Genomic approaches to plant disease resistance

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Genomic approaches are beginning to revolutionize our understanding of plant disease resistance. Large-scale sequencing will reveal the detailed organization of resistance-gene clusters and the genetic mechanisms involved in generating new resistance specificities. Global functional analyses will elucidate the complex regulatory networks and the diversity of proteins involved in resistance and susceptibility.

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Abbreviations

BAC	bacterial artificial chromosome
EST	expressed sequence tag
LRR	leucine-rich repeat
Mb	million base pairs
NBS	nucleotide binding site
QTL	quantitative trait locus
TIR	toll-interleukin receptor
VIGS	viral-induced gene silencing
YAC	yeast artificial chromosome

Introduction

The transition to a new era of biological research is underway, and both the public and private sectors are moving to exploit the new tools and opportunities presented by genomics. In response to the promise of both fundamental advances and profitable applications, there has been an infusion of funding that is enabling large-scale experimentation and rapid progress. New technologies are also permitting experimentation on a scale that was previously unimaginable. The massive amounts of data beginning to be generated are providing new insights and challenges. The transition will affect most areas of biology, and disease resistance in plants is no exception.

Genomic approaches are already beginning to impact fundamental and applied plant biology. Over the next ten years, there will be paradigm shifts in experimentation (Table 1). A transitory change is occurring from hypothesis-driven research to a period of descriptive study involving the generation of large amounts of data. Subsequently, hypotheses will often be derived from *in silico* analyses of databases, and testing may initially involve computer simulations prior to actual experimentation. Testing of hypotheses will still require detailed phenotyping, but experimental studies will access a broad range of new tools capable of global analyses of RNAs, proteins, and metabolites rather than a gene-by-gene or protein-by-protein approach.

This review considers the trends in genomics and their current and potential impact on disease resistance, particularly on resistance genes and the genes that they regulate. True to the title of this journal, this review has to be a current opinion rather than a retrospective review because the application of genomic approaches to resistance gene research is still in its infancy and there are few papers presenting primary data. Nevertheless, by inference from other fields, particularly the medical and microbial areas, it is obvious that we are entering an era of rapid change and that our understanding of disease resistance will be very different a few years from now.

Definitions

Genomics is the discovery and study of many genes simultaneously on a genome-wide scale. Three interrelated areas have been variously described: structural genomics, which is primarily concerned with the determination of genome structure at the sequence level; comparative genomics, which involves the molecular basis of differences between organisms at a variety of taxonomic levels; and, functional genomics, which focuses on the function of genes. Structural genomics is the discovery engine for the other areas. Comparative genomics provides the allelic variation for functional genomics. Proteomics is an outgrowth of functional genomics that involves global studies of gene expression at the protein level. Bioinformatics is the acquisition, curation and interrogation of large collections of complex biological data.

Structural genomics

Advances in sequencing chemistries and automation as well as computational power and algorithms have revolutionized our ability to generate and analyze immense amounts of DNA sequence data. Technologies currently under development will probably increase this capacity yet further through massively parallel sequencing and microfluidic processing. The complete genomic sequences of a variety of microorganisms and an increasing number of model organisms are being determined, including those of *Arabidopsis* and rice. Large portions of the genomes of crop species will also be sequenced as technologies improve and costs decrease; this will be facilitated by the identification of and focusing on gene-rich islands of the genome [1,2]. Sequencing of resistance gene clusters is an objective of projects recently funded by the National Science Foundation Plant Genome Program [3].

The nucleotide binding site (NBS) is a protein motif that is present N-terminal to a leucine-rich repeat region (LRR) in predicted proteins encoded by the majority of resistance genes cloned from a variety of species (reviewed in [4•,5]). These NBS can be grouped into two distinct classes: those with toll/interleukin-1 receptor homology N-terminal to the NBS (i.e. the toll-interleukin receptor [TIR] class) and those without (i.e. the non-TIR class). The TIR class

Table 1

Plant genomics, now and in five to ten years.

