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Biotechnology and Fruit Growing

S. SANSAVINI[1]

Introduction

Ecologists are calling it the "new green revolution." Advances in biotechnology are seen as ushering in a new era in world agriculture rivaling that of the 1960s and 1970s, when genetics were applied to crop breeding with the result that entire continents became self-sufficient in staple foodstuffs. The enormous increases in grain yields in China, India and Mexico are the most striking examples.

Perhaps because of the inherent complexities in working with tree species, i.e. their having such a lengthy reproductive cycle, the fruit growing industry has lagged behind in taking advantage of the benefits wrought by the new biotechnologies as well as in implementing the substantial changes in crop management that have become standard practice in some modern orchards. The current trend of using biotechnology for crop improvement is geared to replacing the highly chemical-dominated, polluting and costly techniques of high energy input with innovative alternatives that are cleaner and more specifically "targeted." Nor is it simply a matter of yield. Improving the quality and marketing image of products as well as methods of "organic" cultivation and the health issue also are involved—objectives that clearly will be difficult to attain in the short term.

Just as clearly the time is ripe for procedures in biotechnology to lend the fruit growing industry a helping hand. The traditional methods of crossbreeding, mutagenesis and selection are slow, very costly and often have meager results to show for their efforts. This is especially true concerning pear, citrus, olive and grape—plants which have acquired through centuries of domestication a rich genetic heritage. The most important of these traits, expressed in the field performance of each species, continue to be the concern of painstaking research even today. Yet many traits are unfortunately polygenic, of complex heritability and cannot easily be bred by crossing except over several generations.

The objectives being pursued by the more advanced research institutes today are not the same as in the past. These endeavors are mainly focused on resistance to disease (fungi, bacteria, viruses, nematodes, etc.), to environmental stress (drought, waterlogging, high and low temperatures), to soil salinity, and on increasing photosynthetic efficiency and yields (in terms of dry matter, fruit/leaf ratio, etc.) on enriching a fruit's nutritional and dietary value, on extending its shelf life, on improving its firmness, taste and processing qualities.

While the traits controlling disease and pest resistance may manifest themselves and be detected at a very early stage (in a matter of weeks or months of a plantlet's life), those linked to cropping take years to evince. This reveals some of the interest in biotechnology, which is seen not so much for its potential in alternative methods, but rather as a source of techniques complementing traditional breeding programs. Thus biotechnology may be used to accelerate these procedures, to pursue specifically targeted objectives, to augment genetic variability and to

[1]Istituto Coltivazioni Arboree, University of Bologna.

enhance the selection range of new genotypes. It is to be kept in mind that a plant's genetic make-up comprises tens of thousands of genes that have to be combed for different alleles in the best combinations.

Let us take a brief look at the main applications of biotechnology in fruit breeding and at the results attained thus far.

1) *"In vitro" culture*

The new technologies in plant breeding are often based on *in vitro* culture methods. One of these is *micropropagation*, which was introduced commercially in the 1960s for the multiplication of orchid apices but was not applied to fruit species until the 1970s. Our researches in the development of micropropagation techniques for strawberry (the first strawberry workshop was held in Italy at Cesena exactly 12 years ago) and numerous deciduous fruit tree rootstocks (notably the GF 677 peach x almond hybrid and other *Prunus* species) were among the first of their kind in Europe. Today the millions of plantlets produced annually by Italian and other European laboratories are radically changing the nursery industry.

Some of this basic research has also evinced the methodological and economic limits of the technique's application; phenotype variations (epigenetic) and even genotype instability have been found in shoots grown from meristem apices of strawberry (22) and other species. There are numerous advantages, too, as the following summary suggests:

- Vegetative propagation of fruit species with poor rhizogenic capacity (e.g. pear) and of those usually propagated by seed or such costly techniques as green (e.g. mist) and woody cuttings or by stool bed.
- Extremely rapid mass multiplication of clones: the programming in sequential series, free of seasonal limitations, of the stages of proliferation, elongation, rooting and hardening in very restricted space by industrial techniques enabling marked economies of scale.
- Use of meristem cultures to propagate disease-free clones (or a least enhancing the chances of keeping in a healthy state clones that have been restored by heat treatment) of strawberry, citrus, grape, etc. Specific "micro-grafting" techniques for citrus have been developed by Navarro in Spain and adopted for other species. Meristem apices are grown in test tubes and grafted after only two weeks' growth in the dark on in vitro seedlings. The technique admits of several variations and can also be used to pretest grafting incompatibility in vitro (15).
- The introduction of a more reliable system of control and certification of nursery-produced plants (all the more so since it was shown by Edin et al. in 1987 that "micropropagated" rootstocks perform as well as normally propagated ones).

