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AN INTRODUCTION TO SOMATIC CELL GENETICS

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Somatic cell genetics is a relatively new discipline which relies upon various plant tissue culture techniques. This discipline may be defined broadly as any genetic or physiological investigation involving cultured cells, and includes such areas as gene transfer, gene regulation, cell selection for genetic variants, control of development, and regulation of metabolism.

Tissue culture techniques

Tissue culture is a cyclic procedure whereby types of hormones incorporated into a medium and their concentrations can be modified to force either differentiated or undifferentiated growth (4, 19, 33) (Fig. 1). Differentiated growth involves the production of either shoots or roots, while undifferentiated growth is the production of a tumorous mass of cells called callus.

Callus usually can be produced from any differentiated structure (e.g., leaf, stem, root) by placing explants on media containing relatively high levels of auxin and low levels of cytokinin. Once produced, the callus can be grown either as large, multicellular masses on solid media or as small cell aggregates in rotated liquid media. By using high levels of cytokinin and low levels of auxin in the media, it is sometimes possible to stimulate the undifferentiated callus into producing differentiated structures which eventually turn into shoots.

Tissue culture also can involve the production of protoplasts, wall-less plant cells. The presence of a cell can make many genetic and biochemical studies nearly impossible. Protoplasts are produced easily by incubating either differentiated or undifferentiated tissue in a mixture of cellulase + macerase. These enzymes digest the cell wall (cellulose) and surrounding pectins and hemicellulose. The protoplasts then are cleaned of cellular debris and enzymes by differential centrifugation. Cell walls usually reform on the individual cells within one to 2 days when protoplasts are plated on a callus-forming medium. Cell division begins within 1 to 2 weeks. The callus which forms from the protoplasts then can be shifted to media stimulating redifferentiation.

There are now over 40 species in which procedures have been worked out for regenerating protoplasts into whole plants. In addition, there are hundreds of species in which techniques have been developed for regenerating whole plants from callus, and thousands of species where whole plants can be produced in culture from differentiated tissue. A quote from J. Torrey is appropriate: "Lack of success in culturing a tissue is usually attributable to lack of trying" (32). A number of excellent reviews discuss this subject in detail (4, 19, 29, 32, 35). Tissue culture procedures can be used in many areas of research, the principal areas being propagation, embryo culture, anther culture, germplasm preservation, and somatic cell genetics.

Use of somatic cells in biochemical studies

There are 2 major advantages in using cultured cells when studying biochemical or physiological events. First, one can obtain a uniform population of undifferentiated, heterotropic cells in culture. When using whole plants or tissues in studies, one is forced to work with a mixed population of differentiated cell types. Some cells are programmed for photosynthesis, water uptake, gas exchange, nutrient movement, continued growth, or physical support. All these different cell types make it almost impossible to determine the underlying biochemical basis for most phenomena.

The 2nd advantage in using cultured cells is that both the physical and chemical environment can be controlled and defined. All of the nutrients and growth regulators must be supplied for continued cell

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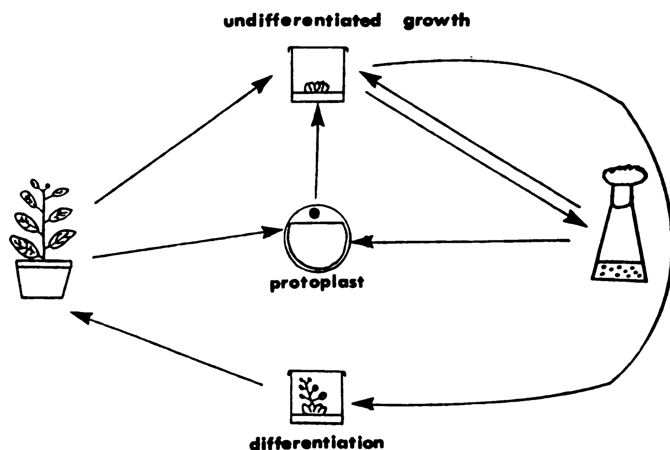

Fig. 1. Tissue culture cycle.

growth. Transport phenomena, differentiation, and cellular associations can affect the distribution and perception of both nutrients and genetic regulatory signals in whole plants, as well as the response of organs and cells to these signals and chemicals.