Activity	Now	Five to ten years
Sequencing	*De novo* generation	Rice, *Arabidopsis* and large portions of other crop species sequenced Resequencing of allelic variants
Genetic mapping	Based on segregation analysis Low density maps for many species High density maps for a few	Hybridization to contiguous arrays of genomic clones Extensive inferences between species based on macro- and micro-synteny
Gene expression	Predominantly at mRNA level Sequencing of random cDNAs Microarray analysis beginning Proteomics in its infancy	Quantitative catalogs of all expressed genes will exist for many species and situations Routine global analyzes using DNA chips, protein arrays and/or other technologies
Gene discovery	Gene-by-gene basis High throughput phenotyping of mutants beginning	Candidate gene approaches by relating phenotypes to sequenced genomes Bulk discovery relating differentially expressed sequences to phenotypes
Comparisons between homologs	Emphasize similarities for functional inferences	Analyses of allelic differences to explain variation in function
Focus of research	Basic biology of model species	Transfer of paradigms from model species to crops and study of crop problems in model species
Traits characterized	Mostly simple Mendelian traits	Complex traits and genotype x environment interactions
Experimental design	Initially based on empirical observations Refined through practical experience	Often derived from *in silico* analyses Simulated *in silico* and refined before being performed

includes proteins encoded by the resistance genes *N* from tobacco, *M* and *L6* from flax, and *RPP5* from *Arabidopsis*. The non-TIR class includes proteins encoded by the resistance genes *RPS2* and *RPM1* from *Arabidopsis*, and *I2*, *Mi*, and *Prf* from tomato as well as *Dm3* from lettuce.

Genomic sequencing of *Arabidopsis* and rice has already yielded interesting insights into the numbers and organization of disease resistance genes. Analysis of 1.9 million base-pairs (Mb) of *Arabidopsis* suggested that ~14% of the genes are potentially involved in disease resistance, encoding either signaling components or antimicrobial proteins [6]. Analysis of ~67 Mb representing > 50% of the *Arabidopsis* genome, detected 120 predicted gene products with similarity to the NBS domain encoded by plant R-genes [7••]. Assuming a similar distribution of genes in the remaining 50% of the genome, ~200 NBS-encoding genes are present in *Arabidopsis* (~150 encoding NBS of the TIR-type and ~50 of the non-TIR type). This would represent close to 1% of all *Arabidopsis* genes. NBS-encoding sequences tend to be clustered in the *Arabidopsis* genome [7••,8,9,10•]; numerous phenotypically defined resistance loci map to the clusters of NBS-encoding sequences on chromosomes IV and V [11,12]. Analysis of the currently available BAC end-sequences, which represent ~5% of the rice genome, suggests that there are probably 750–1500 NBS-encoding genes in rice [7••]; this estimate is several times greater than the number that would be predicted for rice on the basis of the representation of NBS-encoding genes in *Arabidopsis*. All NBS-encoding genes in rice encode non-TIR-type NBS; TIR-type genes have not been detected in genomic or expressed sequence tag (EST) sequences from any grass species [7••,13••]. This raises interesting evolutionary questions as to how a dispersed family of sequences, which is present in progenitors of angiosperms and abundant in dicotyledenous plants, now appears to be absent or diverged beyond recognition in grass genomes.

So far, only a few clusters of resistance genes have been sequenced. The complete sequencing of the *RPP5* (encoding NBS-LRR-type proteins) cluster in *Arabidopsis*, the *Cf-4/9* (encoding LRR-transmembrane-type proteins) and *Pto* (encoding protein kinases) clusters in tomato, and partial sequencing of the *Dm3* (encoding NBS-LRR-type proteins) cluster in lettuce revealed highly duplicated regions containing little more than resistance-gene homologs. The *RPP5* cluster contains 8–10 homologs spread over ~90 thousand base-pairs (kb), interspersed with protein kinase pseudogenes and retrotransposons [14•]. The *Cf-4/9* cluster contains five resistance genes spread over 36 kb; the *Cf-4/9* homologs are interspersed with fragments of *Lox* genes, which may have played a role in the duplication of the region [15]. In the *Pto* cluster, five *Pto* homologs are spread over 60 kb along with a single NBS-LRR gene, *Prf*, that is necessary for the function of at least two members of the *Pto* cluster ([16]; DT Lavelle *et al*., unpublished data). The *Dm3* region in lettuce is the largest resistance gene locus charac-

terized at the molecular level so far; at least 24 resistance gene homologs are spread over at least 3.5 Mb. There was no evidence for functional genes in the *Dm3* region, other than homologs of *Dm3* and transposon-related sequences [17,18•]. Sequencing of resistance gene loci in other species will determine whether the organization of the *Dm3* region in lettuce is typical of species with moderately sized genomes and whether species with larger genomes have correspondingly larger clusters of resistance genes.

