GENETIC ENGINEERING AND CROP IMPROVEMENT[1]

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Most yield advances in crop species have been achieved by individuals whose training and focus have been directly concerned with agricultural production. The past several decades have seen the development of new knowledge and techniques in the basic sciences, genetics in particular, which hold promise for crop improvement, but which have not yet been applied toward that end. If the new approaches can be made routinely available to plant breeders and agronomists, then progress in plant improvement will be enhanced. Restructuring of genetic organization, the process which is currently termed "genetic engineering," has always played a central role in crop improvement. This paper will briefly consider some genetic aspects of crop improvement, and suggest ways that recently developed, novel approaches to genetic manipulation may complement more classical means of genetic engineering.

Classical plant breeding

Plant breeding is an ancient science. The origins of our current crop species are buried in prehistory; all evidence indicates that crop species were domesticated during the Stone Age. Crop species arose from weed species that underwent natural hybridization resulting in increased genetic variability and subsequent selection for desirable phenotypes by prehistoric peoples. Methods of reaping and sowing in the field or methods for storage or preparation can be effective selection screens for plants just as growth on a Petri plate is for a bacterial colony. The early plant breeders searched for, recovered and propagated genetic variants or recombinants which displayed desirable traits. The transformation of weed species into crop plants was accomplished in the absence of modern science or of any knowledge of Mendelian genetics.

Contemporary plant breeders employ essentially the same strategy with success. Their approach involves the production of populations with a broad genetic base followed by selection at the whole plant level for recombinants with positive alterations. Genetic manipulation is practiced without knowing the basis of the separate components which comprise the character being modified. Selection for traits such as yield is practiced at the endpoint of the complex biological processes which produce a whole plant. Mendelism, and a knowledge of transmission genetics, provide a conceptual basis for what is occurring during the breeder's genetic manipulations, but this knowledge is not a requirement for crop improvement.

In most current breeding programs, the availability of genetic variability is not the limiting factor in crop and variety improvement. There is a wide range of genetic diversity in the surviving natural population of most crop species. The focus of breeding efforts is centered on selecting desirable recombinant types that emerge from any particular cross or segregating population. To select desirable recombinants, it is first necessary to decide which alterations or recombinants to select. Currently the assays of agronomic or horticultural utility and subsequent selection are based on observations of whole plant phenotypes. Consequently only major alterations can be recognized. These alterations appear as statistically significant changes in characteristics of bulk populations. Assaying at the endpoint of a number of complex biochemical, physiological and developmental processes hides many potentially useful recombinants in the complexity of the buffered processes producing a whole plant.

The complexity of plant biology and crop productivity yield is also expressed in the genetics of agriculturally important traits. The majority of these traits appear to be controlled by "polygenes" and their transmission is analyzed by quantitative methods. The quantitative inheritance of these traits is a reflection of the complex biological processes which underly their expression and of the lack of well defined mutants with which to analyze these processes. Quantitative inheritance is a phenomenon involving naturally occurring genetic variability and complex biological end products. There is no reason to expect that mutants affecting these processes could not be produced once the individual components are known, nor that the genetics and biochemistry of such traits would be any different from that found in other organisms (e.g., metabolic pathways). For the time being, however, the plant breeder has no choice but to use the phenotype of the endpoint as the basis for selection. Significant progress could be made in the improvement of breeding techniques if we can establish reliable assays at critical points in a number of the component processes of agronomic traits. Examples of such processes are: nitrogen metabolism, photosynthesis, water relations, mineral nutrition, transport within the plant and grain development. With these critical processes individually analyzed and assayed, genotypes demonstrating optimal performance at different steps in a process could be combined to produce a new, highly productive cultivar.

Role of molecular biology

Recent advances in molecular biology have provided methods of genetic manipulation which should be applicable to agricultural plant species. This is certainly an exciting prospect. Despite the rapid progress in expanding our knowledge of basic genetic and biochemical mechanisms in lower organisms this knowledge has had no direct impact on crop improvement. This lack of impact may be ascribed in large part to conceptual and experimental differences between the disciplines of molecular biology and plant breeding.

