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Current Genomics Research: The Prospect of Unraveling Gene Function and Its utilization in Crop Improvement

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Research in the field of molecular biology is now carried out in a multidisciplinary environment. The environment is so diverse that more often than not, researchers in one field lose track of what is happening in a related area and yet the integration of research results is sometimes crucial to understand the complexity of some of the life processes involved. A researcher working on phytohormones may not be well conversant with structural and functional genomics and yet such knowledge is a great help for him to identify the candidate genes, clone them and study their characteristics. Furthermore, the pace of research within the past five years, particularly since the completion of sequencing of the human genome, has been phenomenal. The speed was further accelerated when sequencing of the genome of the model plant *Arabidopsis* was accomplished. Journals like *Plant Physiology* and *Plant Cell* now publish two - three times more materials compared to their previous record in the 1990s.

The assimilation of the required wealth of scientific knowledge is a formidable task, more so for scientists of the developing countries where access to the Internet is not only expensive but also extremely slow and undependable due to interference in all servers and ethernet services. This being the situation, one rarely has the patience to wait for a long period of time for sites of interest to arrive. Realizing the difficulties of scientists from this part of the world, it is planned to publish in this forum a series of articles reporting the recent outstanding development and progress in the multifaceted discipline of molecular biology covering both its fundamental and practical aspects. The current article deals with structural and functional genomics and RNA interference (RNAi).

Genome Research

The genome of any organism is its entire hereditary material represented in the form of a definite set of chromosomes and various types of organelles. The chromosomes that vary in number from organism to organism contain the basic hereditary element, DNA. DNA contains all the necessary instructions for making the molecules that carry out vital as well as all other functions, which make an organism unique and distinct from close and distant forms. Genomics is the study of an organism's genome in its entirety. While the study of traditional genetics concentrates on a few genes at a time, genomics of an organism involves the study of all of its genes present in its genome. These genes may be constitutively expressed or activated in response to an environmental signal or any other factor related to the growth and development of an organism. For instance, if a plant is under attack of a pathogen or is under water stress, a number of genes will go into action to combat the diseased condition while others will remain quiescent. Some genes may also be expressed sequentially. The advantage of studying genomics is that one gets to know the whole gamut of genes which can be expressed under certain conditions and the interplay between different genes. Some of the expressed genes can turn out to be totally unexpected.

Like traditional genetics, there are mainly two aspects in genomic studies: (a) structural- and (b) functional genomics. In structural genomics, the base sequence of all the genes that constitute an organism is determined and analyzed. In functional genomics, the focus is to understand in depth the manner in which specific genes act in carrying out various processes that are vital to an organism.

Choice of material

To facilitate genomic studies, plant scientists selected a small weedy plant known as *Arabidopsis* of the mustard family, *Brassicaceae*. The reasons to choose *Arabidopsis* as a model organism were as follows: (a) it has a small genome size with only five pairs of chromosomes; (b) it has a life cycle of only 60 - 70 days, allowing geneticists to critically analyze their data in the shortest possible time and (c) it lacks the less-informative repetitive DNA sequences that complicate genome analysis in most crop plants. The choice of suitable research material is indeed very important as it leads to successful completion of a project launched to achieve certain objectives.

Extrapolation of the results to agronomically important species

Initially there were questions in breeders' minds whether discoveries of useful genes with *Arabidopsis* contribute to the development of improved crops? Doubts were soon dispelled, when it was demonstrated that the discovery of a gene or genes for a specific trait in *Arabidopsis* makes it not only possible but also relatively easy to identify similar genes in other crop plants through comparison of their base sequences.