Comparative genomics

Only a finite number of chromosomal rearrangements have occurred during the evolution of angiosperm plants. Significant blocks of genetic material may therefore be colinear (i.e. syntenic) among genomes of related species. Macrosynteny based on linkage analysis is becoming increasingly well-documented among monocot species as well as among *Brassica* species and *Arabidopsis* [19]; nevertheless, preliminary data indicate only limited synteny between monocot and dicot species [20,21]. Also, comparison of orthologous regions at the sequence level reveals that the level of microsynteny is variable. As several plant species, particularly rice and *Arabidopsis*, are sequenced and once the extent and pattern of synteny has been established for a particular species, it will become possible to predict the position of some, but not all, of the genes in each part of the genome. One of the challenges in comparative genomics is to distinguish orthologs (i.e. homologous genes with a common ancestor that have been separated by a speciation event) from paralogs (i.e. homologs resulting from a gene duplication event); this is particularly problematic within large diverse multigene families such as the resistance genes.

There have been few studies that have directly addressed the synteny of resistance genes. Resistance genes may be located in less stable regions of the genome in which synteny is poorly preserved. The chromosomal positions of resistance-gene candidate sequences seems not to be preserved between grass species [22]. Homologs of the *RPM1* gene are missing from susceptible genotypes of *Arabidopsis* [23,24]. Attempts to use synteny with rice as part of map-based cloning strategies for the *Rpg1* resistance genes in barley (which has a larger genome) were only partially successful because the *Rpg1* homolog was missing from the rice genome, although flanking markers were syntenous between rice and *Arabidopsis* [25]. Resistance-gene homologs are located in syntenic positions within the Solanaceae but the resistance specificities encoded by these genes are not conserved [26]. In several species, resistance genes seem to be either telomeric or close to the centromere. For example, of the two resistance-gene clusters in lettuce that have been localized by fluorescent *in situ* hybridization, one was telomeric and the other centromeric [27]; *Rpg1* in barley is telomeric [25] whereas the *Mi* gene in tomato is at the border of centromeric heterochromatin [28]. It will be interesting to see if these patterns hold for clusters of resistance genes in many species because chromosome rearrangements often involve changes close to the telomere and centromere; chromosomal position may therefore contribute, at least partially, to the lack of synteny of some resistance genes.

Sequence similarity between cloned resistance genes has allowed the use of PCR with degenerate oligonucleotide primers for the cloning of large numbers of resistance-gene candidate sequences from diverse species [7••,13••,29–32]. These sequences often map to regions containing known disease resistance genes. Over 130 NBS-encoding sequences similar to those of known resistance genes currently in public databases have been identified by PCR [7••,13••,33]. This number will continue to increase as this approach is applied to an increasing number of species and new combinations of primers are used to amplify different subsets of sequences [13••]. As such work progresses, the likelihood that a candidate sequence will be available when a new resistance gene is genetically mapped will greatly increase.

Currently, candidate sequences, with the exception of those from *Arabidopsis*, can only be mapped by analysis of segregating progeny. In *Arabidopsis*, resistance-gene homologs were mapped relative to known resistance genes by hybridization to an ordered array of yeast artificial chromosome (YAC) clones [10•]. Ordered contiguous bacterial artificial chromosome (BAC) clones are now available for *Arabidopsis* [34], but mapping by hybridization to such an array will be superceded as the genomic sequence approaches completion. Arrays of contiguous genomic BAC clones will become available for rice and corn, and later soybean. Hybridization to such contigs will provide a rapid and accurate method for mapping cloned sequences and will replace segregation analysis. Such a hybridization strategy has the added advantage that it does not require polymorphism between the parents of a mapping population. High-throughput genotyping will allow the high-resolution mapping of phenotypic resistance genes relative to PCR-based markers or using DNA chips [35]. Together, these technologies will facilitate the isolation of many resistance genes of known specificity (see below).

High-throughput genotyping will also facilitate the genetic analysis of populations that are large enough to allow the accurate mapping of quantitative trait loci (QTLs) determining quantitative disease resistance and the dissection of genotype x environment interactions. Integration of QTL mapping with genomic sequence data and information on allelic differences will provide the basis for candidate-gene approaches to cloning the QTLs for disease resistance.

Another application of high-throughput genotyping will be in monitoring the dynamics of allelic variation at resistance loci in wild populations. At present, it is difficult to sample enough individuals to allow conclusions about the evolutionary forces influencing resistance-gene diversity

to be reached [23,36•,37]. Nevertheless, functional tests of minor variants will still be needed to confirm that the same resistance specificity is being expressed because a few changes in amino acid sequence may result in different specificities (e.g. [36•]).

