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Frontiers in Plant Science: Transcripts from a Symposium Sponsored by the Minnesota Academy of Sciences

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Current Plant Science Research

Gretchen Hagen

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Introduction

It is always a pleasure for me to talk about current research areas in molecular biology, an exciting area of research which I have been involved in for a number of years. I was quickly overwhelmed, however, when I started to list research areas and methods I could cover here. So, rather than try to mention them all, I will focus on a couple of research areas and the methods used in those areas. To discuss genes and how they are studied, I will talk about some of the macromolecules that concern the plant molecular biologist. I will also describe an example of the elucidation of a gene from corn, the result of Dr. Irwin Rubenstein's research at the University of Minnesota in the Department of Genetics and Cell Biology. From this example, I will discuss some methods using recombinant DNA technologies and briefly address some other current issues in plant molecular biology.

A plant molecular biologist usually begins a line of research with an observation made during the growth and development of a plant. Such observations generally lead to questions like these: what is it that is causing the observed phenomenon; and, more specifically, what genes are involved, how are these genes expressed, and what makes them become expressed at a specific time during the plant's growth and development? In exploring these questions, a plant molecular biologist faces a high level of complexity. For one thing, higher plant organisms are composed of organs and tissues, and even at the cellular level, are quite complex, containing a number of organelles and structures of interest to the plant molecular biologist. Of these, I will focus on the nucleus, the mitochondria, and the chloroplast. The macromolecules that I will be talking about — DNA, RNA, and proteins — reside in the plant cell.

Genetic Material

To examine the basic genetic material of a plant cell, one has to look at the DNA, which is organized into chromosomes in the nucleus and also is found in the mitochondria and chloroplast. A chromosome is composed of a lot of highly coiled DNA which is associated with many proteins and RNA. The DNA itself is composed of two strands that are connected to form a double helical structure. These strands are held together by hydrogen bonds between the bases adenine (A), guanine (G), cytosine (C), and thymine (T). Adenine always pairs with thymine; and cytosine with guanine.

Transcription and Translation

A gene is a specific sequence of these bases within the DNA and carries signals indicating the starting points and end points of its own information. During gene expression, information carried by the specific order or sequence of bases within the gene is first transferred to an RNA molecule through a process called *transcription*. Certain classes of RNA contain the information for the specific structure of a protein, and the transfer of that information to form a protein is called *translation*. One strand of the DNA directs the formation of the RNA. The single-stranded DNA is copied into a complementary single stranded RNA molecule using the bases G, A, C, and uracil (U) in place of thymine (T). The newly formed RNA is then shuttled out of the nucleus into the cytoplasm.

A specific class of RNA called messenger RNA (mRNA), carries instructions for the arrangement of amino acids — the building blocks of proteins. The instructions are contained in the sequence of three bases in the mRNA, and the proteins are formed through the process of translation. Within the nuclear DNA there are also regions, or genes, that code for RNA molecules called transfer RNA, which move into the cytoplasm and pick up the needed amino acids. In another section of the nuclear DNA are genes for ribosomal RNA (rRNA), an important component of cellular structures called ribosomes. All three species of RNA come together to read the information off the mRNA to make proteins.

Proteins

Proteins are extremely important to the cell not only as enzymes, which are involved in many metabolic pathways and processes, but also as structural components. Proteins are often the best place for molecular biologists to begin their research. Protein was the starting point for Dr. Rubenstein, who has studied the storage proteins contained in kernels of corn. The kernels contain several different kinds of proteins, the most abundant of which is a small group called zeins, which are made at a very specific time during kernel development. Dr. Rubenstein's research questions were: what do the genes for zein look like, and what is it that controls the expression of those genes at a very specific time during development of the kernel? After observing the proteins, Dr. Rubenstein chose to study the genes by working backward from the RNA macromolecule. Techniques are available for isolating and identifying the RNA for a specific protein, and in this case, Dr. Rubenstein isolated and identified the mRNA for zein.

