genes. It should be possible to use arrays designed for closely related species for both of these purposes. Spotted arrays may also be effective for mapping ESTs if polymorphisms can be detected. Deletion lines [47] can be used to map ESTs on any array format.

The next five years should see a burst in the number of QTL cloned, thanks to advances in genomics. These QTL will reveal new genes and new alleles of known genes that have evolved in particular genetic backgrounds under specific environmental pressures.

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