Molecular biology is comprised of two basic elements: the *reductionist* world-view of basic science and a powerful set of analytical experimental tools. One example of this approach was the use of defined genetic variants combined with precise biochemical methods to elucidate the mechanisms that regulate metabolic pathways in a variety of organisms. In contrast, plant breeding as currently practiced has of necessity a more *holistic* approach. Plant breeders have to operate within difficult constraints. They have little choice in either their experimental materials or the problems which confront them. No strong correlations have been established between yield and any of the individual processes that contribute to the final product. The experimental and technological requirements of plant breeding and the constraints of the plant system are different from those imposed by molecular biology. The question is, can the novel methods of "genetic engineering" defined in microbial systems really be applied to crop improvement?

Two different approaches are presently being developed that attempt to extend the methods and techniques of molecular biology from microbial investigation to application for crop improvement: one involves cellular manipulations and the other involves DNA manipulations. Cellular manipulations hold the potential for developing an experimental system for crop species suitable for more refined analytical techniques. Using single somatic cells as experimental organisms, it is possible to achieve mutant production, analysis and hybridizations not possible using whole plants. Such techniques may permit important cellular processes to be characterized to the extent that useful directed modification is possible. Engineering at the level of the genetic material itself (i.e., manipulation of DNA) has great potential for the manipulation of defined genetic elements, although much work needs to be done before such sophisticated techniques can be applied to specific agricultural problems.

Although many tools of the molecular biologist are now available to the plant geneticist, some limitations prevent their application to breeding problems. The first problem with these approaches is a technical one. Regeneration of whole plants from single cells is essential for application of the technology of in vitro genetic manipulation to higher plants. However, this step has not yet been routinely accomplished with any major food crop. The second problem arises from the real needs of the plant breeder. In most instances, the availability of genetic variability is not the limiting factor in crop improvement; the ability to recognize and recover useful recombin-

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ants sets the limit. Hence the production of genetic variability via cellular mutation or hybridization provides no uniquely useful tool at present. The third problem results from the developmental biology of agronomic and horticultural characters. Many agronomic traits are tissue-specific; their expression is found in only one or a few tissues within the plant and is often not found in cells cultured in vitro. If a particular trait is not expressed in culture there is no reason to expect that the trait can be altered and screened for via in vitro methods. The fourth problem involves the genetics of agricultural traits. Mutant selection systems and DNA manipulations allow modification of single gene traits. Most agronomic and horticultural traits as they are now defined are polygenic in inheritance. Small additive, stepwise modifications would be difficult to recognize. Currently, genetic modification of crop plants using cellular or DNA manipulations should prove appropriate in cases where the alteration involves single gene traits which are not tissue-specific and for which there are good selective techniques. The technology involved in these approaches will almost certainly be improved to overcome the limitations discussed above. However, at present, single gene traits which are not tissue-specific are rare as are appropriate selective systems. Possible examples of such traits would include disease resistance or tolerance to ion toxicity, but the range is limited.

It would appear that molecular biology is not yet relevant to crop improvement. The difficulty is that current efforts have attempted to transfer the experimental results directly (defined genetic manipulation) without also extending the reductionist approach of molecular biology. The immediate need is not to find new ways to generate genetic variability but to find new ways to screen critically the variability already provided by nature and to identify the biochemical, physiological and developmental components of agronomic traits. Once individual components and the rate limiting steps of important traits are identified, designing methods of selection for altered traits is possible. We identify the first task as the extension of the world-view of molecular biology to agriculturally important characters.

Perhaps an appropriate approach to this task would be to choose a character which is defined from the breeder's perspective and attempt to apply molecular biological tools to its analysis (i.e., attempt a genetic-biochemical study of the character). Agronomic and horticultural traits are vastly more complex than bacterial metabolic pathways (e.g., lactose catabolism). A trait such as yield for example, which represents the integrated product of innumerable specific pathways, would have to be broken down into a large number of subcomponents. At first glance the question of how to begin to dissect such complex traits appears difficult at best. One apparent approach to identifying rate limiting processes important in crop production is by analysis of heterosis.