Then there are the more challenging objectives that go beyond the scope of immediate application in nursery development and renewal. These concern programs of fruit tree breeding, with particular emphasis on such in vitro techniques as embryogenesis, embryo, tissue and protoplast cultures, differentiation, somatic organogenesis, somaclonal selection (with and without mutagenesis), bacterization and microrhization of fruit plants, germplasm identification and storage. The Cadriano Experimental Station of the University of Bologna's Istituto di Coltivazioni Arboree (including CMVF, CRIVE and CNR'S CESTEF laboratories) are among the Italian centres engaged in these investigations.

2) Cell and Tissue Cultures and somaclonal Selection

That cell cultures have been used for quite some time in genetic research can be explained by the frequent occurrence of mutants in nature as well as their relatively high susceptibility to mutagenic treatments and capacity to generate ploidal variations. If the cells are somatic and totipotent, then plant regeneration by organo- or embryogenesis can be achieved.

The mutants involved in these regeneration techniques can be either chimeric or solid (23) and are used for what is known as somaclonal selection. Variability induced in cell lines subjected in vitro to controlled selective pressure is usually quite high. The real limiting factor is in the subsequent regeneration stage. Thus far concrete results have been achieved in the selection of cell mutants of crop species resistant to biotic and abiotic stress, i.e. biological toxins (culture filtrates), pest-and herbicide molecules in general, and high concentrations of NaCl and other salts which can be added to culture media. On the assumption that there is a correlation between in vitro and field resistance, the mutants, selected very precociously, are then regenerated and whenever possible planted in the field. Some examples are tobacco resistant to Paraquat, maize resistant to *Helmintosporium* sp., potatoes resistant to *Phytophthora* sp. (1).

Of far greater importance are the mutations involving the nuclear genes as they can be transmitted sexually. In contrast, if the mutations concern only cytoplasm DNA (Mitrocondrial or chloroplast), then transmission follows the patterns of maternal heredity, although they can be sustained in fruit trees by vegetative propagation.

Another mutant found to be important in terms of application is that concerning resistance to high rates of aminoacids. This obviously would represent an early screening method in the selection of cereals or species of enhanced nutritional value, eg those with more stored proteins. In apple and other species, this could also hold true for any correlations between "heat shock proteins" (of high molecular weight and linked to temperature rises) and "cold shock proteins" (temperature drops) and resistance to high and low temperatures, respectively. These temperature shock proteins supposedly protect the stressed cells.

Precocious "in vitro" somaclonal selection can also be performed on tissues and shoots. It is the scope of research on strawberry that was recently begun at the Cadriano Station by P. Rosati. Once a positive correlation between "in vitro" and field varietal susceptibility of strawberry to *Phytophthora cactorum* and *Verticillium* sp. was established by enriching the medium with a culture filtrate of these fungus pathogens, the method was then applied to the early selection of seedling progenies.

A variation of this technique, which is even more rapid and quite simple, is "ion conductance," i.e. the use of leaf discs for in vitro screening. These are cultured in a liquid medium enriched with cultural filtrates of various pathogens, and then a conductimeter measures their ion loss: the higher the strawberry's susceptibility to the fungus the greater the number of ions lost by the contaminated disc into the liquid. Ion conductance has also been employed on apple to test susceptibility to *Phytophthora,* and, if it proves accurate for tree species as well, the method will no doubt find extensive use in fruit growing.

There have been some spectacular results in applied research too. At Beltsville in the USA, Hammerschlag (10, 11) succeeded in regenerating a pair of peach plants from callus cultures (from

400 young embryos of cv Sunhigh). They were grown in media enriched with toxic metabolites of *Xanthomonas pruni*, a bacterium that causes spotting of fruits and leaves. The F_1 plants are now fully resistant to *Xanthomonas* sp., whereas the original cultivar is very susceptible.

