**Genetic Aspects of Mineral Nutrition: Future Challenges and Directions**

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CLASSICAL AND MOLECULAR GENETICS

There are several avenues the modern plant scientist can use to study the genetic basis of mineral nutrition. The one that has been most widely followed to date could be termed the classical genetic or breeding approach, which involves collecting and screening germplasm for a particular phenotypic trait. For mineral nutritionists, this might involve looking for plants resistant to stressfully low levels of minerals (Gabelman and Gerloff, 1983) or for plants resistant to high levels of a toxic ion or salt (Rush and Epstein, 1981). A second approach, made possible by recent advances in recombinant DNA techniques, could be termed a molecular genetic approach. In this case, the researcher first identifies and isolates a specific gene or gene product that is suspected to play a role in mineral nutrition. Each of these approaches has advantages and disadvantages, as described below.

There are two immediate advantages in the classical genetic approach. First, there are no assumptions made concerning underlying physiological or biochemical mechanisms; therefore, results can be obtained even when the underlying biological mechanisms are unknown. Second, if the trait is inherited through a combination of several genetic loci acting together (e.g., "quantitative trait"), the breeding approach will still succeed, whereas a more specific molecular approach may not. A disadvantage that frequently arises is the inability to transmit useful traits between different species.

A major difference between the two approaches is that the molecular one almost exclusively focuses on individual genes. In using the molecular genetic approach, the investigator will either isolate a single mutant allele from a mutagenized population of plants or cells, or else purify a particular protein of interest and then perform "reverse genetics", i.e., use the protein as a probe to isolate its structural gene. The gene may then be re-introduced into other plants using established genetic transformation techniques (Lloyd et al., 1986). This overall approach can be illustrated by a recent published example. To create tobacco plants that were resistant to sulfonyleurea herbicides, Haughn and Somerville (1986) first isolated a herbicide-resistant Arabidopsis thaliana plant, using standard mutagenesis and selection procedures. Then, since it was known that the cellular target for this herbicide was acetolactate synthase, an enzyme in the synthetic pathway for a common amino acid, these investigators used a microbial gene for this enzyme to hybridize with the gene from a library consisting of DNA isolated from Arabidopsis plants. These studies established that a single base substitution in the acetolactate synthase gene was the cause for the resistance phenotype. Furthermore, when the "resistant" Arabidopsis gene was inserted into the tobacco genome via Agrobacterium-mediated transformation techniques, newly created herbicide-resistant tobacco plants were obtained.

How can one apply this procedure in mineral nutrition studies? As mentioned earlier, molecular genetics is not well-suited for manipulating phenotypes resulting from a complex combination of many genetic loci. However, there is an area of mineral nutrition for which the molecular genetic approach is quite appropriate, and that concerns the genetic basis of membrane transport. When one selects for microbial cells that are resistant to stressfully low concentrations of minerals in the medium, one invariably identifies mutations in structural genes encoding specific plasma membrane transport proteins. For example, mutant Escherichia coli and Neurospora crassa cells that survive in low potassium concentrations have been obtained and single point mutations in genes responsible for potassium transport across the plasma membrane were found to be responsible for the mutant phenotype (Hesse et al., 1984; Slayman and Tatum, 1965). When a similar screening strategy was applied to beans, it was observed that a single recessive gene was responsible for the survival of bean plants in low potassium (Shea et al., 1967). Detailed potassium transport studies have not yet been performed with these plants.

DNA POLYMORPHISMS AND "REVERSE" GENETICS

Restriction fragment length polymorphisms

In the marriage between old and new genetic technologies, the use of restriction fragment length polymorphisms (RFLP) may have wide applications in mineral nutrition studies (Botstein et al., 1980). An RFLP is defined as a change within or near the 4- to 6-base-pair (bp) region of DNA that is recognized and required for cleavage by the class of nucleases known as restriction enzymes. When this

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DNA difference (i.e., a base change, insertion, deletion, or rearrangement) exists between two sexually compatible individuals of a population, it is possible to follow the inheritance of the genetic locus in all subsequent progeny. To visualize this event, the technique relies on a procedure known as Southern blotting, whereby the genomic DNA of an individual or a group of isogenic or even to cross species individuals is extracted, cleaved with a restriction enzyme, and then separated on the basis of size using agarose gel electrophoresis. The separated fragments of DNA are blotted and affixed onto membranes made from nitrocellulose or cationic nylon and then "probed" by hybridizing to a radioactive piece of DNA. After the probe is hybridized to the blot, the position of radioactive bands is determined by autoradiography (Fig. 1). Key components in this procedure are the enzyme used for cleavage and the particular radioactive DNA probe. To discover an RFLP, there are two main variables one adjusts—the DNA fragment probe (typically this is 0.1 to 10 kilobases in length) and the particular restriction enzyme used to cleave the genomic DNA. When a particular probe and enzyme yield a different hybridization pattern with genomic DNA isolated from individuals, an RFLP has been found. Obviously, if the two individuals are evolutionarily well-separated, the chances of finding an RFLP are enhanced.

