ities of competitive grant funding agencies. In his plenary address to our Society, Wittwer (9) stated ". . .it's time that we became competitive" for federal grant funds. Horticulturists can compete successfully through careful presentation of well-conceived research as exemplified by the following contributions.

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Identifying and Manipulating Metabolic Stress-resistance Traits

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Overall plant resistance to environmental stresses is conferred by characters expressed at four levels of organization: developmental, structural, physiological, and metabolic or biochemical (Fig. 1). Characters expressed at the upper levels are usually controlled by many genes. The potential for using recombinant DNA (rDNA) technology to understand and alter stress resistance is presently limited to single-gene traits, especially those expressed at the biochemical level. Such traits may be rare, and the effects on overall crop performance of modifying them cannot be foretold (Fig. 1), but they remain invaluable for basic research. The ability to construct specific mutations in vitro with cloned genes, and then to reintroduce them into the plant via some mechanism of transformation, would provide the power of genetic analysis to test models of molecular function in stress (2, 22). The ability to suppress expression of individual genes by the use of anti-sense messages would serve similar ends (4, 25). Thus, there is much interest in identifying and isolating single genes related to stress resistance or susceptibility. We discuss and criticize three ways of going about this task and show how helpful an understanding of stress physiology and biochemistry can be.

CASE 1: WHEN A MENDELIAN GENE FOR RESISTANCE IS ALREADY KNOWN

Single genes conditioning resistance or susceptibility to an environmental stress are known in a few instances. An example, Al-tolerance in wheat (31), is illustrated in Fig. 2A. Other examples include osmotic adjustment (41) and salt tolerance (27). Isolating such genes—whose molecular function is unknown—may become feasible by genetic and molecular–genetic means alone. One strategy being explored (1) is shown in Fig. 2B. In this hypothetical example, the gene for Al-tolerance is first mapped by linkage analysis, using DNA molecular markers (50). If tight linkage can be established between the desired gene and such a marker, it may be possible to "walk" along the chromosome from the DNA marker to the tolerance gene via overlapping chromosome fragments (see Fig. 2B legend). However, chromosome walks have fairly limited range, and are complicated by blocks of repetitive DNA sequences, so genes that are quite close to molecular markers in a linkage map may be inaccessible. Thus in Fig. 2B where only 2 map units separate the desired gene and DNA marker (tight linkage in Mendelian genetics), the walk would have to span hundreds of kilobases—probably beyond practical reach in plant DNA.

It remains a difficult task to locate the desired gene even in cases amenable to chromosome walking. Knowing where the gene is depends on finding a difference in sequence between chromosome segments of tolerant and susceptible genotypes. Thus, natural variants pose problems since their DNA sequences may differ in sites besides the tolerance gene (1).