Even though cultured plant cells do not reflect any specific, developmental state found within the whole plant, they can be useful in studying the basic processes associated with various cell types (7, 24, 28). Cell cultures, for example, have been used to elucidate some of the steps in the flavonoid biosynthetic pathway.

In vitro anthocyanin synthesis in *Helianthus tuberosus L.* cell cultures was stimulated by a nutrient medium containing kinetin. In addition, high light intensity, low temperature, and media rich in phosphates and inorganic nitrogen were essential for *in vitro* production of cyanidin 3,5-diglucoside (14). Likewise, high light intensity was essential for *in vitro* production of cyanidin 3-rutinoside in *Haplopappus gracilis L.* cell cultures (6). By feeding radioactive precursors to *Haplopappus* cultures, it was concluded that dihydroflavonols are converted into anthocyanins efficiently. It seems that the most feasible pathway for this conversion involves a hydride ion from the C-3 of the dihydroflavonol shifting to the C-4 on the anthocyanin pseudobase. Further studies (6) using dimethyl sulfoxide (DMSO)-permeabilized cells indicated that the microsomal system was capable of catalyzing the hydroxylation of the dihydroflavonones to dihydroflavonols in the presence of NADPH and O_2. Additional data (11) support the following scheme for the biosynthesis of cyanidin. L-Phenylalanine is deaminated in the cytoplasm to cinnamic acid, which then is hydroxylated to p-coumaric acid on some membrane. Activation to the CoA thiolester and condensation with 3-malonyl-CoA to naringenin occurs in the cytosol. Hydroxylation of naringenin to dihydroquercetin takes place on some membrane again. Further studies (8, 13, 15, 16) using protoplasts isolated from flower petals have indicated that the glucosylation of dihydroquercetin and the methylation of the anthocyanidin take place in the cytosol. The final product is transported then into the vacuole where it accumulates.

This example illustrates how biochemical data, obtained using a somatic cell approach, can be combined with classical genetic data on flower color in *Petunia*. The result is a detailed and complete characterization of the pathway for flavonoid biosynthesis (Fig. 2).

An interesting phenomenon occurs when the biosynthetic pathways of cultured cells are compared with those found in cells *in vivo*. Both cyanadin and delphinidin are produced in cultured rose cells, while in intact petal cells, only cyanadin is produced (14). The difference between cyanadin and dephinidin is in the number of hydroxyl groups, dephinindin having one more. These data suggest the presence of an additional hydroxylation gene which is active in the *in vivo* cultured cells, and inactive or nonfunctional in the *in vivo* cells. In addition, many other factors determine the end product of a biosynthetic pathway.

One must be careful in correlating the gene activity of cultured cells with the activity of those genes in differentiated tissues and organs. Many genes in whole plants are expressed only in specific tissues or organs. An important part of plant development involves repressing unneeded genes. Genetic repression can be quite strong, and in some instances irreversible. This developmental trait can make it difficult to characterize many biochemical pathways, for few cells within differentiated tissue contain all of the needed genes for a given pathway in an active, functional state. The use of undifferentiated cultured cells can overcome this difficulty because cultured cells usually are under weak genetic regulation. An example of this weak regulation is seen in the fact that environmental factors can change gene expression in cultured cells. In the case of anthocyanin biosynthesis, a slight change in the concentration or type of hormone, light intensity, or even temperature can change the expression of the genes involved in this pathway (6, 14).

