Gene discovery for crop improvement

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Future improvements of crop plants will benefit from the isolation and characterization of genes that underlie both simply-inherited and polygenically-controlled traits. The molecular isolation of economically important plant genes has been facilitated by the construction and application of genetic maps, transposon-based gene tagging, protein–protein interaction cloning, and the development and analysis of large collections of cDNA sequences.

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Abbreviations

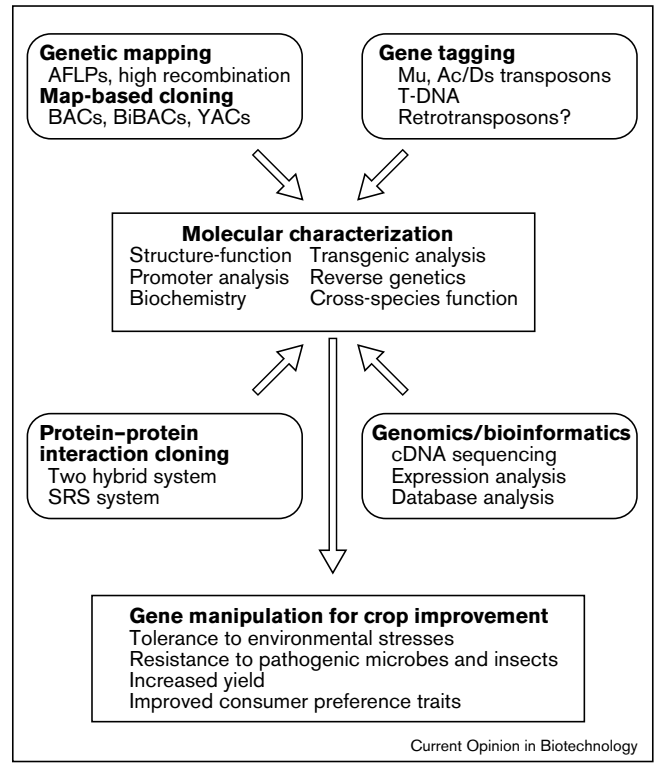
Ac/Ds	activator/dissociation
AFLP	amplified fragment length polymorphism
AIMS	amplification of mutagenized sites
BAC	bacterial artificial chromosome
EST	expressed sequence tag
GEF	GDP-GTP exchange factor
Mu	Mutator
QTL	quantitative trait loci
R	disease resistance
T-DNA	*Agrobacterium* transfer DNA
YAC	yeast artificial chromosome

Introduction

Plant breeding has been remarkably successful in the improvement of both qualitative and quantitative traits that affect agronomic performance and consumer preferences. Our inadequate knowledge about underlying biological processes continues, however, to impede breeding progress for quantitatively-inherited traits such as environmental stress tolerance, yield, and even some simply-inherited traits such as resistance to pathogens and insects. Future improvement of these traits will be facilitated by a deeper understanding of complex metabolic and signal transduction pathways that are involved in their expression. The discovery of new genes provides an important starting point to dissect these fundamental plant processes and to manipulate them for the improvement of crop plants.

There has been much progress in the development of strategies to discover new plant genes. In large part, these developments derive from four experimental approaches: firstly, genetic mapping in plants and the associated ability to use map-based gene isolation strategies; secondly, transposon/T-DNA tagging which allows the direct isolation of a gene; thirdly, protein–protein interaction cloning that permits the isolation of multiple genes contributing to a single pathway or metabolic process; and finally, bioinformatics/genomics — specifically the development and use of large EST databases, search software, and methods to quickly analyze gene expression in response to environmental stresses or during specific developmental stages. Recent progress in these four areas is permitting the isolation of many new plant genes that will have a major impact on crop improvement (Figure 1).

Figure 1



Gene discovery in plants. BiBACs, binary vector/bacterial artificial chromosome; SRS, Sos-recruitment system.

Genetic mapping and map-based cloning

Genetic maps based upon large numbers of cloned DNA markers are now available for all major crop plants and several model plant species (http://probe.nalusda.gov). These maps have revealed that related species often have highly conserved gene order along extensive segments of their chromosomes [1–4]. Such synteny allows cross-referencing among plant genomes, some of which have many mapped agronomically important loci [2–4], and this will expedite the identification of related genes from several species [5••,6••]. The first clear example of synteny was demonstrated between tomato and potato but conservation of gene order is perhaps most compelling among the grasses, where synteny has been found among

segments of the rice, corn, wheat, sorghum, oats and barley genomes [1–4]. In one particularly striking report, it was shown that gene order in small regions of chromosomes may be conserved even over the large evolutionary distance encompassed by the monocot-dicot division of plants [7••]. Cross-genome referencing may thus prove to be a widely applicable gene discovery strategy for linking well-studied model genomes such as *Arabidopsis* with crop plants.