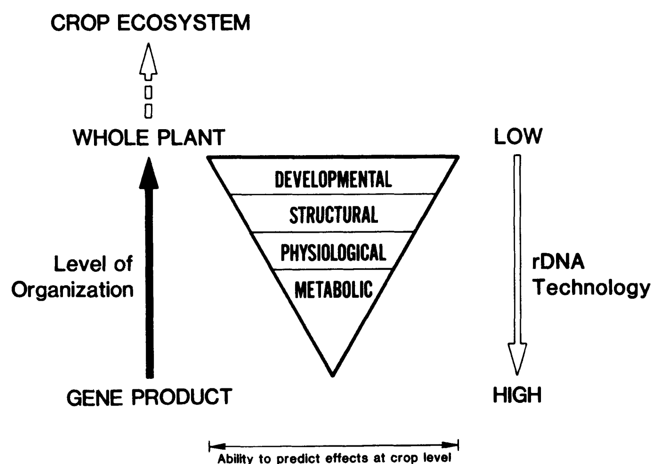

Fig. 1. Some relationships between stress-resistance and rDNA technology. Crop stress resistance can be viewed as the sum of traits expressed at four levels of organization. At the highest level are developmental traits (e.g., time of flowering). Below this come structural traits (e.g., rooting patterns or leaf waxiness). The next lower level comprises physiological traits such as osmoregulatory behavior and water use efficiency. At the lowest organizational level are metabolic or biochemical resistance traits; since these traits can be construed in terms of small numbers of enzymes (gene products), they offer a point of entry to rDNA technology. The organizational levels are framed by a triangle whose width is proportional to our ability to predict the effects of genetic manipulation on stress resistance at whole plant and crop levels. Thus, precise intervention by rDNA technology at the biochemical level has an unpredictable outcome for whole plant and crop stress resistance. This is both its strength as a basic research tool and its present weakness as a breeding tool.

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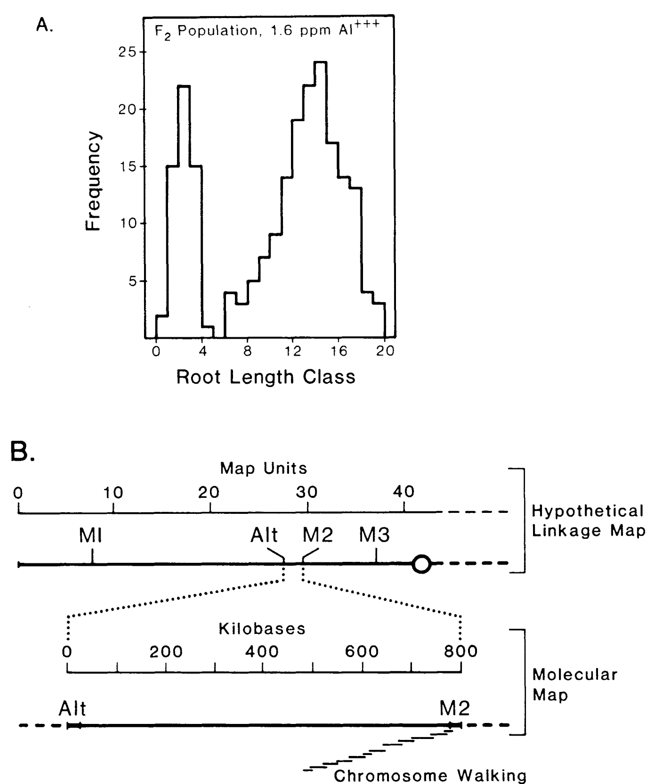


Fig. 2. Mendelian stress-resistance genes and chromosome walking. (**A**) An example of single-gene stress resistance: Al-toxicity in wheat (31). Aluminum at 1.6 ppm severely inhibits root growth (<5 cm) in seedlings of susceptible genotypes, but is without effect in tolerant genotypes. When 'Brevor' (Al-sensitive) and 'Druchamp' (Al-tolerant) are crossed, the F_1 is tolerant, and the segregation of tolerant to susceptible plants in the F_2 is 3:1. (**B**) Isolating a Mendelian stress-resistance gene by virtue of its chromosomal position. First, the Al-tolerance gene could be mapped by linkage analysis using DNA polymorphisms as genetic markers (50). These markers [termed restriction fragment length polymorphisms (RFLPs)] are changes in the sequence of nuclear DNA that add or remove sites for restriction endonuclease attack; they are detected using cloned unique DNA sequences as probes against gels of restricted DNA. The diagram shows a hypothetical chromosome arm carrying three RFLP markers (M1-3), with the aluminum tolerance gene *Alt* tightly linked (2 map units) to M2. The cloned DNA sequence used to detect M2 could be used as a starting point for a chromosome walk towards the *Alt* gene (1). Step one in the walking procedure entails screening a cloned library of large nuclear DNA fragments for those that overlap the M2 probe. These fragments are restriction-mapped and aligned, and sequences furthest from the start point used as probes to find a new set of fragments extending further out along the chromosome. The walk advances by a series of such steps, each of a few kilobases, along the chromosome via overlapping DNA fragments (short overlapping bars). In practice, it is necessary to walk in both directions unless or until some landmark that can orient the walk is reached. The diagram gives a rough correspondence between map distance and DNA sequence (1 map unit = 400 kilobases) based on *Drosophila* work (1, 47); higher plants may have several-fold more base pairs per map unit. The overlapping bars extend to the distance (315 kilobases) covered by a *Drosophila* chromosome walk undertaken to isolate DNA containing genes for two enzymes (1).