In Belgium Viseur (28) selected, by regenerating shoots from root callus cultures of the pear cv. Durandeau, a somaclonal variant that is not receptive to "in vitro" inoculations ("escaped infection") of *Erwinia amylovora*. Other examples include citrus plants seemingly resistant to salinity and cultural filtrates of various pathogens (26) and grape vines that have been precociously screened for selection of seedlings with a high concentration of antimicrobe compounds such as phytoalexine, which is supposedly correlated to mildew resistance (24).

3. *Protoplast Culture*

A new and promising field of research concerns protoplasts, isolated cells deprived of their walls by enzyme digestion. This is relatively easy to accomplish with cells of somatic tissues, usually foliar ones that have been taken from plantlets grown in vitro and hence already sterile.

Potentially protoplast culture opens up avenues of research hitherto considered unapproachable in the genetic engineering of fruit trees. Its aim is the fusion of cells of genetically remote species, or of those that are biologically incompatible of the same species, so as to produce somatic hybrids. Fusion is attained either with the aid of polyethyleneglycol (PEG) or via electric impulses (electrofusion).

The first such hybrid was produced by Carlson in 1972 with *Nicotiana* spp. Figure 2 is a theoretical model of how fusion works. In other words, a hybrid can be derived from complete fusion of the protoplasm, including the two nuclei, resulting in a fertile or, if there is a loss of chromosomes (i.e. aneuploidy) an unfertile plant; or in the fusion only of the two cytoplasms (whence the term "Cybrid," for cytoplasm hybrid) with the nucleus of one or the other cell; and asymmetric fusion in which one of the two nuclei can be devitalized (usually by radiation). The latter technique appears the most realistic in terms of practical results. Fusion hybrids are in actual fact new plants resembling sexual anphidiploid hybrids, as for example the domestic prune. So far, however, nature has found a way to defend itself, and almost all attempts with fruit trees along these lines have met with failure.

Instead the isolation and culture of protoplasts have, though not without difficulty, been successful for several species in regard to simple duplication and formation of callus (13). This contrasts with the limited success that has been reported for the subsequent stages of differentiation and regeneration of the entire plant: only with pear, Colt cherry, apple (19), kiwi fruits and citrus (7, 20).

High-voltage electric impulses (250-500 V), or electroporation, employed on cherry protoplasts have promoted callus growth and the regeneration of shoots so derived (19); the same technique also has been used to transfer genetic information (plasmid DNA) to tobacco mesophyll protoplasts (8). Protoplast culture lends itself both to genetic engineering (*infra*) and to attaining somaclonal variability (in relation to the cell of origin and the type of morphogenesis). Gupta et al. (9) developed a method of regenerating somatic embryos from protoplasts derived from the suspension of embryo cells of immature *Pinus taeda* seeds.

4) *Transgenic Plants*

The transfer of genes or chromosome strands from one cell to another (aside from impollination or sexual means) began about fifteen years ago when Boyer and Cohen developed the technique called "recombinant DNA." The method itself involves the transfer

to the target cell or plant of certain genetic messages or DNA sequence, which is the code for the desired traits. The world's most prominent research centres in molecular biology and genetic engineering vied with one another in developing and applying the technique. The literature already is quite vast.

The procedure is not a simple one, not even from the viewpoint of the type of bacterial cells normally employed in its application. The following is a brief outline of the stages involved.

1. Gene identification and isolation; the use of restrictive enzymes to cut the polynucleotide sequence; cloning the genes making up the DNA molecule.

2. The gene is next introduced (barring rejection) into a similar area (T-DNA) of the TI plasmid (Tumor-inducing) of another bacterium called the vector, which is actually "engineered."

3. Transfer of the gene from the vector to the "target" cell or plant, which must be "infected" with the vector plasmid.

4. Testing to ascertain that the "change" has occurred and the trait has been transferred and accepted, having even passed through the meiotic grid (in successive generations).