Genetic linkage maps on which agronomically important loci have been located are also proving useful for mapping expressed sequence tags (ESTs) or PCR products encoding motifs that are characteristic of certain classes of gene products. Recently, this strategy was used to isolate and map DNA sequences with homology to disease resistance (*R*) genes from plants [8–10,11••]. *R* genes encode various motifs including leucine-rich repeats, nucleotide binding sites, protein kinase domains, interleukin-1 receptor homologies, and other short conserved sequences with unknown function. To isolate homologs of *R* genes, these features were targeted by degenerate PCR or EST database searches. The resulting sequences were placed on genetic maps to determine whether they are located near known *R* loci. This approach successfully identified over 100 *R* gene homologs from potato, soybean and *Arabidopsis* [8–10,11••]. It is important to note that close linkage or even co-segregation with a particular *R* locus is not sufficient evidence that the candidate gene is responsible for disease resistance; confirmation requires analysis of mutant alleles or complementation of the mutant phenotype by transformation of the cloned gene into the mutant plant. Nevertheless, genetic mapping of candidate genes should prove generally useful for isolating many genes whose products display highly conserved motifs.

Probably the most important development in genetic mapping over the past two years has been the demonstration that quantitative trait loci (QTL) can be localized near cloned DNA markers with very high resolution [12,13,14••,15]. QTL often underlie traits of great economic importance and are typically difficult to manipulate in plant breeding programs. Genetic maps based on DNA markers have allowed the dissection of some quantitative traits into single component loci which contribute substantial percentages of the phenotypic variation for a trait. For example, the tomato *fw2.2* locus accounted for up to 47% of the phenotypic variance in fruit weight, and the *fs8.1* locus accounted for up to 37% of the phenotypic variance in fruit shape [13,14••]. Although such associations have been observed in many crops for different QTL, it has now been demonstrated that QTL can be localized very precisely near specific DNA markers using nearly-isogenic lines [13,14••]. A surprising result from QTL mapping involving germplasm of wild species of crop plants is that the allele contributing the positive effect is often derived from the parent that is not especially notable for the trait under study [15,16,17••]. For example, a QTL that contributes to dark red fruit color in tomato was identified in a wild tomato species with fruit that does not even turn red upon ripening [16]. These observations indicate that many useful genes are present in germplasm collections of wild species and primitive varieties of crops. Plant breeders frequently ignore such collections because they do not show obviously valuable phenotypes, they are difficult to screen, and often are not adapted to temperate environments. Identification of DNA markers linked to specific QTL offers the possibility of marker-assisted selection for these traits, and high resolution mapping of QTL will allow map-based cloning of the genes responsible.

Map-based cloning involves the identification of closely linked DNA markers and use of these markers to isolate a genomic clone that spans the target locus. The genomic clone is used to identify candidate genes by sequencing, complementation of a mutant plant with subclones, or by use of the clone as a hybridization probe to isolate corresponding cDNA clones. Key developments in this strategy have been the realization that saturation of the target region with markers and the development of very high resolution maps (with less than 1 cM between the target locus and linked marker) can greatly expedite gene cloning [18]. It is relatively easy in plants to analyze large segregating populations, and the development of high-throughput DNA markers has expedited high resolution mapping of many loci. As a result, map-based cloning, although still not routine, has been successful in plants ranging from *Arabidopsis* (with a genome size of 145 Mb), to rice, sugar beet, tomato and barley (430 Mb, 760 Mb, 950 Mb, and 5000 Mb, respectively; [19–21,22••]).

DNA markers are used to identify DNA polymorphisms among different crop varieties. They have proven useful for following the inheritance of genetic loci in segregating populations. Originally, DNA markers consisted of RFLPs (restriction fragment length polymorphisms detected by hybridization of genomic DNA with either random genomic clones or cDNAs), or PCR-based markers such as random amplified polymorphic DNAs (RAPDs). Recently, other PCR-based strategies have been developed, such as amplification from small DNA linkers attached to restriction fragments (AFLPs; [23]), amplification of simple-sequence repeats (SSR; [24]) or amplification of the spacer regions between these repeats (inter simple-sequence repeats [ISSR]; [25]). These high-throughput marker strategies in combination with near-isogenic lines or bulk segregant analysis now allow a specific region of the genome to be saturated with DNA makers with much less effort than previously. AFLPs in particular have proven integral to the isolation of several *R* genes, including the *Mlo* gene from barley and the *Rx* gene from potato [22••,26].

