'Nif' and 'hup' genes

Raymond C. Valentine

All crops need nitrogen for growth. Nitrogen fixation—the enzymatic conversion of atmospheric nitrogen gas into a form available to the plant—is a trait that only some bacteria have evolved.

If plants such as soybean form a symbiotic association with nitrogen-fixing bacteria, nodules develop on their roots, and the plants can become independent of soil or chemical nitrogeneous fertilizers. Obviously, improving the efficiency of symbiotic nitrogen fixation by leguminous crops and genetically engineering new crop plants that produce their own nitrogen supply would be of tremendous benefit to agriculture. Other University of California scientists at Davis are devising strategies to improve nitrogen fixation efficiency. My primary goal is to use genetic engineering techniques to isolate and study the genes responsible for nitrogen fixation.

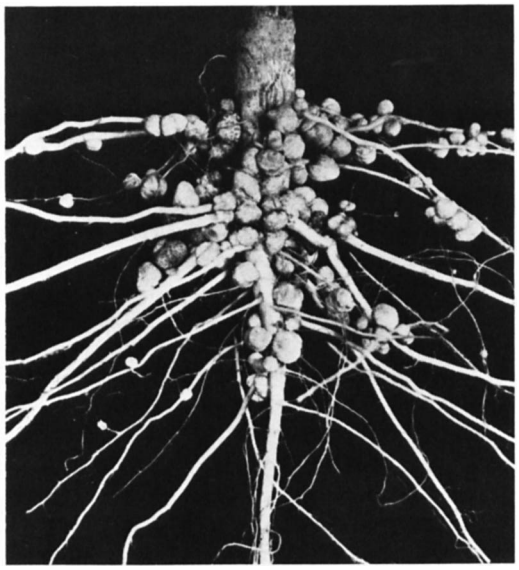
The *nif* (nitrogen-fixation) genes were isolated from an important variety of soybean root nodule bacterium, *Rhizobium japonicum* strain 110. After DNA from this *Rhizobium* species was purified, random fragments were inserted into a plasmid vector, which was used to transform *Escherichia coli*. Calculations showed that the total chromosome of *Rhizobium* should be represented in about 3,000 to 4,000 independent *E. coli* transformants. Among the 3,325 *E. coli* transformants screened, 21 contained a piece of DNA with the *nif* genes. Analysis of the plasmids in these 21 strains showed that all had the same size piece of DNA of 5.8×10^6 daltons. Further experiments confirmed that this piece of DNA carried the *nif* genes.

The availability of cloned nitrogen fixation genes from *Rhizobium japonicum* now makes it possible to study their organization and mode of expression. Ultimately the *nif* genes may be used for genetic engineering of new crop plants, but this is long-range research with many hurdles yet to be overcome.

A lot of energy is wasted during nitrogen fixation through the loss of the hydrogen gas released by many nodules. One researcher calculated that the energy loss in the form of hydrogen from soybeans in the United States alone was equivalent to the total supply of energy used in England over a three-month period.

Some strains of *Rhizobium japonicum* turn out to have genes for hydrogen uptake (*hup* genes), which means that soybean plants whose root nodules contain these strains are more energy-efficient than the other plants. Scientists in my laboratory have recently discovered that the *hup* genes are situated on plasmids, which can be transferred from one bacterial cell to another by conjugation. This discovery raises the possibility of introducing these plasmids, or smaller derivatives of them, into *R. japonicum* strains that lack them, thus increasing energy efficiency without significantly reducing the strain's ability to compete in its original environment. This is important, because local soil and other environmental factors such as pH, drought, salt, toxic ions, and so forth may largely prohibit the introduction of new rhizobial strains where local varieties have evolved to be far more competitive.

Raymond C. Valentine, Professor, Plant Growth Laboratory and Agronomy and Range Science, U.C., Davis.



Soybean root nodules—miniature nitrogen-manufacturing factories—provide the plant with fixed nitrogen from atmospheric N_2 gas. Some nitrogen-fixing bacteria also have genes for hydrogen uptake, recycling H_2 produced by the reaction. Examples of important nodulated crops in California are alfalfa, field beans, and clovers.

Maize anaerobic genes

Michael Freeling

A higher plant has between one and ten million genes, each of which holds information that specifies the structure of a product RNA and, often, a protein. In addition, each has nucleotide sequences involved in recognizing on-off signals. Using recombinant DNA technology, pieces of DNA carrying one or a few genes can now be removed from practically any organism, replicated in microorganisms, and then studied at the level of nucleotide sequence and sequence arrangement. A few genes from higher plants are now being analyzed at this level, and alcohol dehydrogenase-1 (*Adh1*) in maize, the gene studied most intensively in my laboratory, is one of them.