The use of mutants is crucial when applying a somatic cell genetics approach to the study of any biochemical problem. The comparison of the behavior of crippled mutant cells with that of normal cells allows a more thorough characterization of a given biochemical event. Cultured carrot cells, for example, which were resistant to 5-methyltryptophan, have been selected (35). By adding intermediates of the tryptophan biosynthetic pathway to the culture medium, it was determined that 5-methyltryptophan inhibits the production of anthranilic acid. The enzyme anthranilate systhetase then was isolated from the 5-methyltryptophan-resistant cell lines and characterized. The resistant phenotype was due to a lack of feedback inhibition. Further studies comparing the defective enzyme from several different mutants with the normal enzyme will allow one to characterize the mechanism involved in feedback inhibition. It would be difficult, if not impossible, to study this type of enzyme activity without the use of mutants. Cultured mutant cells also have been used to characterize phenylalanine ammonia lyase (1); they also are important in genetic studies.

Use of somatic cells in genetic studies

It is possible by using single plant cells as experimental organisms, to use procedures adapted from microbial studies to analyze and modify higher plants. These procedures include mutant induction and selection, gene transfer, somatic hybridization, and haploid production.

The advantages of using somatic cells in genetic studies are the same as those in biochemical studies—more precise environmental control and more uniform cell populations. It is possible to elucidate some of the mechanisms involved in gene regulation by controlling the cellular environment around mutant cells. Mutants, for example, can be isolated in cultured carrot cells which are blocked at different stages in somatic embryogenesis (31). Several antimetabolite-resistant mutants (e.g., resistance to 5-fluorouracil or 5-methyltryptophan) were isolated from carrot cell cultures. Several of these mutants were lacking in regenerative capability. Mutant W001, for example, regenerated at a frequency of 1×10^{-5} that of the control. Further investigations have shown that W001 has an increase in tryptophan synthesis. This alteration leads to an accumulation of endogenous auxin which inhibits regeneration. The synthetic pattern for the embryonic proteins also is disrupted in the W001 mutant. Another mutant, F5R82, displays a normal induction of the embryonic proteins, but the maintenance of these proteins is disrupted. Several other mutants also have been identified which are impaired in other steps in regeneration. It will be possible to obtain a broader understanding of the control of basic plant development by identifying the specific genes expressed during the regeneration process, and by studying the expression of these genes in mutants.

Cultured cells are under little genetic regulation and have a relatively short generation time; thus, a wide array of variants can be isolated (26). A number of amino acid auxotrophs have been selected in both tobacco and carrot cell cultures. Tobacco cells also have been selected which are able to grow in the absence of cytokinins (26, 27). These habituated cells are, however, not true genetic mutants, for the habituation occurs at high rates and is regularly reversible. In addition, the altered phenotype is not transmitted sexually. This nongenetic type of variation has been termed epigenetic.

Epigenetic variation may be produced through a number of different processes. The migration of transposable elements, for example, is known to alter reversibly the transcription of both structural