Heterosis and biology of crop improvement

Effective genetic manipulation of agronomic and horticultural traits, especially if utilizing in vitro selection techniques, requires identification of the relevant metabolic processes and specific rate-limiting steps. Many such traits, including yield, drought tolerance, time of maturity, protein content and tillering ability are complex quantitative traits under the control of multiple genes (polygenes) (1, 3). The final phenotype is separated from the basic biochemical steps, the units of selection, by several levels of biological organization and environment-genotype interactions. Unfortunately, the definition of polygene and statistical methods for its analysis are not compatible with the analytical approaches of molecular genetics. Likewise, biochemical approaches have been frustrated by the complexity of quantitative traits even when they include the analysis of divergent genotypes. For example, despite considerable effort, no strong correlation has yet been found between final yield and the productivity of any distinct biochemical pathway (2).

Characterization of heterosis will provide information about the processes that limit expression of quantitative traits. Heterosis (hybrid vigor) refers to the beneficial effects (increased size and productivity) which are observed in most F_1 hybrids derived from crosses between unrelated parental lines (1, 3). This classically defined genetic phenomenon is described almost entirely by statistical methods and remains essentially uncharacterized in molecular terms. Known genetic and molecular mechanisms can account for the phenomenon of heterosis (6). However, there is no direct evidence that any of these mechanisms are in fact involved in heterosis. Several cases have been described where the activity of a specific enzyme (e.g., nitrate reductase, mitochondrial oxidative phosphorylation) was higher in the hybrid than in either parental line (4, 7). However, no causal relationship has been established between such increases in enzyme activity and overall increases observed for growth or yield. Despite uncertainty concerning the mechanisms involved, it is clear that constraints in one or more of the systems that limit yield in the parents have been relieved in a superior hybrid. Such hybrids offer an opportunity to identify physiological and developmental traits that are of direct importance to yield and the rate limiting steps contributing to these traits. This information would eventually result in development of techniques to predict the combining ability of prospective parental lines.

We need an experimental system dealing with a specific character that satisfies both the classical definition of a heterotic effect and the requirements for effective manipulation and analysis of the discrete genetic and biochemical components involved. One approach would be to use specific stress conditions to modulate the magnitude of the hybrid advantage. For example, if the superior performance of a hybrid genotype is due in part to more efficient conversion of a particular input (e.g., light, CO_2, nitrate, phosphate, etc.) to a useful product, then, 1) the advantage should diminish in the absence of any essential input because this condition will now limit the final phenotype; 2) the hybrid advantage should be magnified under limiting conditions where growth is directly related to the level of that specific input; and 3) the relative response of the hybrid and parental genotypes should be unaffected if the heterotic effect is not related to the stress conditions. It should therefore be possible to take a hybrid clearly evidencing heterosis under field conditions, raise it under

Table 1. Relative growth response of F_1 hybrids and inbred parental cultivars to different levels of nitrate.[z]

Hybrid genotype	Trait	Relative growth (%, hybrid/better parent)			
		Nitrate concn			
		6mM	None	6μM	60μM
C0113 x C0109	Dry wt	125[y]	107[x]	187	157[w]
	Fresh wt	120[y]	98[x]	187	173[w]
	Height	118[y]	102[x]	118	126[w]
OH43 x B14	Dry wt	144[w]	69[w]	64	109
	Fresh wt	144[w]	75[w]	61	111
	Height	100[w]	88[w]	79	110
M107 x N28	Dry wt	134	163		196
	Fresh wt	230	192		204
	Height	142	126		135
H93 x VA26	Dry wt	202	104		78
	Fresh wt	172	130		97
	Height	130	93		88

[z]The values in the Table represent an average of 1-4 experiments. In each experiment the relative growth is indicated as a percentage in which the performance of the hybrid is compared to the better of the two inbred parental lines. The performance of each genotype is based on the average of measurements of 9-12 plants grown in 30 x 46 cm flats. Flats containing 13 to 15 cm washed. sterile sand over 10 cm of Perlite were sown with 24 seeds (2 genotypes) at a uniform spacing. Flats were maintained in growth chambers with 20 hr days (32.4 klx) at 1.2 m from lights, from 4:00 AM to 12:00 PM, 54 klx from 11:00 AM to 7:00 PM and 77.8 klx from 2:00 PM to 5:00 PM at 27°C and 4 hr nights at 27°. Flats were watered twice daily with deionized water.