A gene serves the organism not only by encoding a product, but also as an integral part of the organism as a whole. For example, the alcohol dehydrogenase enzyme is formed in some organs and cell types of maize but not in others. In some organs *Adh1* is expressed naturally, but in others it is expressed only under anaerobic conditions. The *Adh1* gene is woven into a complicated net of gene circuits that respond both to internal, developmental cues and to external, environmental stimuli. To work backwards from specific genes to the now unknown levels of gene regulation is my research focus.

Adh1 enzyme activity is absolutely required if a maize seed or seedling is to survive drowning for more than a few hours. When maize seedlings are subjected to an anaerobic environment, most protein synthesis halts. After a few hours, *Adh1* and about nine major and ten minor proteins are synthesized. To prove that some or all the anaerobic proteins cause flood tolerance is extremely difficult. The only approach that might suffice, and the one we are using, is to obtain maize mutants or cultivars that are flood-tolerant and to find out how many and which genes are involved. We have been particularly successful in isolating mutants of *Adh1*. Some of our mutants alter the stability of enzyme expression, others the quantity. Some are restricted in their organ specificity, others show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Geneticist Michael Freeling (right) thinks we will see quite a few monsters like the knotted maize plant shown above before we engineer any improvements. This dominant structural phenotype can result from DNA insertions that inactivate the alcohol dehydrogenase-1 (*ADH1*) gene.

complicated alterations of quantitative expression involving different tissues. Several mutants were caused by insertion of a piece of DNA into the normal gene, others were induced with radiations. Because each of these mutants can now be studied at the nucleotide sequence level, *Adhl* has become an example of how classical genetics and molecular biology can complement one another.

Two of our insertions in the *Adhl* gene simultaneously obliterate ADH enzyme synthesis and generate a grotesque, vascular overgrowth specified by a dominant allele called "knotted," identified by classical genetic analysis and situated less than 0.1 map unit from the *Adhl* gene.

Since recombinant DNA technology makes it possible to isolate, clone, and study any gene as a sequence of DNA, some researchers have argued that molecular biology is nearly sufficient to solve the important problems of gene regulation during development, with little help from the tools of classical genetics such as mutational analysis, genetic fine structure, chromosomal position effects, and aneuploidy. The strategy is called *in vitro* genetics—adding, subtracting, or rearranging nucleotide sequences of cloned genes, then introducing them into a biological system for expression.

The most primitive expression system might be one that permits transcription: the most elegant would be one where the altered gene is introduced into a zygote, germ-cell, or plant protoplast so that gene expression might be assessed in the whole organism. The primitive system is exceedingly limited in the sorts of gene behaviors that can be seen. The whole-organism expression system, on the other hand, is identical to the whole organism in which traditional Mendelian mutants are recognized.

Since there are good reasons to believe that whole-organism transformation systems will soon be routinely utilized, it is prudent to ask

whether or not geneticists should continue to use the traditional methods of inducing and recovering mutants in whole, complicated plants. The answer will depend on what sort of mutant is desired and the ability of the biological assay system to permit recognition of it. When the phenomenon to benefit from mutational analysis involves sequential action of molecules compartmentalized in space or time, or all but the most simple molecular interactions, classical genetic technology is the cutting edge. For example, there is little question that classical genetics would have the greater chance of finding a mutant altering the anaerobic gene program itself. Since we know so little about the rules or mechanism governing how development controls groups of genes, it is wise to allow the organism maximum freedom to give us clues. Once particular DNA sequences are defined, then *in vitro* genetics becomes the approach of choice.

Michael Freeling, Associate Professor, Genetics, U.C., Berkeley.

Leaf protein synthesis

William C. Taylor
Timothy Nelson
Mark Harpster
Lino Fragoso
Belinda Martineau
Steven Mayfield
Judy Yamaguchi

The most abundant proteins in the leaves of higher plants perform specialized functions in photosynthesis. Many of these proteins are located within the chloroplast. Some are encoded by the chloroplast genome, and some by the nuclear genome. The synthesis of several of these proteins has been shown to be controlled by light.

We are interested in how synthesis of these proteins is regulated during leaf development. Our studies have shown that environmental factors interact with the leaf's developmental program to influence the quantity and timing of synthesis of several abundant proteins. We have also discovered that synthesis of the same protein is regulated in very different ways in two different plants.