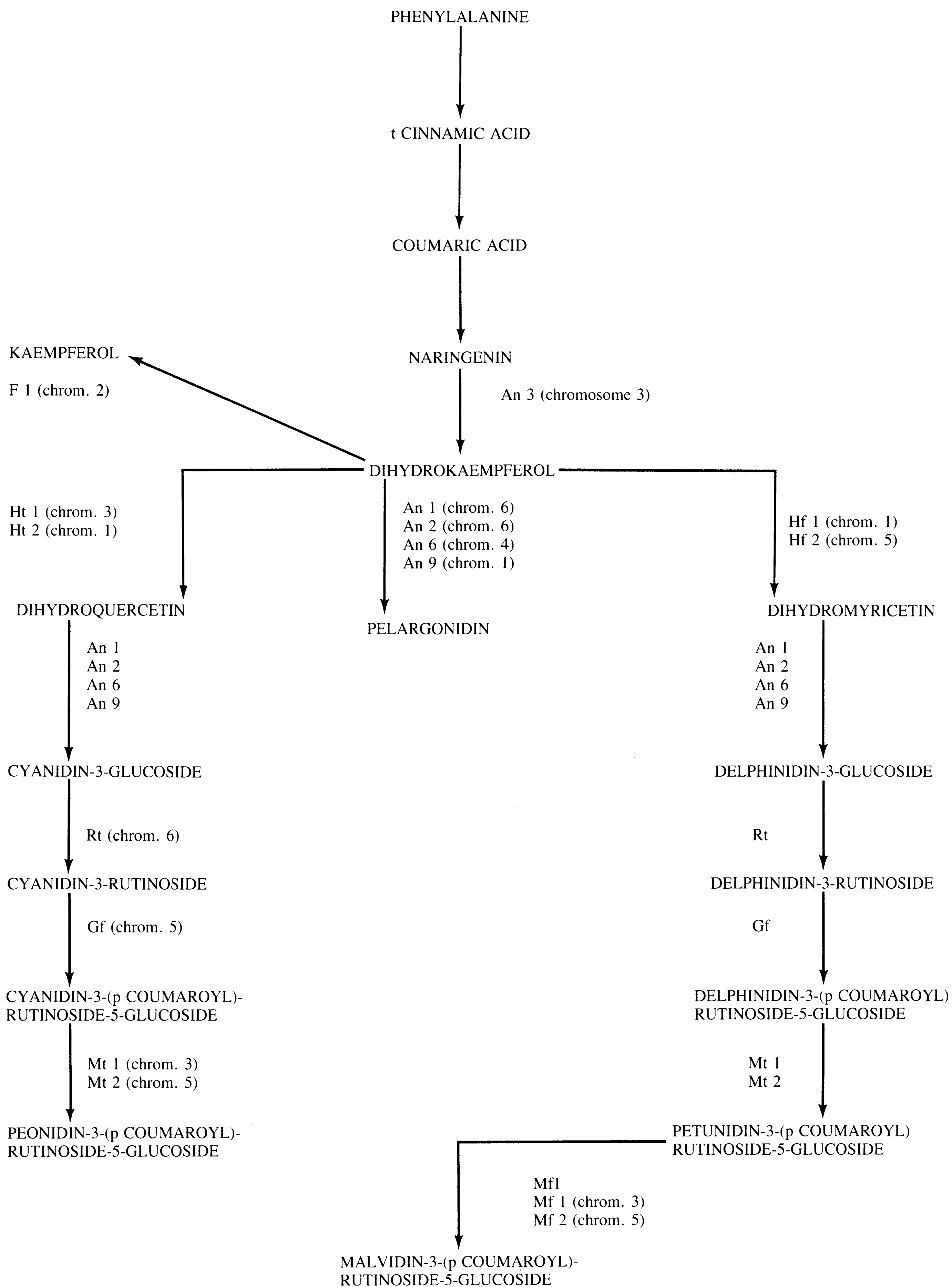

Fig. 2. The flavonoid biosynthetic pathway found within the genus *Petunia*. The genes and chromosome location of these genes are listed for each enzymatic step. (Adapted from 6, 8, 11, 13, 14, 15, 16).

and regulatory genes. Epigenetic variation, even though reversible, can be quite stable; it raises the possibility that many so-called mutants which have been isolated in cell cultures are produced through changes in gene expression, and not through changes in the gene itself. Besides epigenetic changes, another tissue culture specific change occurs, which has been termed somaclonal variation (20).

Somaclonal variation involves stable genetic changes produced during a tissue culture cycle. These genetic changes may be caused by one of 2 processes. First, the tissue culture cycle itself may induce the variation. It is well-known that many of the chemicals included in tissue culture media are mutagenic. For example, 2,4-D can induce chromosomal structural changes. Second, tissue culture releases cells from a specific developmental fate. Spontaneous mutations are occurring continually as developmentally predetermined cells undergo division to reach their differentiated state. Deleterious mutations in those genes which are not expressed in the differentiated state will tend to accumulate. When this differentiated tissue is then placed in culture, additional deleterious mutations will tend to accumulate in those genes which are not expressed in the cultured state. Therefore, it should not be surprising if genetic variation is obtained in plants regenerated from either differentiated or undifferentiated tissues which have been in culture for a long period of time.

Somatic cell genetics also can be important in gene transfer. It is possible to modify the genetic makeup of a single cell through the use of cultured cells and then reproduce an entire plant with the modified phenotype from this modified cell. There are numerous methods for introducing foreign genetic material into plant cells (12, 17, 23). A number of reviews (4, 33) discuss the use of somatic hybridization or cell fusion in producing hybrid plants from sexually incompatible species. Techniques are now available for fractionating protoplasts into various components and using these components in fusion. It is possible to create cells which are called cybrids by fusing protoplasts which lack a nucleus with protoplasts which contain very little cytoplasm (3) (Fig. 3).