Such an understanding led to launching of intense research activity in early 1990 by several world-wide leading genetics laboratories. About six years later, a coordinated effort in the sequencing project was initiated in late 1996. Well over 100 scientists from Belgium, China, France, Germany, Italy, Japan, the Netherlands, Spain, UK, USA, took part in the project. By the end of 1999, the nucleotide sequence of the *Arabidopsis* genome was published in the prestigious journal Nature (Mayer et al. 1999; Xiaoying et al. 1999). The results, regarded as a milestone in the world of genetics, were a sensational revelation. Nearly 60% of the *Arabidopsis* genes were found to be homologous to their counterparts in human and the model nematode (*Caenorhabditis elegans*). In other words, a number of predicted protein-coding genes have been functionally characterized on the basis of their homology to known genes in human and *C. elegans*. For instance, a comparison between genes that induce pest resistance in *Arabidopsis* and crop plants, reveals certain common features. The discovery of such genes in *Arabidopsis* facilitates subsequent identification of their counterparts in major crop plants, thereby helping breeders to use these genes for improvement of crops such as corn, soybeans, cotton, and potatoes.

Sequencing of the rice genome completed

Some laboratories in Japan, China and the USA focused their attention to sequence the genomes of both the *japonica* and *indica* subspecies of rice. Although it was a much more laborious undertaking because of its larger genome size, rice was chosen mainly for the following reasons. It is a model monocot providing food to the majority of world population that inhabit Asia and Africa. Unlike wheat it is a diploid with 2n = 24 and most importantly the extent of repetitive DNA in rice genome is minimal. Availability of a faster method called shotgun sequencing helped rice genomic scientists complete base sequencing of rice in much shorter time than expected. The shotgun sequencing protocol employs a method which shears the entire genome bases into several thousand long DNA pieces. The protocol was devised by the outstanding scientist by the name Craig Venter of Celera Genomics and was originally used to sequence human genome. The method proved so efficient and fast that the human genome project had been completed nearly two years ahead of time. In two separate papers published on the 5th April edition of *Science*, (Yu et al.

2002) a group of 100 scientists in China headed by Dr Yu Jun at the Beijing Genomics Institute and another group of 55 led by Dr. Stephen Goff at the Torrey Mesa Research Institute (a research division of the Swiss agro-chemical company Syngenta International), have published the draft of entire DNA base sequences of the two most important subspecies of rice, *Oryza sativa*. Since the two genomes were sequenced and assembled using the shotgun cloning sequencing, the results are considered "draft" versions since many small gaps remain to be identified. An international consortium led by Japan is expected to finish a more complete version of the *japonica* rice genome later this year based on mapped overlapping clones.

While the megabase size in *indica* was reported to be 466, that of *japonica* was 420. The number of genes in *indica* genome has been estimated to be between 46,022 to 55,615 , while in *japonica* the suggested range is from 32,000 to 50,000. It may be recalled that the genome of the model plant *Arabidopsis thaliana* contains a smaller number of genes, i.e. 25,000. The above scientists discovered 98% synteny (conserved large chromosome segments among related species) between rice and other cereal genomes such as wheat, maize and barley. In contrast, the synteny between cereals and *Arabidopsis* was found to be limited. Commenting on the immense benefits that rice breeders may derive from the unraveling of base sequences, it was pointed out that the genes for synthesis of vitamin A are already present in the *japonica* rice genome in an inactivated state. In light of present findings, all is needed is to activate those genes by means of suitable promoters instead of inserting foreign genes from an altogether unrelated species, such as daffodil as was done by Ingo Potrykus to evolve Golden rice. The rice genome project will usher in a new era in the improvement of cereals in general and rice in particular.

The first step in the genomics research in an organism is the identifica-tion of all the genes in terms of nucleotide sequences. The next step, which is as important as the first, is to determine the specific function of all the genes that constitute the genome of an organism. Determination of the function of each individual gene is the objective of functional genomics. Almost all of the 25,000 *Arabidopsis* genes have been sequenced but currently scientists have been able to discover the functions of only 60% of the genes either experimentally or by comparison with other known genes. The huge task ahead is to determine what characters or traits the rest of the genes control. The task is further complicated when we take into consideration characters such as drought-, salt-, heavy metal tolerance-, disease and pest resistance. Under such stressed conditions, a number of genes, dispersed throughout the genome, are activated making it extremely difficult to pinpoint the role of individual genes that respond to a biotic or an abiotic stress simultaneously. Fortunately, the scientists have developed a powerful technology called DNA Microarray Analysis (of gene expression).