Starting 10 days after planting the seedlings were watered three times a week with a nutrient solution. One liter of solution per flat was applied and allowed to drain through.

Control flats received a Long Ashton nutrient solution composed of the following mineral salts (concn in mg/liter):

Macronutrients: KNO_3, 202; $Ca(NO_3)_2 \cdot 4H_2O$, 472; $Mg(SO_4) \cdot 7H_2O$, 184; $Mg(SO_4) \cdot 7H_2O$, 184; $NaH_2PO_4 \cdot H_2O$, 184.

Micronutrients: $MnSO_4 \cdot H_2O$, 1.69; $CuSO_4 \cdot 5H_2O$, 0.125; $ZnSO_4 \cdot 7H_2O$, 0.29; H_3BO_3, 3.1; NaCl, 5.85; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.0088.

Iron: Ferric tartrate, 5.0.

The experimental flats received a modified nutrient solution which contained either low levels of nitrite or no nitrite. The macronutrient composition of these modified nutrient solutions is listed in Table 3. The concentration of the micronutrients and iron remained unchanged. Fifty six to 65 days after planting the aerial portion of each plant was measured, harvested and weighed. Dry weights were determined after 6 days of drying at 95°C.

[y]Avg of 4 experiments.

[x]Avg of 2 experiments.

[w]Avg of 3 experiments.

controlled, specified stress conditions in the laboratory and thereby identify parameters which alter the relative performance of the hybrid and parental genotypes. Using this approach it should be possible to pinpoint specific biochemical pathways that contribute to hybrid vigor. Extended study can focus on individual steps within those pathways, elucidate the mechanisms responsible for heterosis and identify the rate limiting steps underlying yield.

Experimental approach

This experimental approach was tested by growing 8 inbred strains of corn and their F_1 hybrids in a controlled environment with nutrient solutions containing different levels of nitrate. We sought to discover whether increased efficiency in the processes of nitrate uptake and utilization could account for heterosis in any of these hybrids. Preliminary results and the experimental procedures are presented in Table 1. Each set of inbred strains and their hybrids were fertilized either with a nutrient solution containing standard levels of nitrate (6mM) or a modified nutrient solution containing either no nitrate or reduced levels of nitrate. The height, fresh weight and dry weight of the aerial portion of the plants were measured after about 60 days of growth; the ratios between the values for the hybrid and the values for the better of the two parental strains were used as measures of heterosis. All four hybrids surpassed the better parent for each of the traits measured (except height, OH43 x B14) (Table 1). In 3 of 4 cases the heterotic response disappeared as predicted when nitrate was removed from the medium. Continued superiority of the M017 x W28 hybrid at no nitrate presumably reflects higher levels of nitrogen in the hybrid seed. One of the 4 hybrids, C0113 x C0109 showed increased heterosis at low levels of nitrate indicating, according to our hypothesis, that increased efficiency of uptake and/or assimilation of nitrate contributes in part to this heterosis. The H93 x VA26 hybrid does not show a response at low levels of nitrate and the OH43 x B14 hybrid responds slightly at 60μM nitrate compared with 6μM nitrate, but there is no stimulation of heterotic effect above control levels despite the higher levels of nitrate reductase reported in the hybrid (8). An attempt was made with C0113 x C0109 to localize further the heterotic effect within the nitrate assimilation pathway by using the intermediates, nitrite, ammonium and L-glutamine as nitrogen sources (Table 2). The magnitude of the heterotic effect decreases when later intermediates are used; this suggests that the overall advantage results from the cumulative effect of small increments at several sites rather than a large advantage at a single enzymatic step. The results are not strictly comparable however, since alternate uptake pathways are important for these different nitrogen sources (5, 9). The enzymes that catalyze these steps are being characterized in current studies.