Cybrids are a type of hybrid in which only the organellar or cytoaplasmic genomes are in a hybrid state, but the nuclear genome is not in a hybrid condition. This, potentially, can be a valuable method for transferring ctyoplasmicly encoded traits. For example, the resistance to triazine herbicides is encoded in the chloroplast genome (21). It should be possible to transfer herbicide resistance into a wide array of different crops by fusing enucleated protoplasts from triazine-resistant plants with protoplasts from triazine-sensitive species. Because the nuclear genome will not be in a hybrid state, a complex, introgressive breeding program will not be necessary to produce a commercially, useful plant type from the fusion event.

Techniques also are available for introducing foreign nuclear genes into host cells. There is a large array of different techniques. Some

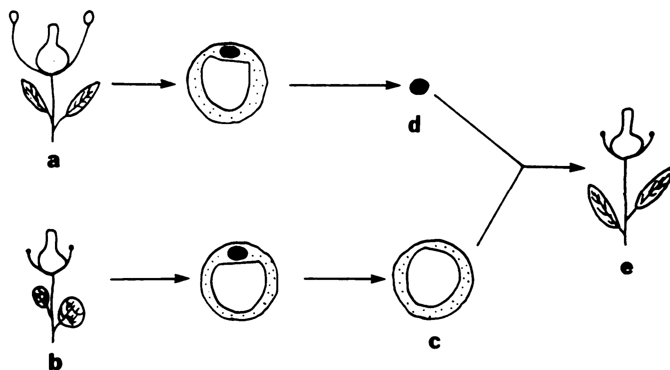

Fig. 3. Transfer of cytoplasmically encoded traits: **(a)** species A is male fertile; **(b)** species B is male sterile; **(c)** production of enucleated protoplasts from the male sterile species B; **(d)** the isolation of nuclei from the male fertile species A; **(e)** the uptake of isolated nuclei from the male fertile species A by enucleated protoplasts of the male sterile species B; the resulting hybrid will express the nuclear genome of species A and the cytoplasmic genome of species B.

of the primary methods include the microinjection of protoplasts (9); the chemically induced uptake of DNA (23), nuclei (22), or chromosomes (10); the use of bacterial plasmids as vectors, especially the Ti plasmid of *Agrobacterium* (12); and the use of oil droplets (2) or artificially created lipid vesicles (25).

Somatic cell genetics can be used effectively to produce new genetic variability; however, care must be taken in selecting the appropriate means for producing specific types of variation. We now have an abundance of technology, but we still lack the basic understanding of the development and physiology required to apply this technology to plant improvement. One problem is that many of our horticultural characteristics are not defined adequately at a biochemical level. Modification through somatic cell genetics cannot occur without this biochemical definition. In addition, somatic cell genetics will allow transfer of genes between any 2 species; however, many of the engineered plants will need further classical breeding before they are of economic value.

Application of somatic cell genetics to stress physiology

A combined genetic, physiological, and biochemical approach at both the whole plant and single cell levels is needed in order to understand clearly the effects of a given stress treatment. For example, where do ions accumulate when plants are exposed to salt stress? In what part of the plant and in what cells do the ions accumulate? Do the ions stay in the cytosol or move into an organelle? These data are important in developing an approach for selecting resistant cells.

A mutation which reduces the uptake of these ions, or which increases the transport of these ions into the vacuole, might confer resistance to salt stress (5). After determining where in the cell the salt has its effect, then what the effect is must be determined. For example, does the excess salt cause plasmolysis? If so, does the plasmolysis itself kill the cell or does the salt impair the activity of some essential enzyme, change the permeability of membranes, or alter active transport? Each of these effects would require a different approach in creating or selecting cells tolerant to salt stress. If salt is affecting the permeability of a membrane, for example, then the substitution of a different lipid or degree of saturation into that membrane might lead to salt tolerance (30). Resistance to high salt concentrations also might be created by changing the active site on some enzyme so that it could function at a higher salt level (18).