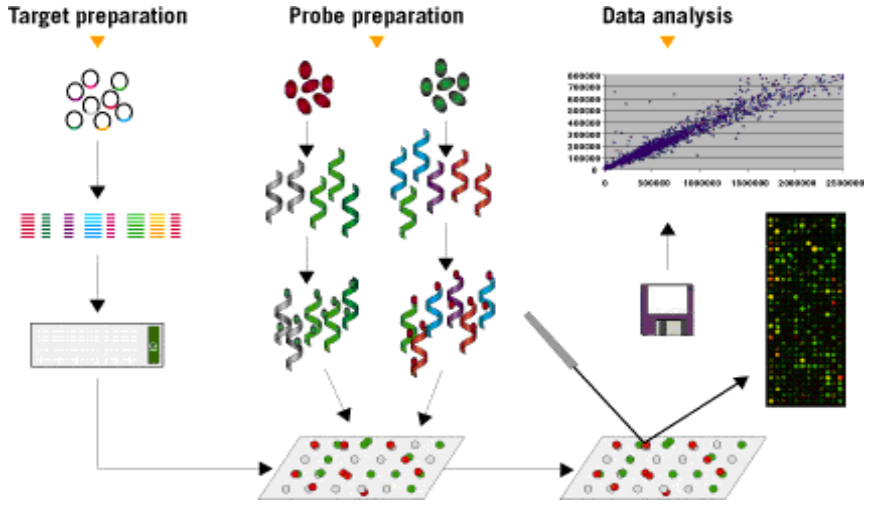
Microarray Analysis

Microarray analysis has proven to be a high-throughput method for analyzing expression levels of multiple genes simultaneously.

Analysis of several thousand genes can be carried out all at once in one experiment. This method is most suitable for identifying and analyzing genes that express differently in normal tissue and tissues under biotic or abiotic stress. The technique is described briefly as follows:

cDNA fragments extracted from all the individual genes of an organism are arranged in the form of tiny square grids in a glass slide by a computer-aided robot. Fluorescently labelled probes, prepared from cellular RNA, are targeted to cloned cDNA or gene fragments that have been immobilized on a solid microarray slide. Probes are prepared from two different plant tissues, or the same type of tissue exposed to two different conditions, and then labeled with different dyes.

The labeled probes are then incubated with the DNA already on the slide. Each probe binds only to those samples of DNA that represent expressed genes in the tissue under investigation.



Overview of the microarray procedure borrowed from Amersham Biosciences (http://www.amershambiosciences.com/cyscribe/kits.htm#Microarray)

Using a special reader slides are analyzed. This analysis allows the determination of genes that are expressed in each sample. A computer screen displays the output of the reader; the massive quantities of data produced in such experiments can also be analyzed using appropriate software developed for this purpose.

In order to determine which genes are turned on, or expressed, in various plant tissues, dyes of different colours are used. Supposing a red dye is used to label genes expressed in seeds and a green dye to label genes expressed in leaves, yellow spots would indicate expression of the same gene in both locations. Genes that are not expressed in either tissue will show unlabeled spots.

Microarray technology has proved a powerful tool to study the class of genes whose expression changes during environmental stresses. These genes differ greatly in their ability to tolerate various stresses such as cold or drought. For instance, mild frost kills some plants, such as tomatoes, while crops such as winter wheat are not affected by freezing weather. Professor Michael Thomashow and his colleagues in the Department of Crop and Soil Sciences at Michigan State University have identified a number of genes which are activated by exposure to cold (Thomashow, 2001). Genes such as *COR* (cold responsive), acting in unison with a family of cold-regulated transcriptional activators: CBF1, CBF2 and CBF3 protect the plants from cold and frost damage. Research is under way to determine whether the *CBF* genes can be used to increase the freezing tolerance of crops of agronomic importance. DNA microarray analysis can be employed to identify which genes in a plant are controlled by the CBF regulatory genes. New insights gained through such studies will help understand the mechanisms of cold and possibly drought tolerance making it possible to breed varieties of crop plants with enhanced stress tolerance. With further studies, more genes contributing to yield increase and enhancement of nutritional qualities will be discovered. Use of such genes will help breeders to launch projects aimed at breaking the yield plateau reached in crop productivity with built in resistance against virulent pests and diseases and stress tolerance.