The identification of very closely linked markers potentiates the direct isolation of a large-insert genomic clone spanning the target locus without the need for chromosome walking [18]. Such chromosome 'landing' greatly simplifies gene isolation by eliminating the difficult procedure of isolating overlapping clones spanning a region. The availability of extensive physical maps of rice and *Arabidopsis* is further simplifying this step of map-based cloning in these species [27–29]. An impediment to map-based cloning in the past has been the relative difficulty of constructing and manipulating yeast artificial chromosome (YAC) clone libraries. The development of bacterial artificial chromosome (BAC) vectors has allowed the construction of large BAC libraries for many crop species [28,30,31]. It is easier to isolate large amounts of DNA from BAC clones than YAC clones; thus, subsequent isolation of end-clones and subclones for complementation is expedited. Complementation of a mutant phenotype by transformation of a clone carrying the putative target locus, or comparison with multiple mutant alleles, remains the definitive proof that the target gene has been cloned. The development of a BAC vector and a YAC system that can be used directly in *Agrobacterium* transformation promises to facilitate this often time-consuming step [32•,33•].

Map-based cloning strategies usually culminate in the need to identify the target gene within a large region of genomic DNA (30–400 kb). This process can be simplified if the gene is 'marked' by a deletion as sometimes results from fast-neutron or gamma irradiation [34]. Such deletions are often detectable by using the candidate gene as a probe on genomic DNA or mRNA blots. In some cases, a simply scorable phenotype, such as resistance or susceptibility to a disease will permit identification of plants that have an intragenic recombination between mutant alleles giving rise to the wild type trait [22••]. Other promising approaches include the capture of cDNAs by using hybridization to the BAC/YAC DNA bound to nitrocellulose or latex particles, or sequencing of the entire BAC/YAC insert followed by computer analysis for regions with high probability of protein-encoding sequences [22••,35••]. In the syntenic cereal genomes where there is little conservation of repeated sequences, it appears feasible to identify potential genes by hybridization of a BAC/YAC from a related species to a cDNA or genomic library of the target species [5••,6••].

Transposons/T-DNAs

Gene discovery based on gene inactivation by insertion of a transposon or T-DNA into an open reading frame or regulatory sequence continues to be successful in many plant species [36–38,39•]. A particularly powerful approach (and one which can also shed light on gene function) is the use of large maize populations that contain active Mutator (Mu) transposons [36]. Mu transposons are especially amenable to gene discovery because they cause high mutation rates, insert randomly throughout the genome rather than preferentially to linked sites, and are stable once inserted. Several groups have developed populations that contain thousands of Mu-insertions for determining the function of genes that have already been cloned. However, such a 'site-selected insertion'strategy requires knowledge of the gene sequence in order to develop PCR primers.

A related strategy has recently been developed to aid in the discovery of previously uncharacterized genes that are known to carry a Mu insertion [40•]. Previously, such Mu-tagged alleles were identified by the co-segregation of a Mu-hybridizing restriction fragment with the mutation. Determining co-segregation is often difficult, however, because Mu can be present in many copies in the genome complicating analysis by hybridization. A new approach termed 'amplification of mutagenized sites' (AIMS) incorporates the AFLP approach of adding DNA linkers to restriction fragments followed by PCR with linker-targeted primers and Mu-specific primers [40•]. Mu-tagged fragments that differ by as little as a few base pairs are visualized by this method, and products that co-segregate with the mutation are used as probes for further mapping and cDNA isolation. This clever combination of techniques promises to greatly simplify identification of tagged alleles and should be applicable in any plant species where routine insertional activation is possible.

The maize activator/dissociation (Ac/Ds) transposon system is also functional in species such as *Arabidopsis*, tobacco, petunia and tomato. As in maize, Ac/Ds elements preferentially transpose to linked sites on the chromosome in these species. Thus, for gene identification by transposon inactivation it is an advantage if an Ac/Ds insertion is present near a target gene. *Arabidopsis* and tomato populations are being developed in which transposon insertions have already been mapped to facilitate targeted gene tagging [37,38,41]. Potential limitations to heterologous transposon tagging are the relatively low efficiency of transformation in some plant species which makes generation of large transposon-tagged populations difficult and, in *Arabidopsis*, the observation that Ac shows reduced levels of activity. The recent identification of *Arabidopsis* mutants that show increased Ac excision (although low levels of re-insertion) may lead to a clearer understanding of host factors involved in controlling transposon activity and may improve their utility in gene discovery [42].