Once he had the mRNA, he encountered several problems familiar to most molecular biologists. First, mRNA is not very abundant, yet large quantities of a specific mRNA are needed to do further characterization. The second problem also involves the mRNA; because it is single stranded, mRNA is not very stable and tends to be chewed up quickly by enzymes in the cell.

cDNA

A molecular biologist can use various *in vitro* techniques to address both problems. The first step involves making a more stable double-stranded DNA from the mRNA. This is done in the test tube using the bases, sugars, and phosphates needed to make DNA, and enzymes such as reverse transcriptase and DNA polymerase, which have been purified from viruses and bacteria. The product is a more stable, double-stranded, complementary copy of the mRNA called cDNA. This cDNA can then be used to study the gene and the RNA.

To end up with the necessary large amounts of cDNA, we can take advantage of an observation made by bacteriologists a number of years ago. There are some strains of bacteria that, in addition to containing the bacterial chromosome, contain a small, double stranded circular DNA called a *plasmid*. These plasmids are present in one or more copies within the bacterial cell and are important to bacteria because they contain genes that code for proteins that make the bacteria resistant to antibiotics. For our purposes, bacteria can be grown up very quickly in large quantities and then the plasmid DNA can be separated from the bacterial chromosomal DNA. The idea, then, is to take the double stranded cDNA, open up the plasmid, insert the cDNA into the plasmid DNA, take this recombinant plasmid and reinfect it into the bacteria, and grow up the bacteria. Under this technique, it is possible to get a large amount of cDNA for further characterization.

The problem then becomes how to reopen the plasmid DNA. The method we use was derived from an observation that there are specific enzymes, primarily isolated from E. coli, that recognize specific sequences of double-stranded DNA. For instance, the enzyme Eco RI recognizes the sequence G A A T T C and will cause a break between the G and A of the sequence on the inner side of a double-stranded DNA helix. The break leaves a double stranded part of the molecule with single-stranded overhanging ends. These overhanging ends can "heal" back together again, using temperature and another enzyme. The technique involves cutting the plasmid DNA with an enzyme and using the same enzyme on the cDNA to create the overlapping "sticky ends," then putting them together with heat and the additional enzyme to reseal the whole thing. We infect bacteria with these recombinant plasmids, grow the bacteria up, and isolate the plasmid, obtaining large quantities of the cDNA that we started with.

What can we do with this cDNA? How can we get back to the genes, and what information can we get from the cDNA? We can actually determine the order of bases within this cDNA. This is of interest to biologists because the cDNA is a reflection of the RNA, and, as such, reveals the RNA structure.

We can also use this cDNA to hunt for the genes from which the RNA was made. To look for a gene, one has to go back to the chromosomal DNA, and in plants and animals, chromosomal DNA is very large and very unworkable. So it is necessary to make clones of various chromosomal DNA fragments by using the same basic techniques. We isolate the nuclear DNA, use restriction endonucleases to chop up the DNA into various sized pieces, and put the DNA back into plasmids. In this way, we can separate certain sections of the chromosomal DNA in individual bacteria cells. Using radioactively tagged cDNA, we can then determine which of these bacterial cells contains the chromosomal DNA segment with the particular gene being investigated. After isolating the plasmid that contains the gene of interest, we can sequence that DNA to determine the sequence of the gene.

Signal Sequences

Dr. Rubenstein used this method with corn storage protein (zein) genes. He found that the coding information for the zein protein was in the base sequence but also identified some sequences at either end of the gene that are now called "signal" sequences. Examples of such signals include the sequence CAAAAT, called the "cat box," and the sequence TATAAATA, the "tata box," which have been found in many genes and are extremely important for gene function. Tata boxes are found at the beginning of many genes. Other signals within the coding sequence for proteins are found on the ends of the gene and also are important for gene expression.

Reviewing these signals, we find that a number of signals have a primary role in the regulation of gene expression, suggesting that it is extremely important for us to understand the sequence of a gene before we try to put it into a plant

before we try to engineer it.

Another interesting feature revealed through sequencing the cDNAs (again, a reflection of the mRNA) is that the sequence of the RNA is not necessarily an exact copy of the sequence found in the gene (in the DNA). Biologists have found that in many genes there are sequences of DNA bases that interrupt the coding sequence in the mRNA. During transcription (RNA production) these intervening sequences initially are found in the mRNA, but by the time the mRNA gets out of the nucleus, these sequences have been spliced out of the RNA that codes for protein. Once again, this points out the importance of understanding the processes that regulate the final expression of a gene particularly signals involved in transcription and translation.