Reverse Genetics and T-DNA knock out

Since late 1990s a few more techniques have been developed to determine specific gene functions. One of the techniques is, 'Reverse Genetics'. It is an approach different from that of traditional or forward genetics. Unlike forward genetics, in which the genotype of the mutant is determined, in the new approach i.e. in reverse genetics, a mutant gene sequence is first determined and then the effect of the base sequence change in phenotype is determined. One of the protocols used in reverse genetics is to disrupt the physical structure of a gene by means of T-DNA. This is done by the insertion of a piece of DNA of known sequence. The insertional element could be a transposon. T-DNA insertion is a random event, and that the inserted genes are stable through multiple generations.

Let us now describe in brief the most commonly used technique to integrate T-DNA into the plant genome. Whole plants of interest e.g. *Arabidopsis* are dipped into suspensions of *Agrobacterium tumefaciens,* the bacterium containing T-DNA. It has proved to be a powerful method in that it results in the production of the hundreds of thousands of insertional mutations, necessary for saturation of the genome. It is to be borne in mind that the consequences of insertion of a T-DNA element into the *Arabidopsis* genome, depends on the nature of the T-DNA as well as the precise site of insertion. In an illustrated article, Patrick et al. (1999) have shown that T-DNA insertion may occur anywhere in the gene of interest: in the coding region, at the beginning or at its end, at the promoter region or at the 3' end untranslated region. The result i.e., whether the insertion has occurred at the right place, depends upon where a T-DNA element has landed: within or very close to the gene of interest. Out of six possibilities described by Patrick et al. (1999), only in one, the gene of interest will be silenced or mutagenized, if T-DNA is inserted in its coding region. A PCR (a device that can generate millions of copies of a specific DNA strand) product is formed, using a set of primers from a specific gene and the T-DNA, if the gene is knocked out resulting in the production of a null mutant.

Characterization of phenotypes

Plants that are homozygous for the mutated gene as a result of T-DNA insertion are identified and crossed to ensure that only one copy of T-DNA element is present in the selected individuals. The trait of the mutant is then compared with the wild type in terms of its altered trait, including that of growth and development. The mutated gene will be marked with a recognizable DNA sequence difference together with its altered phenotype. In other words, insertion events within specific gene is correlated with changes to a particular phenotype leading to the isolation and identification of unknown but affected gene and determination of its function. There are a number of private and public research institutes, which provide collection of insertional mutants. For instance, Torrey Mesa Research Insitute has generated approximately 100,000 individual T-DNA mutagenized *Arabidopsis* plants (Columbia ecotype) to boost up genomics research.

Realizing the importance of genomics research, several institutions have been established worldwide. One of such important institutions is: The Institute of Genomic Research (TIGR), located in Rockville, MD, in the greater Washington, D.C. Founded by Craig Venter (one of the two main architects behind the completion of human genome project) in 1992 TIGR's primary research interests are in structural, functional and comparative analysis of

genomes and gene products from a wide variety of organisms including *Arabidopsis* (see their web site: http://www.tigr.org). TIGR has joined a well-known microarray company, Affymetrix in order to develop an *Arabidopsis* whole genome chip. The work on the whole genome chip has been completed recently (May 14, 2002) and the chip is now being tested. The new technology will immensely speed up functional genomics work and before long, the function of most of the *Arabidopsis* genes will be in public databases for use in other crop studies or for crop improvement.