The integration of *Agrobacterium* transfer DNA (T-DNA) into plant genomes (primarily *Arabidopsis*) has also proved useful for tagging genes [39•]. Populations of over 40,000 T-DNA-tagged lines are now available in *Arabidopsis* and are being used to isolate large numbers of new genes. Estimates with these populations indicate that as many as 35–40% of the mutant lines carry a T-DNA insertion within a gene. T-DNA tagging combined with a gene trapping approach may increase this number to 80% [43•]. Based on mapping studies, it appears that T-DNA

insertions occur randomly within genes and throughout the genome. Thus, it should be feasible to use this approach to saturate the *Arabidopsis* genome. By using a combination of a site-selected insertion (or AIMS-style) PCR strategy with multiplexing of DNA pools derived from these lines it should soon be possible to identify an insertion in practically any gene in the *Arabidopsis* genome.

Finally, retrotransposons (which transpose by using an RNA intermediate) have been proposed as a possible tool for gene discovery in rice [44]. Although these elements are typically inactive during normal growth conditions, certain subclasses of the elements are active during tissue culture. One rice retrotransposon, Tos17, appears to be a good candidate as it can be activated by tissue culture, induces mutations at high frequency, and its copy number is quite low in some rice cultivars making it easier to identify new insertions [44].

Protein–protein interaction cloning

Gene isolation methods often rely on the ability to detect a mutant phenotype in the plant; however, genes that are indispensable or that give no discernible phenotype when mutated may be overlooked by such approaches. For example, sequencing of the yeast genome revealed that, despite the intensive study of this organism, 35% of the open reading frames had not been uncovered by genetic analysis. One approach to overcome this limitation is to use protein–protein interaction as a basis for gene isolation [45]. This approach relies on the fact that protein–protein interactions are fundamental to many cellular processes, including signal transduction, transcription, and metabolism. Thus, to identify genes contributing to a particular phenotype, a single gene is first identified that plays a critical role in a biological process, and the gene is then used as a 'bait' to capture other genes whose products physically interact with the protein encoded by the bait gene. In the past, assays of protein–protein interactions relied on biochemical techniques. Recently, genetic approaches, such as the yeast two-hybrid system, have proven extremely powerful in isolating new plant genes [45,46].

The yeast two-hybrid system relies on the physical interaction of a 'bait' protein fused to a DNA binding domain with a 'prey' protein fused to a transcription activation domain. Interaction of the bait and prey proteins activates expression of a reporter gene by localizing the transcriptional activation domain near the cognate promoter [46]. The two-hybrid system has been successful in testing interactions between specific plant proteins that were predicted to interact [47,48]. In addition, new genes have been discovered by using 'interaction hunts' involving a characterized bait protein and a cDNA library fused to the transcription activation domain. Using this approach, genes have been isolated that play a role in signal transduction [49,50•,51], transcription [52] and a metabolic pathway [53].

Despite this progress, the yeast two-hybrid system has several shortcomings. Particularly large or hydrophobic proteins often do not make good bait proteins, presumably because they do not fold properly or have difficulty entering the yeast nucleus. In addition, proteins with regions rich in acidic amino acids are sometimes found to activate the reporter genes autonomously when fused to the DNA-binding domain precluding their use as bait proteins. Introduction of a transcriptional repressor domain in-frame with the bait protein has been shown to suppress autoactivation of a reporter gene by the p53 protein [54] and may prove useful in identifying interactors with plant transcription factors and other 'autoactivators'. It might also be possible to surmount reporter gene autoactivation by truncating the bait protein, although this could interfere with the identification of interacting proteins that require the complete protein for interaction. To circumvent some of these limitations, new protein–protein interaction systems have been developed that do not rely on transcriptional read-out [55•,56].

The Sos-recruitment system avoids the requirement for reporter gene transcription by relying on protein–protein interactions directly in the yeast cytoplasm [55•]. The system depends on restoration of growth to a temperature-sensitive yeast GDP-GTP exchange factor (GEF) mutant, by expression of a truncated mammalian GEF (hSos) that must be localized to the plasma membrane. A bait protein is fused to hSos and the prey protein (or cDNA library) is fused to a myristylation targeting sequence. When the two fusion proteins are expressed in the yeast GEF mutant, cells grow only if the bait protein physically interacts with the myristylated prey fusion protein, thus recruiting hSos to the plasma membrane. The Sos-recruitment system was tested using the previously known interaction of c-Jun and c-Fos, and was also used to isolate new c-Jun interacting proteins [55•]. A potential drawback of the system is the large size of the truncated hSos protein (115 kDa) which, in fusion with large bait proteins, might lead to low expression or hinder physical interactions.