The use of this approach thus is limited by the scarcity of single genes known to control stress resistance, the fact that the few known genes involve natural variability, and the need to find flanking DNA markers. Physiology could help in the future by devising methods for screening mutagenized populations in order to find and map mutations relevant to stress-resistance (e.g., ref. 3). Were large collections of such mutants available, some of the genes involved would probably be within walking range of DNA markers. Note that induced mutants are preferable to natural variants, since the former are more likely to differ simply enough from the wild type at the DNA level to identify causal changes.

Developing screens for mutants could help bridge the void between Mendelian genes and DNA sequences in another way. It is becoming possible to exploit mobile genetic elements (transposons) to inactivate genes by insertion events; DNA probes for transposons can then be used to isolate the genes into which the transposon has inserted (7, 8, 10). Since transposon mutagenesis in plants is presently a random process, the ability to test large numbers of plants for interesting mutations is central to its use.

One caution in searching for mutations in stress-related genes is that these mutations are much more likely to entail changes from resistance to susceptibility than the converse. Mutations conferring such hypersensitivity to stress could occur in many genes whose normal products do not limit performance in stress. Hence, not all stress-sensitive mutants can be expected to be equally informative.

CASE 2: SEARCHING FOR STRESS-INDUCIBLE PROTEINS AND mRNAs

The most widely used approach in seeking genes related to stress resistance entails the assumption that novel mRNAs and proteins that appear in response to an environmental stress, typically a sudden shock, help the plant survive the stress. Stresses to which this approach is being applied include heat shock (32), anaerobiosis (44), water deficit (20), salinity (46), heavy metal exposure (26, 53), and freezing (13). The approach has the great advantage that it can lead quickly to gene isolation, either via cDNA clones (11) or via amino acid sequences of the stress-related proteins, which can be used to construct synthetic oligonucleotide probes (48).

There are some drawbacks, however. Foremost is the difficulty of determining the functions of the induced proteins, which creates two types of problems. The first concerns the distinction among reactions to stress that favor survival of the whole plant vs. survival of the cell. Sacrifice of individual leaves, flowers, or developing fruits can be beneficial to the stressed plant as a whole (18), and shedding of excess leaf area is often preceded by withdrawal of assimilates and orderly differentiation of abscission zones (28). This stress-induced senescence is likely to require changes in gene expression (52); hence, it follows that mRNAs and proteins that peak in stressed tissue may not be products of "stress-resistance genes" in a cellular sense. There is thus a risk of confounding stress-induced genes that enhance the survival of a cell with those that hasten its altruistic demise.

The 2nd drawback of stress induction as a criterion for identifying stress-resistance genes can be illustrated with the anaerobic proteins (ANPs) of maize (Fig. 3A). In maize roots deprived of O_2, the synthesis of most proteins ceases, and a novel set of about 20 proteins (ANPs) appears. There is a corresponding increase in the level of mRNA encoding at least some of the ANPs (11). A com-