RNA interference in plants

One rigorous way to analyze the function of a specific protein is to test the effects of suppressing the expression of the gene that encodes this protein. Reverse genetic techniques are used to "knockout" or reduce gene function of a specific target gene with a known DNA sequence. There are two reverse genetics techniques namely antisense suppression and cosuppression by overexpressing a gene to supress mRNA levels of a specific gene and examining the phenotype of the transgenic plant. In the past several years, RNA-induced gene silencing has emerged as a novel and promising reverse genetics approach for analyzing protein function in eukaryotic cells (for general reviews see Fire 1999; Sharp 2001). This method is effective in both plants and animals and has been generically referred to as RNA interference (RNAi), although the mechanism of silencing appears to be somewhat different in plants than in animals (Vaucheret et al. 2001). We will explain the RNAi terminology here in this review. Its discovery in plants arose out of observations that cosuppression of gene expression or gene silencing was occurring in transgenic plants. From the applied side, this unwanted gene silencing phenomenon in transgenic plants caused serious complications for plant genetic engineering. In plants, it appears that the RNAi-induced silencing exists to provide natural virus resistance. According to this method, the introduction of double-stranded (ds) RNA into cells induces a kind of defense response in which cells selectively destroy not only the dsRNA but also single-stranded (ss) mRNA strands containing the same sequence as in the sense strand of the dsRNA. As evidence that this new technique is growing in interest and use, RNAi was first runner-up for *Science* magazine's breakthrough of the year for 2001 and there are over a dozen RNAi abstracts being presented this summer at the American Society of Plant Biologists annual meeting.

There has been a considerable effort to understand the molecular basis of RNAi. Many of the molecular components required for RNAi are now being characterized. It has been documented that cleavage of dsRNA into many

small RNAs of 21 - 25 nucleotide fragments, is required for RNA silencing in animals and plants (Waterhouse et al. 2001). The RNase enzyme that is responsible for this first degradative step has been identified in *Drosophila* and is called 'Dicer' (Bernstein et al. 2001). Another key enzyme needed for RNAi is an RNA-dependent RNA polymerase and this enzyme has been identified in *Arabidopsis* and is required for gene silencing induced by transgenes (Dalmay et al. 2000a). A requirement for this enzyme appears to be a characteristic feature of RNAi in other systems as well (Nishikura 2001). The RNA-dependent RNA polymerase acts catalytically to amplify the initial dsRNA signal allowing a very small amount of dsRNA to completely suppress the target gene. Although there have been many advances to our understanding of how cells achieve this regulation in plants, the main focus of this review will be applications of this technique in the field of plant molecular biology.

Much of the early success in applying RNAi to study the function of specific gene products was obtained in two model genetic systems, *Caenorhabditis elegans* and *Drosophila melanogaster* (Fire et al. 1998; Kennerdell and Carthew 1998). In plants, the application of RNAi is certainly aided by the availability of genomic sequence data. To use RNAi in a particular plant species requires only sequence data for the gene of interest and a method to successfully introduce synthesized dsRNA that is homologous to this gene into the plant. The next step is to document the downregulation of the endogenous gene's mRNA to determine if the dsRNA treatment was successful. Northern blot analysis is one method that can be used to monitor the success of an RNAi treatment. Alternatively one can use quantitative RT-PCR if gene specific primers are available. Finally, the consequences of downregulation of a particular mRNA can be analyzed with respect to a physiological process/response that the target gene is suspected to play a role in.

To optimize the effectiveness of RNAi for gene suppression in a particular plant system, further characterization of RNAi in that system may also be required. Specifically, one may need to experimentally determine: (1) the optimum concentration of dsRNA that will allow the most specific suppression for each gene that one wishes to silence; (2) how rapidly dsRNA suppresses the expression of genes; i.e., how rapidly does the cognate sense mRNA and the protein encoded by that mRNA disappear after treatment? (3) how long does the dsRNA-induced suppression last. Is the suppression strictly post-transcriptional or is it also transcriptional (Mette et al. 2001). (4) does a particular dsRNA designed for a single member of a multigene family act specifically or does it affect other related genes. Another important question to address for applying RNAi to suppress a target gene in order to study its

function is to determine which parts of a gene sequence are most effective for silencing. Two studies in tobacco support the idea that dsRNA is most effective when it corresponds to the open reading frame (ORF) of a gene. Using the basic -1,3-glucanase gene it was demonstrated that internal sequences from the ORF regions were more efficient targets for the silencing machinery than 3′ and 5′ untranslated regions (UTRs) (Jacobs et al. 1999). Additionally, Hutvagner et al. (2000) did not find any small RNAs in their GUS silenced lines with homology to the 3′ or 5′ UTRs.