The bacterium most commonly used is a strain of *Agrobacterium tumefaciens*. It is rendered harmless (incapable of generating the well-known tumors) while preserving unaltered its capacity to transfer TI plasmid, carrying the foreign gene, to protoplasts, bacteria, viruses, or even to leaf discs, small shoot segments or "in vivo" plants (Fig. 4) that are to be contaminated and genetically engineered.

It is not a simple operation. Gene markers that can be detected by biochemical analysis are used to make certain that gene transfer has taken place. In the case of *Agrobacterium*, the strain can be the carrier of resistance to an antiobiotic like kanamycin. It is thus sufficient to add kanamycin to the culture of the target cell: if the cell survives, it means transfer has probably occurred. When the transformation is confirmed then one proceeds to regeneration and the expression of the acquired trait in the so-called "transgenic" plant. In other cases gene markers encoding for opine systhesis are used.

Instead of *A. tumefaciens* use is sometimes made of *A. rhizogenes*, which enhances rhizogenesis in the infected plant. Here the genetic information in the T-DNA region is carried in the RI plasmid (Root-inducing); the transfer mechanism is the same.

There are numerous examples of transgenic plants, eg cotton, *Solanum* spp. and several legumes, in which monogenic traits have been introduced (usually resistance to diseases or to express quality characters); some have even caused quite a stir. This is the case of tobacco and tomato made resistant to the herbicide glyphosate or to certain insects, i.e. the plants were engineered with the coding gene for endotoxins of *Bacillus thuringensis* so they became in effect bioinsecticidal (27). Sensational in this respect was last year's case of the tobacco plant made luminescent by transfer of the gene controlling this trait from the firefly.

There are still very few examples concerning fruit trees. Their traits are mainly polygenic, quantitative and thus not easily transferrable via this technique. Nor are their genes easy to relocate in the same sequences in the host plant. At Kearneysville in the USA researchers are attempting to identify the ripening genes in peach, and at Plant Cell Research Institute, Dublin in California they are trying to clone the gene of the Brazil nut which controls a protein rich in S-Aminoacids so it can be transferred to soybean and other oil-producing plants which are poor in these aminoacids.

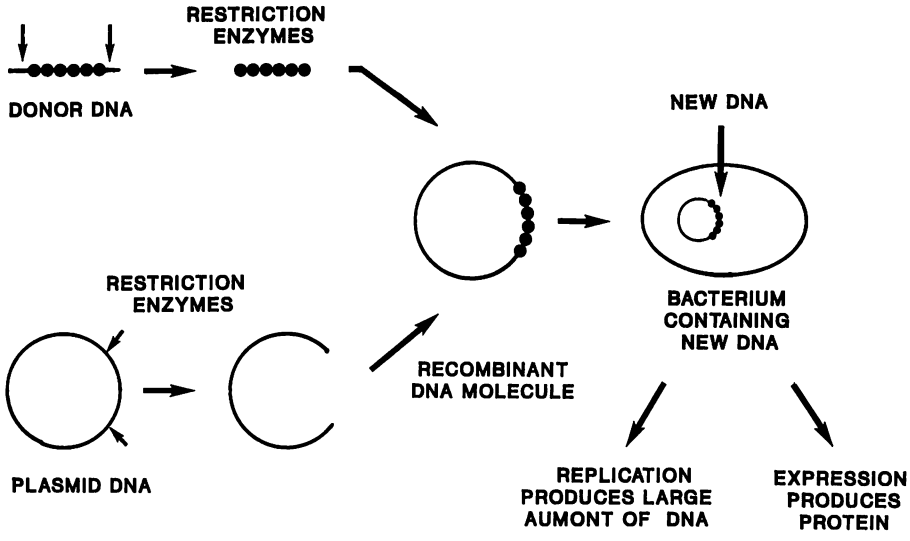

Figure 1. Recombinant DNA: the technique of recombining genes from one species with those of another (1) (Source: Office of Technology Assessment, 1984).

Here are a few of the more numerous examples of transformation concerning fruit species. James et al. (14) in the UK transferred genes of harmless *A. tumefaciens* with the engineered binary vector "Bin 6" to apple shoots regenerated from leaf disc callus. This is a so-called chimeric gene having the coding sequences for the production of nopaline and for an enzyme (phosphortransferase of bacterial neomycin) controlling resistance to kanamycin.