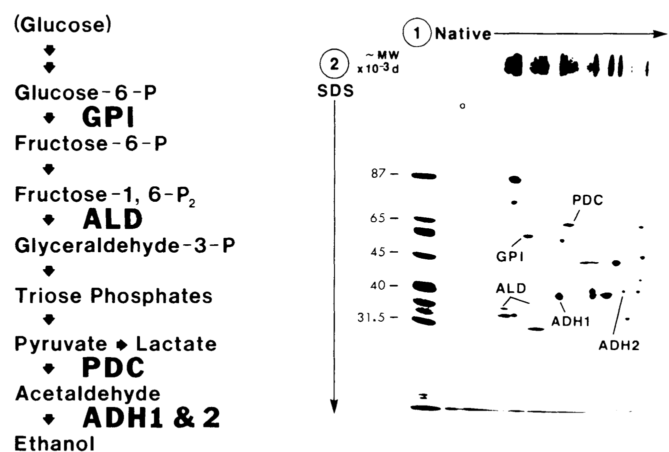


Fig. 3. Identification of the maize anaerobic proteins. (**Right**) the standard ANP profile of maize seedling roots [after Sachs et al. (44)] as seen in a fluorograph of a two-dimensional PAGE separation of [^{3}H]leucine-labeled proteins synthesized during anaerobic treatment. Spots corresponding to identified ANPs are marked as follows: ADH 1 and 2, alcohol dehydrogenases 1 and 2 (44); ALD, fructose-1,6-diphosphate aldolase (30); GPI, glucose phosphate isomerase (29); PDC, pyruvate decarboxylase (35). (**Left**) metabolic map of glycolysis, showing reactions catalyzed by ANPs.

bination of genetic and biochemical work has led to the identification of 6 ANPs—all are enzymes associated with glycolysis (Fig. 3B). It is thus reasonable to suppose that simultaneous expression of at least six out of the 20 anaerobic genes is required to enhance and maintain the capacity for glycolysis during anoxia. But is any one of the six a "stress-resistance gene" in the sense that increasing its expression alone would improve the capacity to glycolyze and hence survive oxygen deprivation? Probably not.

In interpreting ANP induction, the value of understanding anaerobic metabolism is obvious. It is important to note, however, that we understand anaerobic metabolism far better than most other types of stress metabolism (16). For these others, we have no way of knowing whether or not stress-induced proteins form part of a functional complex.

CASE 3: WORKING BACK FROM CHANGES IN METABOLISM TO ENZYMES AND GENES

The 3rd approach for isolating stress-resistance genes relies throughout on physiology and biochemistry. It starts by identifying metabolic changes in stressed plants that are likely to be beneficial, rather than mere injury symptoms. The next step is to infer the key enzymes involved, and then to isolate these proteins. From purified enzyme proteins, amino acid sequence data can lead back via synthetic oligonucleotide probes to the corresponding genes. Sometimes short cuts are possible, using cloned genes for similar enzymes from other organisms, or antibodies against such enzymes, as probes. We use the example of betaine accumulation in water- or salt-stressed chenopods and grasses to illustrate the 3rd approach.

Distinguishing beneficial responses from injuries

The initial inference that a metabolic response is beneficial can be strong when the metabolic problems facing the stressed plant are clear (16). One problem for water- and salt-stressed plants is to lower solute potential without suffering inhibitory effects of salts on metabolic functions, especially protein synthesis (54). Wyn Jones has proposed that this may be achieved by accumulating compatible or benign osmolytes such as betaine in the cytoplasm, while salts accumulate in the vacuole (55). In support of this concept, there is evidence for cytosolic localization of betaine (15, 36), and high concentrations of betaine have been found not to interfere with various metabolic activities in vitro (12, 43). There is thus a sound framework of cell physiology from which to interpret betaine accumulation.

The physiology of betaine accumulation in whole plants is symptomatic of a protective response rather than of a metabolic derangement. For example, the betaine level in salinized plants is strictly proportional to external salinity (Fig. 4; ref. 5), and betaine accumulation, although metabolically expensive, continues during many weeks of severe water stress (21). Comparative physiology and

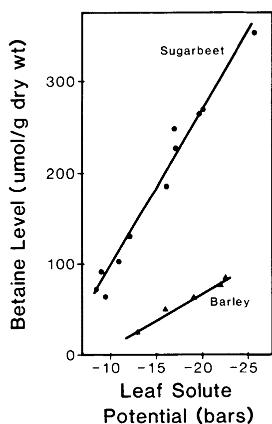
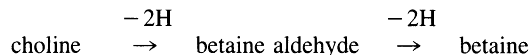


Fig. 4. Physiology of betaine accumulation. The linear relation between solute potential and betaine level in leaves of sugarbeet (data from ref. 19) and barley growing in a range of NaCl concentrations.

biochemistry provide further indirect evidence for a beneficial role for betaine. Betaine is a major osmoticum not only in some halophytic higher plants (14, 55), but also in halophilic bacteria (9) and blue-green algae (40); and supplied betaine will protect bacteria against salt (37).