One of the more exciting uses of RFLP is to "map" a genome. For example, there are now several hundred fragments of DNA that produce RFLPs in corn and tomato, and these have been mapped to specific locations on the various chromosomes by simply comparing the segregation of individual RFLPs with respect to each other and to the segregation of other phenotypic markers (Helentjaris et al., 1986). Thus, one can locate the RFLP not only with respect to distance from all of the other RFLPs, but also from other phenotypic markers (e.g., seed color). The RFLP offers several advantages over other chromosomal markers since the principle involves specific DNA base changes, insertions, and rearrangements, and because the heritability of these "traits" is 100%. Another real advantage is that the RFLP markers are co-dominant and do not result in creating difficult phenotypes, which may affect expression of the trait of interest.

It is now a commonplace event to read of RFLPs that map close to, or are even coincident with, genetically inherited human diseases (e.g., see Tsui et al., 1985). These DNA markers have clinical applications, e.g., in predicting whether an individual is susceptible to disease and, in addition, providing a path by which researchers can eventually isolate and sequence the specific gene whose product causes the disease. The utility of this approach relies on obtaining a battery of different RFLPs as well as having a detailed pedigree of individuals that segregate for a particular trait.

With higher plants, RFLPs have already been used to investigate the genetic basis of complex phenotypes such as insect resistance (Nienhuis et al., 1987) and total soluble solids content in tomato (Osborn et al., 1987). As RFLP markers become more plentiful for each species, it will become easier to pinpoint the location of the genetic locus for almost any phenotype that shows a degree of heritability. For species that contain small amounts of nuclear DNA, as the RFLP density approaches 500 or 1000, it will become routinely possible to "walk" along the chromosome from any phenotypic marker to the specific piece of DNA that controls that trait. For example, Arabidopsis thaliana is a crucifer that contains only 70,000,000 bp per nucleus. Thus, with 1000 randomly placed RFLPs, each one will be <70,000 bp from its nearest neighbor. Phage libraries have been constructed that contain DNA fragments of >35,000 bp in size, per clone. Thus, only 16,000 such phage clones need to be screened to give a 99% chance of locating a single-copy sequence (Meyerowitz and Pruitt, 1985). One can map each of the RFLPs to a particular phage clone by a blotting procedure similar to that shown in Fig. 1. It is now possible to sequence 35,000 bp in <1 week, using either manual or automated DNA sequencing procedures. Thus, with a high enough RFLP density, it should be possible to associate a particular DNA sequence with any heritable phenotypic trait. New procedures for routine cloning of very large segments of plant DNA offer the potential for reducing the time and effort involved in isolating genes (Burke et al., 1987). These advances in DNA handling and sequencing have occurred so fast that it is reasonable to speculate that, within our lifetime, the DNA sequence of an entire plant nuclear genome could become known. If this occurs, then the limiting factor for progress in understanding and modifying the genetic basis for mineral nutrition may well become the discovery of useful heritable phenotypes or the transfer of these traits into agriculturally important crops.

Reverse genetics

Using standard techniques in molecular genetics, it is now possible to work backwards from a specific protein to isolate its structural gene. Two of the most commonly used methods involve either producing an antibody directed against the purified protein or direct determination of the amino acid sequence by automated Edman degradation. Great advances in protein purification (e.g., high-performance liquid chromatography) and order-of-magnitude improvements in the sensitivity of protein sequence determination have decreased the amount of protein needed to obtain this information (Matsudaira, 1987). In the past, these procedures could be performed only with the more abundant proteins, such as seed storage proteins or ribulose bisphosphate carboxylase. However, it is now possible to perform these feats with rare proteins with essential enzymatic functions (e.g., membrane transport proteins).

Once an antibody is obtained, the gene is isolated by screening an expression vector library containing randomly cleaved plant DNA. The most commonly used expression library is agt1. Alternatively, if only sequence information is available, the gene can be isolated by using a synthetic oligonucleotide probe to screen either plasmid or phage libraries containing plant DNA. Once a fragment of DNA containing the structural gene is isolated, it may be used as a probe to identify a restriction polymorphism within the fragment, or in a contiguous region. Once this RFLP is identified, the DNA fragment can be used in a breeding program as a "probe" to follow the inheritance of that gene in a segregating population that is expressing many phenotypes. In addition, using genetic transformation techniques, it is possible to reintegrate the gene at other locations within the same genome or even to cross species barriers and insert the DNA into the germplasm of unrelated plants.