One of the surprising results from inter-species comparisons of resistance-gene-related sequences is that orthologs tend to be more similar than paralogs [4•,7••,13••,32]. This evidence has led to the idea that resistance genes are not evolving rapidly in order to keep pace with changes in the pathogen, but rather are evolving fairly slowly to provide resistance against pathogen populations that are heterogenous in space and time [4•]. The same conclusion was reached using analysis of sequences flanking *RPM1* in *Arabidopsis* [23]. These results do, however, contrast to those obtained from analyses of the *Cf-4*/*Cf-9* and *RPP5* clusters in tomato and *Arabidopsis*, respectively [14•,15]; considerable haplotype diversity was observed within these clusters that was interpreted as indicative of high rates of instability. It is clear that a variety of genetic mechanisms, including point mutation, recombination, unequal crossing-over and gene conversion, generate diversity in resistance-gene clusters and may prevent a reliable designation of homologs within a cluster as orthologs or paralogs [4•,14•]. The relative contributions of each of these mechanisms to generating diversity in resistance-gene specificities remain to be determined.

Comparative genomics also provides allelic variation for research into the molecular basis of specificity. So far, only a few domain-swap experiments have been reported [38••] and their results suggest that the LRR region of NBS-LRR-encoding genes is an important, but not the only, determinant of specificity. As the efficiency of sequencing improves, libraries of resistance-gene-related sequences will be generated from such studies. These sequences will also act as templates for gene-shuffling experiments [39,40] for the generation of new resistance-gene specificities.

Functional genomics

A variety of methods for global analyses of gene expression combined with predictions from DNA-sequence data are greatly increasing our ability to make inferences on gene and protein function [41,42••,43]. Methods for global analysis of protein profiles and cataloging protein–protein interactions on a genome-wide scale are technically more difficult but improving rapidly, although they have yet to be applied extensively to plants. Genetic stocks encompassing insertions or deletions in nearly every potential gene will become available for the analysis of phenotypes in model species such as *Arabidopsis*. Catalogs of genes expressed under a range of different conditions, in different organs, or in different individuals will become available within a few years. The global analysis of plant gene expression is still in its infancy and its full potential is still far from being realized. Both the technology and the algorithms for collecting, displaying and analyzing the vast amounts of quantitative expression data are still being developed [43]. Careful standardization and replication are required to provide robust data sets and to allow comparisons within and between experiments.

Genes that have altered expression in compatible and incompatible plant–pathogen interactions have been targeted for characterization by microarray analysis [42••,44•]. These analyses will provide comprehensive data on expression profiles, both for genes already implicated in plant–pathogen interactions as well as for many genes that were not previously known to be involved in resistance or susceptibility. A first-generation proprietary maize GeneChip (Affymetrix, Pioneer), representing 1500 ESTs or genes, identified 117 genes that were either induced or repressed six hours after challenge with the fungal pathogen *Cochliobolus carbonum* [42••]. Comparison of the regulatory regions of groups of co-regulated genes will indicate potential regulatory sequences and the regulatory networks that control their expression [45–47].

Data generated by expression profiling may imply the function of a particular gene but function will still have to be confirmed on a gene-by-gene basis. This confirmation will be aided by existing data for genes and proteins that are induced in compatible and incompatible interactions (e.g. [48,49]); although many proteins are known to be induced, few have been shown to be causal in resistance [50]. Candidate gene approaches that map phenotypes onto sequenced regions will complement gene profiling data; they will not, however, have sufficient resolution to unambiguously identify individual genes. High-throughput reverse genetics approaches for testing gene function are therefore required. Potentially powerful approaches include viral-induced gene silencing (VIGS) [51] or viral over-expression [52] as well as the use of gene knock-out libraries and promoter-trap strategies [53]. In addition to testing the function of individual genes, all three of these strategies can also be used with libraries of anonymous sequences for *de novo* gene discovery. It is likely that each of these approaches will successfully demonstrate the function of some but not all genes: a combination of approaches may be required to overcome gene redundancy or lethality associated with manipulation of some genes.