Inferring the enzymes involved

Radiotracer work with intact tissues (Fig. 5A; ref. 16) has shown that betaine is synthesized in the leaves by a two-step oxidation of choline:



Betaine is subject to little or no additional metabolism in vegetative tissues (19, 34), implying that betaine level can be regulated effectively via the enzyme(s) for choline oxidation, which would be an uncommonly simple and favorable case for molecular–genetic work. In the more usual case, activities and amounts of both synthetic and degradative enzymes have to be considered. These considerations, taken with those above, suggest that the enzyme(s) of choline oxidation are crucial to cytoplasmic osmoregulation, and thereby to stress resistance.

Exploiting comparative biochemistry

Much is known about the enzymes of choline oxidation in mammals and microorganisms (for review, see ref. 16). Choline oxidation in mammalian liver is mitochondrial; the choline → betaine aldehyde step is catalyzed by an inner-membrane flavoprotein dehydrogenase, which has been purified to homogeneity (51). The second oxidation step in mitochondria is via an NAD^+-linked dehydrogenase of the matrix. On the other hand, some microorganisms have oxidases that catalyze both steps. These enzymes are flavins that donate electrons to oxygen and generate H_2O_2; several have been purified (24, 49). Note that such a solid comparative biochemical background is unusual, but that when it exists, it is possible to test antibodies against analogous enzymes from other organisms as probes for plant enzymes (39), and cloned foreign genes as heterologous probes for the plant genes (45).

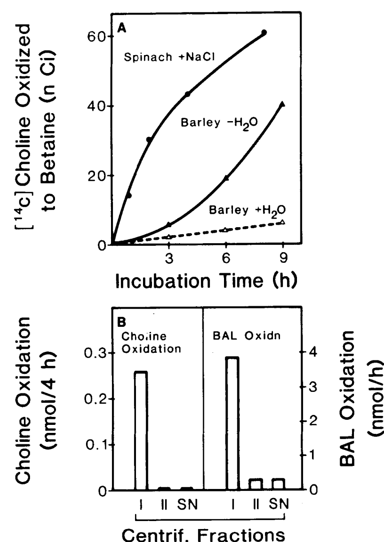


Fig. 5. Biochemistry of betaine accumulation. (**A**) In vivo radiotracer evidence for choline as a precursor of betaine in barley and spinach. Barley leaves fed substrate amounts of [^{14}C]choline converted it to [^{14}C]betaine more rapidly when water-stressed (▲) than when turgid (△). Salinized spinach leaves also showed a high rate of [^{14}C]choline oxidation (data from ref. 6). (**B**) Evidence for chloroplastic oxidation of choline in spinach. Spinach protoplast lysates were fractionated by differential centrifugation as follows: 500–1000× *g* pellet (I); 15,000× *g* pellet (II); supernatant (SN). Fractions were assayed in darkness for oxidation of choline and betaine aldehyde (BAL). Choline-oxidizing activity was present only in pellet I (chloroplasts); BAL-oxidizing activity was also mainly in chloroplasts. Data from (refs. 17 and 38).

Isolating plant enzymes

Like many enzymes of intermediary metabolism, those of choline oxidation are unlikely to be abundant proteins. Calculations based on the specific activity of microbial choline-oxidizing enzymes and rates of choline oxidation in plants suggest an upper limit of 0.01%. The minimum amount of purified protein required for amino acid sequencing is 5 to 10 pmol (23), and, for raising antibodies, $<<10$ μg (33), but it remains a major technical challenge to apply these methods to minor plant proteins.