As mentioned previously, in order to use RNAi one must have a way of introducing the dsRNA into the cell and there are a wide variety of techniques that have been used to achieve this goal in plants. A very simple approach has been successfully employed in fern systems. By supplying dsRNA in solution when imbibing dry spores, the spores readily take up the water and the dsRNA. Using this method, RNAi has been demonstrated to work in the fern *Marsilea*, and is being used to study the regulatory components of the plant cytoskeleton (Klink and Wolniak 2000; Tsai and Wolniak 2001). They found that centrin was required for formation of the motile apparatus during spermiogenesis. Interestingly, they also noted that in their system dsRNA was effective at a tenfold lower concentration when compared with ssRNA. Following protocols developed in Wolniak's laboratory, Stan Roux and his associates at the University of Texas, Austin have demonstrated that this method also works in the water fern *Ceratopteris*.

Another method for introducing dsRNA into plant cells is particle bombardment of epidermal cells. This approach has been successfully used in a study with the cereals, maize, barley, and wheat (Schweizer et al. 2000). In this study, an essential enzyme in the anthocyanin biosynthetic pathway was knocked out leading to reduced accumulation of red anthocyanin pigments. In addition, treatment with dsRNA homologous to *Mlo*, which encodes a negative regulator of race non-specific resistance to powdery mildew in barley, resulted in increased resistance in transformed cells. Another group using the same approach identified a RAC small GTP-binding protein that plays a role in resistance to powdery mildew in barley (Schultheiss et al. 2002).

Cell culture systems are an important research tool in both animals and plants and application of RNAi in these systems could make them even more useful. Several groups have successfully adapted RNAi to *Drosophila* cell cultures. Caplen et al. (2000) established that RNAi is useful in *Drosophila* tissue culture systems using Green fluorescent protein (GFP) as a marker in their assay. Another group has used RNAi in *Drosophila* cell culture for analyzing

the role of four different genes in the insulin signal transduction pathway (Clemens et al. 2000). More recently, there is a report of the effectiveness of RNAi in tobacco BY-2 cell culture using electroporation, another method to introduce dsRNA into the cells (Akashi et al. 2001). Using a dsRNA expression plasmid containing the luciferase gene under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter, RNAi effects were observed within 24 hours. One advantage of using cell culture for RNAi experiments is that it is possible to produce populations of cultured cells that are uniform and divide synchronously.

Development of additional vector systems for application of gene silencing in higher plants is a promising new area. Vectors derived from the RNA viruses potato virus X (PVX) (Dalmay et al. 2000b) and tobacco rattle virus (TRV) (Ratcliff et al. 2001) are able to cause antisense homology based silencing. More recently another group has developed a geminivirus based vector system that allows for more effective silencing in *Arabidopsis* and other plant species of both endogenous genes and transgenes (Turnage et al. 2002). Using novel transformation vectors with dsRNA-expressing constructs linked to a strong viral promoter, RNAi was demonstrated in *Arabidopsis* (Chuang and Meyerowitz 2000). In this case, the dsRNA-expressing vectors were introduced into the genome of *Arabidopsis* via *Agrobacterium*-mediated transformation. This study was done using one gene from each of four major categories of genes that play a role in flower development. The dsRNA-induced phenotypes of each gene were similar to previously reported reduction-of-function or loss-of-function mutants. *In situ* hybridization analysis was performed to confirm that production of the mRNA corresponding to one of the flowering genes was indeed blocked.

Originally, the RNAi phenomenon hindered progress for scientists working with transgenic plants in plant biotechnology, but it has now developed into a new application in plant molecular biology for obtaining knockout phenotypes of specific genes. In fact RNAi technology may replace antisense strategy and become a widely used and powerful tool for reverse genetics in plants when attempting to assign a specific function for a particular gene product.

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