Although >20 resistance genes have now been cloned (reviewed in [5,7••]), this has required extensive map-based cloning or transposon-tagging efforts focusing on individual genes. The cloning of resistance genes will progress beyond such slow, gene-by-gene strategies. Resistance-gene discovery will become much faster and less expensive as resistance phenotypes are matched to candidate sequences identified by genomic sequencing or PCR using degenerate oligonucleotides (see above). The rate-limiting step will be the confirmation of the function of candidate genes. Antisense inhibition or sense suppression can be used to demonstrate whether a member of a multigene family encodes a particular specificity [54];

when suitable viral vectors become available for crop plants VIGS may become the preferred method by which this can be achieved [51]. The identification of genes with individual specificities is still going to require careful experimentation involving a combination of mutation and transgenic analyses. As transformation efficiencies increase, particularly if the floral-dip procedures that are now routine for *Arabidopsis* [55] can be developed for crop species, it will become possible to clone individual specificities by shotgun transformation. Nevertheless, caution is required with this approach as ectopic expression of resistance genes may result in non-specific resistance, as in the case of overexpression of *Prf* or *Pto* [56,57].

Genes encoding NBS-LRR-containing proteins are one of the most prevalent classes in plant genomes (see above) but little is known of their function. Their sequence motifs indicate that they are involved at the beginning of signaling pathways [7••,58,59]. So far, the only demonstrated role for NBS-LRR-encoding genes is in disease or pest resistance. Nevertheless, it is possible that they are involved in other aspects of plant biology including development and responses to the abiotic environment. From the few available data, most characterized NBS-LRR-encoding genes seem to be constitutively expressed at low levels. As such, their function will probably not be directly implicated by global expression analysis. Characterization of the genes induced downstream of NBS-LRR-encoding genes will, however, provide an informative way to distinguish different classes of resistance genes and provide clues as to the variety of functions performed by NBS-LRR genes.

We are embarking on a ligand-independent, gain-of-function approach to determining the function of NBS-LRR-encoding genes in *Arabidopsis*. We have used a similar approach to dissect the function of the *Pto* resistance gene [60]. Such a gain-of-function strategy is likely to be more informative than gene knock-out approaches because the pathogen target and the ligand are usually unknown; in addition, potential gene redundancy problems will be avoided. Expression array data will be generated to provide an 'induced expression signature' for each gene that will indicate their function as well as allowing us to assign them to (possibly overlapping) functional classes. Genetic data indicates that there are at least two classes of NBS-LRR genes (reviewed in [61•]). Microarray data will define these classes further and identify any new classes that exist.

Bioinformatics

Bioinformatics is critical to structural, comparative and functional genomics. There is already a vast amount of DNA genomic and EST sequence data in the public domain and an even greater amount in private databases. The amount of quantitative expression data becoming available in the public and private sectors will increase exponentially and rapidly dwarf the DNA sequence data; quantitative expression data will be considerably more difficult to manage and exploit. Improved and preferably semi-intelligent algorithms are therefore required to acquire, curate and query the data.

A specialized, thematic database of plant NBS-encoding sequences has been developed at the National Center for Genomic Research [33]. This internet database includes links to the underlying database records, data source, BLAST (i.e. basic local alignment search tool; National Center for Biotechnology Information) scores relating the NBS-encoding sequences to known R-genes, organism, map positions in *Arabidopsis* when known, and graphic descriptions of motif organization.

The existing databases can be used to search for homologs of known molecules from other signaling pathways and defense responses in other organisms. The increased power of algorithms such as PSI-BLAST (i.e. position specific iterated BLAST) [62] affords the opportunity for identification of distant homologies (e.g. for caspases) that can be the basis of functional testing of plant genes. Homologies with genes of known function in other organisms can predict the function of newly identified plant genes and provide opportunities for utilization of model systems to demonstrate function. For example, the sequence of the recently cloned *Rar1*, a gene required for Mla-1 activity in barley, implicated its involvement in a cell death pathway [63]. Likewise, similarities between the NBS region of plant resistance proteins and CED-4 and Apaf-1 in nematodes and mammals, respectively, have led to inferences of functional parallels between the cell death pathways of the hypersensitive resistance response in plants and apoptosis in animals [64,65].

Conclusions

We are experiencing a period of rapid change. We already have unimagined tools and capabilities compared with those available few years ago. There is a shift away from the identification and manipulation of individual genes to the global characterization of resistance phenotypes. The cloning of many specific resistance genes can be anticipated in the near future. Sequence comparisons and functional analysis will allow dissection of the molecular basis of specificity; and this in turn will lead to the *ex planta* generation of new resistance gene specificities.

The use of large-scale approaches will provide new opportunities for defining targets for manipulation to achieve disease resistance. Ideally, disease resistance genes control pathogens at a low metabolic cost by inducing defense responses only in those cells that are challenged by the pathogen. In the future, resistance genes will be designed that recognize essential components of pathogens and then induce the appropriate response pathways.

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