Plant enzymes for choline oxidation have not yet been isolated, but in spinach they have been located in the chloroplast (Fig. 5B), and there is evidence that the betaine aldehyde → betaine step is catalyzed by a stromal pyridine nucleotide-dependent dehydrogenase (38, 42). The choline → betaine aldehyde step is promoted by light, but its mechanism is not yet known (17). The chloroplast location of choline oxidation is unexpected in view of the mitochondrial location in mammals, and perhaps a caveat against overreliance on comparative biochemistry.

CONCLUSION

The application of rDNA technology to problems in animal, yeast, and bacterial biology has required background information from several disciplines, especially genetics, biochemistry, biophysics, and physiology. Because such information is much scarcer for plants, molecular–genetic research on plants has until now been focused on a few areas where the necessary background exists. It will be necessary to increase our understanding of basic plant biochemical and physiological processes in order for plant molecular biology to move out from these foci into classical agricultural problems such as stress resistance.

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Cellular Mechanisms of Salinity Tolerance

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Salinity is a significant limiting factor to agricultural productivity, impacting about 9×10^8 ha of the land surface on the earth, an area about 3 times greater than all of the land that is presently irrigated (17, 18). Reduced productivity occurs as a result of decreased yields on land that is presently cultivated [about one-third of all irrigated land is considered to be affected by salt (18, 45)], as well as due to the restriction of significant agricultural expansion into areas that presently are not cultivated. In the United States, salinity is a major limiting factor to agricultural productivity, and as the quality of irrigation water continues to decline this problem will become more acute (1, 56). About 1.8 million ha of land are salt-affected in California (56), the major agricultural state in the nation. Annual losses to crop production in the salt-affected areas, including the Imperial, Coachella, and San Joaquin valleys, are substantial and are increasing at a significant rate each year (56).

Problems of salinity may be addressed using technological and/or biological approaches (18, 61, 68). Technological approaches to cope with salinity include advances in water and soil management (61), irrigation methodology (61), and perhaps desalinization (21). Biological approaches include the identification of halophytes that are potential crop plants and, if necessary, the introgression of more desirable horticultural or agronomic traits into them (57); the introduction of salt-tolerance characteristics into crop plants, the majority of which are glycophytic (17, 18, 56, 67, 68, 78, 79); or the manipulation of glycophytic crop plants to adjust and produce under conditions of moderate or low levels of salinity (2, 51, 52). Today, great effort is being directed towards the development of salt-tolerant crop genotypes through the use of plant breeding strategies involving the introgression of the genetic background from saline-tolerant wild species into cultivated plants (56, 67, 68, 78, 79). With recent developments in biotechnology, there is also the potential for obtaining salt-tolerant crop genotypes by the use of somatic cell selection or protoplast fusion methodologies (19, 38, 72, 73) or by gene transformation using recombinant DNA methodologies (9, 28).

The detrimental effects of salinity are due to the influence of ions on the water activity of the external solution, which affects the water status of the plant, and/or to the direct effects of the ions on the physiological and biochemical functions of the cell (14, 20, 22, 53, 85). These effects can result in turgor reduction, inhibition of membrane function or enzyme activity (20, 22, 85), inhibition of photosynthesis (66, 81), induction of ion deficiency due to inadequate transport/selectivity mechanisms (34), or increased use of metabolic energy for nongrowth processes involved in the maintenance of tolerance (62, 89).

Tolerance mechanisms used by plants to adapt to salinity can be separated into those that allow the growing cells of the plant to avoid high ion concentrations and those that permit the cells to cope with high ion concentrations upon exposure to salt (20, 22, 85). Salt avoidance mechanisms involve exclusion at the root (22, 40), absorption by xylem parenchyma cells (40), xylem–phloem exchange systems (20, 22, 40, 85), distribution of ion gradients between nongrowing and growing portions of the plant (85), and, in the case of halophytes, sequestration of ions into salt glands or trichomes (20). In general, exclusion mechanisms are effective at low to moderate levels of salinity, while ion accumulation is the primary mechanism used by halophytes at high salt levels, presumably in conjunction with the capacity to compartmentalize ions in

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