Cellular Communication

I want to abruptly change gears here and talk about another area that has been under investigation in a number of different plant systems. The research involves studying the communication between the nucleus and chloroplast and between the nucleus and mitochondria. Many questions have arisen from

the observation that there are a number of proteins found in the chloroplast whose genes are actually in the nucleus. One example of this is a protein (an enzyme) called "Rubisco" that is involved in carbon-fixation in the chloroplast. The Rubisco enzyme is made up of eight large protein subunits and eight small protein subunits. The genes for the large subunit are found in the chloroplast DNA, but the genes for the small subunit protein are found in the nuclear DNA. This means that the small subunit protein comes from cytoplasmic RNAs and is transported into the chloroplast. Plant molecular biologists have tried to discover what it is that tells this protein to go into the chloroplast. Because only certain proteins go into the chloroplast, there must be specific information allowing a protein to do so. Scientists have found, often through this recombinant DNA technology, that the protein transported into the chloroplast contains a small group of amino acids called the transit peptide. This peptide channels the protein into the chloroplast and allows it to be pulled into the chloroplast. The transit peptide is clipped off from the protein, and the protein then associates with the large subunit to form the active enzyme. This is a fascinating area because scientists are interested in bringing specific peptides into the chloroplast. This now can be approached using recombinant DNA techniques — by hooking the peptides onto the chloroplast-specific transit peptide in hopes of pulling those proteins into the chloroplast. This has actually been done, although the work has not been published. Research has been directed to determine which specific sequences of the transit peptide are important for targeting it to the chloroplast. This kind of study is also going on with the mitochondria because some proteins are made in the cytoplasm from nuclear genes that are brought into the mitochondria.

New Techniques of Plant Tissue Culture and Their Potential for Plant Improvement

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Introduction

Let me begin with a question: Is genetic engineering really new? I believe it is not. Crop improvement — the engineering of plants to suit specific needs — is as old as agriculture itself. As primitive peoples made the switch from hunting and foraging to the cultivation of crops, they continuously improved the plants we now use for food and fiber.

This early kind of genetic engineering depended upon two techniques to improve plants. The first was selection. Ancient farmers probably selected plants with desirable traits — such as grains that yielded an increased number of kernels, or trees that bore larger fruit. They probably kept the best seeds for another year's crop, perhaps because they had a rudimentary awareness that the "best would beget the best." They selected and isolated plants for cultivation thus narrowing the gene pool and increasing the chances for successful cross-pollination and transfer of desirable genetic traits.

The second technique was breeding. Farmers would select two plants and deliberately cross-pollinate them in an attempt to combine the characteristics of both parents in the progeny. This technique was certainly hit-or-miss because people did not understand the principles of genetic inheritance and could not accurately predict the outcome of a particular cross. Yet, in some instances, valuable characteristics did arise in plants which could then be maintained in a population. This primitive approach to plant breeding has evolved into a powerful technology forming the basis of plant improvement in modern times.

What is genetic engineering in a more modern sense? Very loosely defined, genetic engineering is a collection of new techniques for genetically changing plants. These techniques no longer rely on pollination but instead involve genetic manipulations at the cellular and molecular levels. They promise to be powerful allies of modern plant breeding, and, as the title of this presentation suggests, many of these techniques revolve around plant tissue culture.

The history of modern techniques in tissue culture is very brief. The application of plant tissue culture to plant improvement began in 1960 when it was demonstrated that single cultured cells plated in an agar medium had the potential to divide and produce calluses. In 1976 it was demonstrated that single plant cells were totipotent, meaning a single isolated somatic plant cell could develop into a complete and fertile plant. Shortly afterward, it was shown that haploid plants could be produced from the immature pollen of cultured anthers. In 1977 plants were regenerated from single cultured protoplasts, followed by the demonstration that the somatic cell protoplasts from two different species could be fused to produce a hybrid plant. The important aspect of these discoveries was not necessarily the experimental results but the realization that plants could be manipulated in a manner similar to microorganisms.