Another promising approach for detection of protein–protein interactions in the cytoplasm uses intracistronic complementation of the *lacZ* gene [56]. The system relies on the creation of bait and prey fusion proteins with mutant α and ω domains of the β-galactosidase protein that are active yet show reduced affinity for one another. Interaction of the bait protein with a prey forces interaction of the β-gal peptides and reconstitution of β-galactosidase activity. The system was developed using mammalian cells but it seems likely that it might be adapted for screening cDNA libraries against a specific bait protein in yeast. A similar approach for testing protein–protein interactions directly in plant cells would be especially useful, but the high

endogenous β-galactosidase activity in plant cells would probably preclude the direct application of this system.

Finally, because protein–protein interaction screens often give rise to many candidate interactors, it is important that strict criteria be developed for testing the biological significance of putative partner proteins. Such criteria include testing specificity of the interaction by substituting a closely related but non-functional partner protein in the assay [49,50•] and demonstrating interaction *in vivo* by co-immunoprecipitation or other biochemical techniques [53]. Ultimately, the most convincing evidence for biological significance is to show that perturbation of the candidate gene expression by mutagenesis, antisense, or overexpression alters the phenotype under study [49].

Genomics/bioinformatics

Bioinformatics — the application of high volume information technology to biological databases — has created a paradigm shift in gene discovery. Large-scale sequencing of cDNA and genomic clones and the analysis of databases that contain this information underlie many gene discovery approaches [57,58••,59]. A clear match to an anonymous cDNA or previously characterized gene in the database simplifies the identification of transcribed regions within large inserts of BAC or YAC clones, candidate transposon-tagged genes, or genes encoding specific protein-interactors. A recent effort to identify overlapping ESTs derived from the same gene and group them into cDNA contigs further increases the utility of the *Arabidopsis* database for gene identification [60•].

To date, large numbers of cDNA sequences are publicly available for rice and *Arabidopsis* and numerous EST projects are underway for other model organisms and economic species (see http://probe.nalusda.gov, and the dbEST database at http://www.ncbi.nlm.gov/). Large cDNA databases for corn, soybean, and probably other crops have been developed by private industry but, to date, these have not been released publicly. Refinements in optimizing the sequence information in the *Arabidopsis* database and in search software have accelerated progress in identifying large numbers of genes based on specific motifs [11••,59]. For example, it has recently been possible using a search of the EST database, to identify a large number of cDNA clones that encode proteins with similarity to bacterial cellulose synthase [59]. Previous to this work, cellulose synthase genes had been notoriously difficult to isolate and this 'cloning in silico' promises to facilitate research on plant cell walls.

The pattern of gene expression in different tissues and developmental stages, and in response to specific stresses provides insight into gene function and, thus, plays an important role in discovery of new genes. In the past year, there has been exciting progress in the development of new techniques to analyze gene expression on a genome-wide basis. Two especially promising methods are hybridization to microarrays of characterized cDNAs [61,62,63••], and serial analysis of gene expression [64••]. The power of cDNA microarray technology when applied to the full collection of yeast genes and a large collection of human genes has been recently demonstrated [62,63••]. *Arabidopsis* and rice, with their large cDNA collections and EST databases, are prime candidates for similar analyses. The serial analysis of gene expression technique has been used to analyze gene expression differences between cancerous and normal human cells [64••]. From an examination of over 300,000 transcripts, 500 were identified that were differentially expressed. A similar approach to examine gene expression in plants during pathogen infection or environmental stresses, for example, would be expected to yield large numbers of new genes which may play a role in plant response to these events. Genome-wide expression differences in plants carrying specific mutations or overexpressed transgenes should similarly aid in gene discovery as well as functional analysis of previously characterized genes.

Conclusion

Convergence of genetic and physical maps, transposon tagging, protein–protein interaction screens, and the burgeoning information from genomics projects all point to rapid progress in the identification of new genes for crop improvement. The challenge will be to elucidate the function of these genes at the molecular level and to manipulate the genes in crop plants to produce cultivars with enhanced disease resistance, stress tolerance, quality traits, and the potential for production of useful new products.

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