The most abundant protein in green leaves of the bean plant (*Phaseolus vulgaris* 'Red Kidney') is ribulose-1, 5-bisphosphate carboxylase (RuBPCase). This is the enzyme responsible for fixing atmospheric carbon dioxide, thereby providing a carbon source for photosynthesis. It is found in the chloroplast and consists of eight small subunit polypeptides encoded by a gene in the nucleus and eight large subunit polypeptides encoded by a gene in the chloroplast. It has been known for some time that light influences the amount of RuBPCase in developing leaves of many plants. We have isolated synthetic DNA copies of small subunit mRNA (cloned cDNA) using recombinant DNA technology. This cloned cDNA gives us a very sensitive probe with which we can detect small quantities of small subunit mRNA and measure changes in its relative concentration within the cell. We were unable to detect small subunit mRNA when seedlings were grown in the complete absence of light. However, when seedlings that had been grown in the dark were shifted to continuous illumination, there was a rapid and dramatic accumulation of small subunit mRNA. Light induces a greater than 1,000-fold stimulation of small mRNA synthesis in bean primary leaves.

Fixation of atmospheric carbon dioxide is more complex in corn (*Zea mays*, inbred line B73, donated by Pioneer Seed Company, Des Moines, Iowa). The process involves two different enzymes located in different cell types. RuBPCase is restricted to the chloroplasts of vascular bundle sheath cells; phosphoenolpyruvate carboxylase (PEPCase) is found only in the cytoplasm of mesophyll cells. We have measured the accumulation of both proteins under a variety of dark and light growth regimens using sensitive antibody probes. Synthesis of both proteins begins about four or five days after germination. This time of synthesis initiation is independent of light. Accumulation of both carboxylases proceeds rapidly, even in the absence of light. The only measurable effect of light is to increase the rate of accumulation of both enzymes.

Contrasted to this light-independent regulation of PEPCase and RuBPCase during corn leaf development is the regulation of the chlorophyll a/b binding protein (chl a/b protein). This protein forms a complex with chlorophylls a and b and is responsible for harvesting the light energy that drives photosynthesis. Our preliminary studies indicate that light regulates synthesis of chl a/b protein in corn. In the absence of light there is no immunologically detectable chl a/b protein.

Our studies of leaf protein synthesis in corn and in beans demonstrate that genes encoding the same protein can be regulated in different ways in different plants. This fact, while interesting to the plant developmental biologist, has profound implications for the genetic engineer. Studies by plant physiologists indicate that, in a number of instances, efficiency of carbon dioxide fixation could be a limiting factor in crop yield. Transformation of a plant with a gene encoding a more efficient RuBPCase might therefore improve crop yield. Once the host plant has been transformed with a cloned gene, however, it is necessary that this new gene be expressed in the correct quantity in the correct leaf cells. Problems may arise if the cloned gene is regulated by a different set of signals than those employed in the host plant. It is clear that attention must be paid to the details of regulation of a gene when it is passed into the genome of a different plant.

William C. Taylor, Assistant Professor of Genetics and Assistant Geneticist; Timothy Nelson, American Cancer Society Postdoctoral Fellow; Mark Harpster, Lino Fragoso, and Belinda Martineau, graduate students, Genetics; Judy Yamaguchi, Staff Research Associate, Genetics; and Stephen Mayfield, graduate student, Plant Physiology. All are at U.C., Berkeley.

Storage protein genes

R. William Breidenbach
Robert B. Goldberg
Scott E. Taylor

Interest in the developmental and molecular biology of the proteins that accumulate as reserves in seeds has become keen in recent years. Although most plant cells contain large numbers of different proteins, each present only in small quantities, food chemists, using criteria of size and solubility, long ago found that most of the protein in seeds of the soybean and other legumes appears to be composed of only a few different kinds. In the late 1960s we recognized that, if this were true, it was likely that a correspondingly small number of different kinds of messenger RNA (mRNA) molecules encoded to direct the synthesis of the storage proteins would also be present in higher concentrations in seed tissue cells. Higher concentrations of specific mRNA's would make it much easier to learn how these intermediaries between genes and their protein products are modulated and, in turn, how they control the rates of protein production.

These two aspects of the regulation of gene expression are among the most important unanswered questions in biology and are fundamental to the practical problems of plant improvement by either conventional plant breeding or molecular genetic engineering. To learn how to move genes from one organism to another and have them usefully ex-