As an example of a practical application of reverse genetics in the study of plant mineral nutrition, consider the proteins responsible for transporting minerals across the plasma membrane and tonoplast (Fig. 2). Based on analogy with results obtained from microbial systems, it is expected that these proteins are the products of genes that increase or decrease the capability of plants to survive in stressfully low concentrations of soil minerals and other nutrients. There are basically five ways to isolate these genes: 1) purify the transport protein and, from this, generate the antibody and sequence information used for screening libraries containing plant DNA; 2) isolate mRNA during periods of nutrient stress, to identify genes
whose transcription is rapidly and specifically increased by deficient amounts of a particular mineral; 3) use heterologous DNA (or antibody) probes, i.e., fragments of DNA known to encode transport proteins in microbial systems in cases where there is sufficient homology between plants and lower organisms; 4) isolate single-point mutations in plant species that are easily manipulated through classical genetics (e.g., *Arabidopsis*), and then use these plants to isolate the responsible DNA fragment through chromosome “walking”; and 5) use RFLP mapping to identify specific loci associated with a useful phenotype (e.g., mineral “efficiency”), then use chromosome walking to isolate the gene. We, and others, are pursuing each of these approaches, and these efforts are described below.

The first method is basically a brute force approach; simply purify these proteins to homogeneity, make antibodies or use the Edman degradations to obtain protein sequence data, and then isolate the structural genes from a suitable library of plant DNA. As a general procedure, there are two problems with this approach. First, it may be difficult or impossible to set up an easy assay for these transporters to allow their purification. Second, some of these proteins may be present at such low concentrations in the plant membrane that it is difficult or impossible to obtain amounts sufficient for biochemical analysis.

We have pursued this brute force approach with recent success in the cloning and sequencing of plasma membrane proton pump genes from *Arabidopsis thaliana* (Harper et al., 1989). This enzyme, also known as a H+-ATPase, contains a single polypeptide of 100,000 Da molecular weight and generates all of the proton-coupled transport processes. Although plant transformation is rapidly becoming more and more routine, it is unlikely that such transformation experiments will become as feasible as they are with fungi and so, we are left with the option of using fungal-derived genes as probes for screening plant libraries.

We have recently pursued the fourth approach in attempts to isolate mutant *Arabidopsis thaliana* plants with altered mineral transport properties. In addition to having a small genome and a rapid generation time, this species has small seeds (=1000 seeds per 20 mg). Thus, it is possible to screen 10^6 seeds on 50 petri
plates to obtain a few mineral transport mutants. For these initial experiments, we have focused on a simple selection criterion; namely, resistance to transport inhibitors. We have chosen vanadate and cesium because these two compounds specifically block phosphate and potassium transport, respectively, across the plasma membrane of fungal cells (Bowman et al., 1983; Conklin et al., 1987). Resistance to these compounds in such a simple system usually reflects an alteration in genes encoding plasma membrane transport proteins.

For these experiments, it is critical to use a suitably mutagenized seed population. We have followed a standard protocol to obtain a mutagenized and selfed (M2) population of *A. thaliana* seeds that contain ~0.6% albinos, which is substantially higher than the frequency in a normal wild-type population, and corresponds to ~50 mutations introduced per nucleus (Browse et al., 1985). The minimum concentration of vanadate or cesium needed to kill several hundred thousand wild-type seeds was then determined and used to isolate putative vanadate- or cesium-resistant plants from the M2 population. Out of ~200,000 seeds, 30 to 50 ‘escapes’ were obtained for each inhibitor. Of these plants, ~20 produced progeny that displayed inhibitor resistance in the following (M3) generation. Since this species is obligately self-pollinating, it is only necessary to keep planting seeds to increase the seed stock. Once sufficient material is available, it will be possible to test phosphate and potassium transport properties to determine if these are indeed the proteins responsible for the resistant phenotype.

Finally, we are pursuing the fifth approach in trying to find an RFLP that shows a tight association with a segregating population of tomato and bean plants with known heritable differences in the efficiency of using phosphate, potassium, and calcium. Tomato is an especially useful organism for these studies, since >300 RFLPs are already available, and heritable differences in mineral efficiency usage are known (Makmur et al., 1979).

**CONCLUSION**

In our attempts to “marry” the old and the new genetic techniques, it is essential that we not lose sight of their limitations. The molecular genetic approach offers great potential in identifying and isolating individual genes, but, by its very nature, it may not provide useful inroads into phenotypic traits that show complex inheritance patterns. In this respect, RFLPs provide an exciting solution to deciphering the individual genes involved in “quantitative” traits, as shown by progress already achieved in analyzing complex genes involved in tomato insect resistance and soluble solids content.

One of the most interesting differences between higher plants and procaryotic organisms lies in the use of specialized transport cells to move nutrients between all of the plant’s component cells and tissues. When one considers the incredible transport problems that had to be overcome in evolving a system to transport water to the top of the tree and the nutrients, or to maintain a balance between phosphate and potassium transport, it is easy to marvel at the final result. There is no question that the genetic analysis of mineral nutrition represents a formidable challenge—one that necessitates the use of any and all weapons in our arsenal of techniques. However, just as all of the specialized cell types of a higher plant have evolved a means by which they can work together to occupy a favorable niche, the present-day researcher has become so specialized that it is important to consider specific ways in which interdisciplinary research can occur. Plant breeders, molecular biologists, biochemists, cell biologists, and organismal physiologists all bring unique and useful approaches to the study of the genetic basis for mineral nutrition. Probably our greatest challenge for the future lies in determining ways by which the integration of these approaches is encouraged.

**Literature Cited**