The results are not surprising: 1) heterosis can result from increased efficiency for nitrate uptake and assimilation and 2) the metabolic processes responsible for heterosis are different in different hybrid genotypes. These preliminary experiments do show, however, that this is a productive approach for the identification of specific processes contributing to hybrid vigor; these processes include the rate-limiting steps of yield. Once these steps have been identified, it is then appropriate to use the experimental system of molecular biology to undertake genetic modification in the service of crop improvement.

Table 2. Relative growth response of C011 x C0109 hybrid and inbred parental cultivars to different nitrogen supplements.[z]

	Relative growth (%, hybrid/better parent)					
	N supplement					
Trait	6mM NO_3	0 NO_3	60μM NO_3	60μM NO_2	60μM NH_4	60μM Gln
Dry wt	125[y]	107	157c	115	97	148
Fresh wt	120[y]	98	173c	149	105	159
Height	118[y]	102	126c	118	110	124

[z]Values are an average of 2 or more experiments. The values in the first 3 columns are included from Table 1 for comparison with the results obtained when the C0113 x C0109 hybrid received nutrient solution supplemented with alternate N sources. These N compounds were added to the modified nutrient solution containing no nitrate as indicated in Table 3. Plants were grown following the procedures described in Table 1.

[y]Avg of 4 experiments.

[x]Avg of 3 experiments.

Table 3. Macronutrient composition of modified Long Ashton nutrient solutions with different nitrogen supplements.

	Concn (mg/liter)					
	N supplement					
Macronutrients	60μM NO_3	6μM NO_3	0 NO_3	60μM NO_2	60μM NH_4	60μM Gln
KNO_3	2.02	0.202	–	–	–	–
$Ca(NO_3)_2 \cdot 4H_2O$	4.72	0.472	–	–	–	–
KNO_2	–	–	–	5.1	–	–
$(NH_4)_2SO_4$	–	–	–	–	7.92	–
L-glutamine	–	–	–	–	–	8.76
KH_2PO_4	269.3	272	272	272	272	272
$Ca(H_2PO_4)_2 \cdot H_2O$	499	504	504	504	504	504
$Mg(SO_4) \cdot 7H_2O$	184	184	184	184	184	184
$NaH_2PO_4 \cdot H_2O$	184	184	184	184	184	184

Literature Cited

1. Allard, R. W. 1960. Principles of plant breeding. Wiley, New York.
2. Brown, A. W. A., T. C. Byerly, M. Gibbs, A. San Pietro. (eds.) 1975. Crop productivity – research imperatives. Mich. Agr. Expt. Sta. and Charles F. Kettering Foundation.
3. Gowen, J. W. (ed.) 1962. Heterosis. Iowa State College Press, Iowa City.
4. Hageman, R. H.. E. R. Leng, and J. W. Dudley. 1967. A biochemical approach to corn breeding. *Adv. in Agron.* 19:45-86.
5. Heimer, Y. M. and P. Filner. 1971. Regulation of the nitrate assimilation pathway in cultural tobacco cells. *Biochimica and Biophysica Acta.* 230:362-372.
6. Rice, T. B. and P. S. Carlson. 1975. Genetic analysis and plant improvement. *Annu. Rev. Plant Phys.* 26:279-308.
7. Sarkissian, I. V. and H. K. Srivastava. 1973. Some molecular aspects of Mitochondrial complementation and heterosis. p. 53-60. *In* A. M. Srb. (ed.) Genes, enzymes and populations. Plenum Press, New York.
8. Schrader, L. E., D. M. Peterson, E. R. Leng, and R. H. Hageman. 1966. Nitrate reductase activity of maize hybrids and their parental inbreds. *Crop Science* 6:169-173.
9. Sims, A. P., B. F. Folkes, and A. H. Bussey. 1968. Mechanisms involved in the regulation of nitrogen assimilation in microorganisms and plants. *In* E. J. Hewitt and C. V. Cutting (eds.), Recent aspects of nitrogen metabolism in plants, Academic Press, New York. p. 91.