But what does this new technology offer that existing plant breeding technology does not? In quantitative terms, it offers a potentially tremendous savings in time and space. With the new technology it may become possible to engineer in a single, short step a specific change in a plant that would require several years in a breeding program. Plant scientists could potentially grow and evaluate hundreds of millions of cells in a single flask, each a potential plant, in place of planting and evaluating the progeny of conventional crossing experiments on many acres of land.

New Plant Tissue Culture Techniques

The range of genetic variability currently available to the plant breeder is quite large and might be imagined as a series of concentric circles. At the center is a valuable cultivar to

which a plant breeder desires to transfer a valuable genetic trait. The first circle from the center represents backcrosses to the same species. The second circle represents sexual hybridization (with reduced fertility) to closely related species. The third circle represents sexual hybridization to closely related species with special facilitating techniques needed to ensure that viable progeny will result (e.g., embryo rescue). The fourth circle represents transfer of genetic characteristics from unrelated species to the cultivar using somatic hybridization and gene isolation and transfer. Finally, the fifth circle represents synthetic or molecular techniques in which all potentially important genes could be transferred to a cultivar in a single step. As one would expect, while the overall range of genetic variability increases as the genetic distance increases, the difficulty of transferring genetic information also increases.

The genetic engineering methods I will describe here are useful from the level of the cultivar to the fourth level — the transfer of characteristics from unrelated species. These new methods, which involve the use of tissue culture, are somatic hybridization, the use of vectors to transfer genes directly into plants, somaclonal and gametoclonal variation, embryo rescue, and the production of secondary metabolites in culture.

Somatic hybridization

The simplest way to combine the genetic information of two plant cells is through fusion of their protoplasts. Plant cells are normally surrounded by a rigid and complex polysaccharide wall. Plant organs, especially leaves, may be treated with enzymes, usually a combination of pectinases and cellulases, to dissolve the cell wall, liberating millions of naked cells, or, as they are more commonly known, naked protoplasts. When placed under the proper cultural conditions the protoplasts will replace their cell walls and divide again. At this point they may be maintained as a mass of callus or regenerated into plants.

The fusion product of two protoplasts contains the sum of the nuclear and cytoplasmic genomes of the parent plants. However, subsequent elimination of genetic material from one or both parents often occurs, resulting in death of the hybrid cell, inability of this cell to divide, or inability of the tissues to regenerate.

This fusion technique has been used in my laboratory in attempts to produce hybrids between the Christmas poinsettia, *Euphorbia pulcherrima*, and the annual poinsettia, *E. heterophylla*. The red bracts of the Christmas poinsettia and the green leaves of the annual poinsettia are treated with the enzymes cellulase and pectinase. Thousands of protoplasts are produced after six or seven hours. These protoplasts (some pigmented red and some green) are placed on a microscope slide and fused with the chemical polyethylene glycol. Both single and multiple fusions occur, with single fusions recognizable as a single cell that is half green and half red. These cells can be isolated using a micropipette and then cultured individually. We have found that the somatically hybridized cells remain viable, develop new cell walls after several days, and grow into small lumps of callus tissue. However, we have not been able to regenerate plantlets from the hybrid tissue.

These manipulations have been attempted for a large number of plants but have been successful in only a small number of cases and for plants that have absolutely no economic importance. Apparently, mixing two entire genomes in a single cell results in disturbed development and many cytogenetic abnormalities. Other problems also occur, such as the inability of cereal protoplasts to divide in culture. Many other crops, such as soybeans, are said to be recalcitrant, that is, they do not regenerate plants from culture. The techniques are promising, but we know too little about the fundamental processes of plant development and gene regulation to exploit them at this time.

Gene transfer by vector

In contrast to somatic hybridization where entire genomes are combined, very small amounts of DNA may be transferred to plant cells by the direct injection of DNA or through the use of vectors. Vectors are small pieces of DNA that have had a specific gene spliced into them. These gene vectors may be plant DNA viruses, bacterial or yeast plasmids, or plant organelle DNA.