Another important question concerns the ions themselves. At lethal levels of NaCl, does the Na^+ or the Cl^- kill the cell? A mutation which changes the balance of Na^+/K^+ within the cell might lead to increased resistance if the Na^+ is responsible. If, on the other hand, the Cl^- is responsible, then a mutation which increases the level of a positively charged protein which could bind Cl^- might lead to an increase in salt tolerance (34). This approach could get quite complicated if the Cl^- accumulates in an organelle, for the Cl^--binding protein then must be produced in or transported to that organelle.

Once a mutant cell which is resistant to salt stress has been biochemically and genetically identified and defined, then one needs to look at how this mutant responds to salt as a whole plant. A cell which is resistant to stress when undifferentiated in culture may not be resistant when differentiated in a whole plant. Salt tolerance could be due to the activity of a protein which absorbs Cl^- in the cholorplast. This gene may not be transcribed in all differentiated cells. It is conceivable that this gene might be "turned on" in root cells and not in leaf cells. Because this gene is not active in those cells which contain chloroplasts (leaf cells), the whole plant would not be expected to be resistant. This clearly illustrates why a thorough genetic and biochemical characterization at both the whole plant and single cell levels is critical to understanding the mechanism of stress tolerance or avoidance.

A somatic cell genetics approach to studying stress physiology should allow problems to be attacked in a simple and controlled manner. Many problems cannot be addressed at the whole plant level. How would one investigate the effects of the cell wall on the movement of water and ions across the plasmalemma in a saline environment at the whole plant level? At the same time, there are limitations to using a somatic cell genetics approach, for not all

questions can be answered at the single cell level. How would one investigate at the single cell level the uptake and transport of salts by roots? Thus, a whole plant and somatic cell genetics approach is needed to develop an understanding of stress physiology and to select for avoidance or tolerance to stress. Alone, neither approach will be successful.

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CELLULAR MECHANISMS OF TOLERANCE TO WATER STRESS

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Water availability is one of the principal environmental limitations of crop productivity throughout the world. The water deficits, which are a consequence of either continuous or transitory periods of drought, cause significant yield reductions on presently cultivated land, and greatly restrict the cultivation of crops on over one-third of the earth's land surface considered to be arid or semiarid (25). These restrictions on yield potential are rapidly becoming of great concern in the face of the food demands of an ever increasing world population. The problem becomes complicated further by the fact that supplies of suitable irrigation water are dwindling rapidly, and that the costs of irrigation are becoming prohibitive. As a result, studies on the effects of water stress on plant survival and yield are attracting added interest in plant science research.

[1]Supported by Purdue Univ. Agr. Expt. Sta. Program Improvement Funds and by funds from the Israel-United States Binational Agr. Res. and Dev. Fund (BARD). Journal Paper No. 9767, Purdue Univ. Agr. Expt. Sta.

Plants exposed to water deficits invoke several mechanisms which allow them to alleviate detrimental effects of stress. According to Kramer (25), these mechanisms can be placed into 2 general categories, drought avoidance and drought tolerance. Drought-avoidance plants exhibit growth habits which allow them to avoid water stress conditions. Plants which initiate and complete their life cycle during periods when water is plentiful are examples of this. Drought-tolerant plants can be separated into 2 groups: those that postpone dehydration and those that tolerate dehydration (25). Dehydration postponement is accomplished by mechanisms which either reduce transpiration or increase water absorption, thus preventing the cells of the plant from reaching detrimental, low, cell water potentials (ψ). Dehydration tolerance involves processes which allow the plant to survive and/or grow after the cells actually have been exposed to water deficits and low cell ψ. If the water deficit is severe enough, plant cells either lose turgor (ψ_p), or ψ_P is reduced to a point restricting cell expansion (growth). The cells must adjust their internal osmotic potential ($\psi\pi$) and sufficiently increase ψ_P to resume cell