In the preparation of DNA for direct injection, foreign genes are spliced to a bacterial plasmid. (A plasmid is a circular, extrachromosomal piece of DNA capable of autonomous replication and present in some bacterial cells.) The hybrid plasmid is then amplified in the bacterial cell under controlled conditions to produce millions of copies of the foreign gene. Finally, the amplified hybrid plasmids are isolated, purified, and injected directly into protoplasts, which in turn are regenerated into entire plants.

When a vector is used to transfer the DNA, genes are inserted into the virus vector, for instance, into the DNA of the cauliflower mosaic virus (CMV). Plants are then infected with the virus with the chance that the foreign gene will be incorporated into the plant's genome and thus be expressed.

Some problems have arisen with using the CMV vector. One is that only small genes or portions of genes can be spliced into the virus vector. Also, the principal hosts of this particular virus are members of the family Cruciferae, and the prospect for infecting other crop plants is small.

Another promising gene vector is the Ti plasmid found in the crown gall bacterium, *Agrobacterium tumefaciens*. Ti-plasmids are large, circular DNA molecules about 30 times larger than the DNA of the cauliflower mosaic virus. This vector is extremely interesting because the plasmid of this bacterium can insert itself into the genomes of cells of hundreds of plant types representing more than 90 families, and it causes the formation of a tumor in the infected plants. Although the mechanism by which these tumors arise is not completely understood, it is known that *Agrobacterium tumefaciens* naturally introduces the Ti plasmid DNA into the plant cell during infection. A portion of the plasmid called the T-DNA is incorporated into the nuclear DNA of the cell. The T DNA portion carries the genes for the synthesis of phosphorylated sugars and unusual amino acids called opines. The crown gall bacterium genetically "colonizes" the plant by converting normal cells into tumor cells which are then directed to produce the opines, which in turn, are used as carbon substrates by the infecting bacterium.

As with CMV, there are problems with using *Agrobacterium* as a vector. Many transformed plant cells do not express the inserted gene or do not regenerate from culture, an essential step in the method. Consequently, stability of the T-DNA must be enhanced in successfully transformed plants.

Somaclonal and gametoclonal variation

Successful application of cell and tissue culture methodology to crop improvement depends upon the ability to regenerate plants of known genetic constitution. For example, if tissue culture techniques are used as a method to clone

large numbers of individuals, it is essential that the cloned plants be similar or identical to the donor plant. On the other hand, when this methodology is used to develop a new plant variety, a selection scheme would be devised that theoretically would select only cells with altered genotypes at the target loci but which are genetically identical to the donor plant at all other loci. In other words, we do *not* want genetic variability *in vitro*.

It has recently come to light that spontaneous genetic variability occurs in both cultured cells and plants regenerated from culture. This phenomenon is called somaclonal variation — *soma* referring to somatic cells and *clonal* referring to genetic differences among and between cloned cells. (Gametoclonal variation refers to variations arising from cultured gametic tissues.)

Somaclonal and gametoclonal variation depend upon the occurrence and recovery in regenerated plants of Mendelian and non-Mendelian genetic variation from cell cultures. The genetic variation seems to result from both preexisting variation in the explant donor tissue and from culture-induced variation. Changes in the integrity of the genome are attributable to induction of mutations, mitotic crossing over and mutation, and sorting of organelles.

Although the genetic basis of somaclonal and gametoclonal variation is not completely understood, the technique has been used successfully in several cases. Promising results already have been obtained by selecting for resistance to host-specific pathotoxins, for herbicide resistance, and for tomato breeding.

What are the advantages of somaclonal variation in a breeding program? Table 1 compares somaclonal variation, gametoclonal variation, and a normal backcross program. Two categories illustrate the potential benefits of using somaclonal variation for breeding: the rate of progress and time for breeding line development. For both gametoclonal and somaclonal variation, the time required to successfully develop a new variety is significantly reduced.

Embryo rescue

This technique might be called a new concept in whole-plant genetics. Some plants have simple inherited characteristics that would be desirable to transfer to another species, but because of sexual incompatibilities the plants cannot be crossed. A Danish worker in the 1970s discovered a simple embryo rescue technique that enhances the production of hybrids between species. In this case, researchers were attempting to transfer resistance of a disease called barley yellow dwarf virus from barley to wheat. A single gene called Yd2 in barley confers resistance to barley yellow dwarf virus. A worldwide search failed to find a resistant wheat variety, so it appeared necessary to transfer the Yd2 gene from barley to wheat.

The wheat x barley cross is very difficult to make successfully because the two species are not closely related. Wheat has 21 pairs of chromosomes and barley has 7. If wheat is pollinated with barley pollen, fertilization may occur, but embryos will abort unless excised and placed on a nutrient medium. Even then, less than 1% of the embryos will survive, and they rarely produce mature plants.

The Dane discovered, however, that a hybrid embryo would survive if it were placed on the immature endosperm excised from the developing seed of one of the parents. He found that the endosperm served as a nurse tissue for the hybrid embryo, and it turns out barley endosperm is the best nurse tissue.

Still, the technique may not be entirely successful. Of 50,000 wheat ovaries pollinated by barley, only 440 showed embryo development (about 0.88%) and only 270 of those were rescued (about 0.54%). Of the rescued embryos, only 20 (about .04%) developed into plants that were true wheat x barley hybrids. The hybrids were completely male-sterile but did produce seeds when pollinated with wheat. These seeds produced plants that had at least one of the 7 barley chromosomes and 21 wheat chromosome pairs. It has not yet been determined if these plants are resistant to barley yellow dwarf virus.

Secondary metabolism

Higher plants are a source of several important medicinal substances, and the supply is dwindling at an alarming rate due to exploitation, disappearance of habitat, governmental regulation, and difficulties in cultivation. The production of medicinal substances by plant cells *in vitro* is considered a viable alternative. Cell cultures have the following advantages over natural cultivation: 1) chemical compounds could be produced year-round under controlled conditions; 2) regulation of cellular metabolism could maximize yields; and 3) cells could be genetically engineered to accumulate specific intermediates or end products.

Many of the important pharmaceutical substances pro-

Table 1. Comparison of procedures for variety development, from Evans et al.

	Somaclonal variation	Gametoclonal variation	Backcross program
Source of variation	spontaneous and induced	spontaneous and induced	natural populations
Likelihood of success	undirected variation	some direction, high percentage of success	guaranteed except where linkages not broken
Alteration of quantitative traits	possible	possible	rarely successful
Rate of progress	more than 1 trait per generation	more than 1 trait per generation	one trait in 5-7 sexual generations
Chimerism	none or low frequency	none or low frequency	none
Species limits	in all species that can be regenerated	in all species that can be regenerated	only sexually propagated crops
Time for breeding line development	one generation	one generation	up to 6 generations

duced by plants are secondary metabolites—substances that appear to be end products of metabolism in plants and have no apparent function in the plant. They are often sequestered in specialized cells in the plant such as latex-producing cells, canals, or glands. A few examples of the many important secondary metabolites in plants that have pharmacological uses include alkaloids such as the morphinane alkaloids, codeine and morphine, both important painkillers; vincristine and vinblastine, two important cancer drugs; and saponins, widely used medically outside the U.S. and often used as precursors to metabolically active steroids.

Unfortunately, the undifferentiated cells that proliferate in tissue cultures often do not produce significant quantities of a desired metabolite, or they lose the ability to synthesize the desired substance in a short period of time (usually a few months). Plants apparently must differentiate and develop rudimentary tissues or organs before they are capable of synthesizing complex secondary metabolities.

My colleague, Dr. Kathryn Wilson of Purdue University, and I are researching methods that might be used to identify cells *in vitro* that are capable of synthesizing secondary metabolites. We are currently searching for cells that are laticifer-like in culture by using antibodies to specific secondary metabolites present in the latex of the common milkweed, *Asclepias tuberosa*. We have been very successful in developing a fluorescent, immunocytochemical stain for detection of laticifer cells in whole tissues and are now turning our attention to finding these specialized cells in cultured tissues of of this weed species.

Conclusion

In conclusion, it is fair to say that the new techniques I described here will not supplant current plant breeding technology in the near future. But the problems currently associated with using plant tissue culture for plant improvement are probably not insurmountable. Perhaps with a little luck and a lot of work, we'll make tissue culture work for us in ways we never dreamed were possible.

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