Another potential application regards the transfer of genes codifying the auxin and agropine in the TR-DNA of RI plasmid (18) for use in rootstocks of poor rhizogenic capacity. Lambert and Tepfer (17) employed a simpler method to induce root formation on M9. They infected the rootstock itself with cultures of *A. rhizogenes*, a procedure that also can produce genetic chimeras. Preliminary transformation experiments by infecting seedlings of several species testing (cherry, apricot, peach, walnut, strawberry) with a strain of *A. tumefancies* carrying the PtiA6 plasmid were conducted in California by Dandekar (4). Up to 40% of the seedlings were infected; the transfer was detected biochemically by the presence of octopine.

Aside from these initial successes, the bacterial vector technique is still largely unexplored. This is partly due to both the intrinsic difficulties of obtaining useful genes for practical application and in transferring the simple cell models on which experiments are conducted to fruit species. This explains why other gene transfer techniques are being investigated:

a) Replacing bacterial plasmid with infected virus as vector. While this technique was also proposed by Brisson et al. (2), it would restrict the range of host plants to those species susceptible to infection by the virus employed.

b) Direct transfer of the gene from bacterial plasmids to protoplasts or

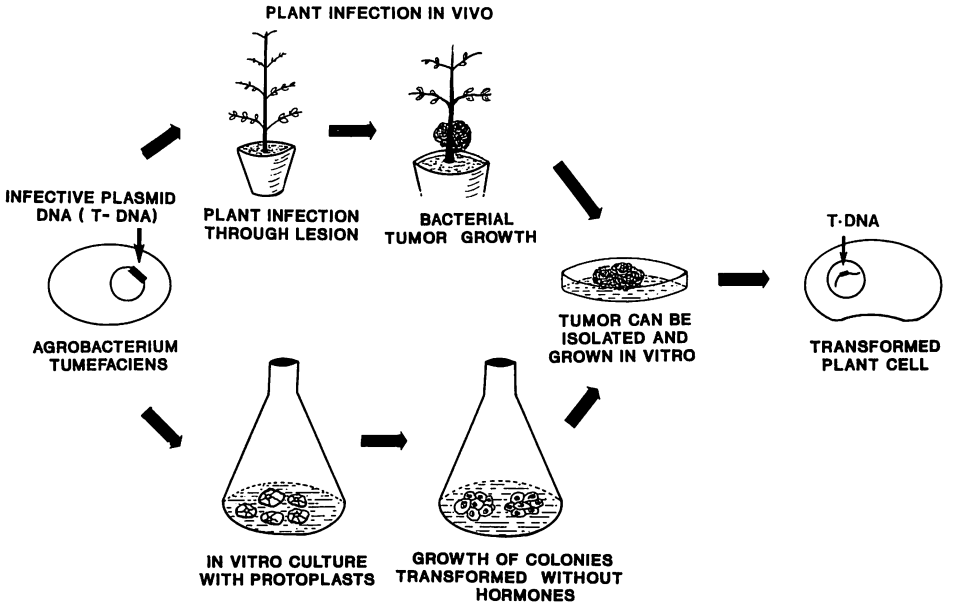

Figure 2. Gene transfer via plasmid vector (T-DNA) of *Agrobacterium tumefaciens*, agent of plant root and collar tumors. Infection can be either *in vivo* or *in vitro* with protoplast culture.

even gamete cells (25). This method is very promising, although its drawback lies in the subsequent regeneration from protoplasts. In their work on *Nicotiana* spp., Castiglione et al. (3) found a pronounced specificity in protoplast transformation. Regardless of the technique employed, by changing the Mg salts in suspension and the polyethylenglycol, or by inducing the pores to dilate via electroporation, the protoplast population divided itself into "competent" and "not competent" for direct gene transfer.

c) Another direct gene transfer system was developed at the Max Planck Institut (5). It does not involve in vitro culture and targets rye, which like other cereals cannot be infected by *A. tumefaciens*. The method is apparently simple: a DNA fragment (of the aminogycoside-phophotransferase gene II carried by specific plasmid) in aqueous solution was injected by hypodermic syringe above each node of the rye culms when the influorescence measured about 2 cm long, ie 14 days before meiosis. This was followed by pollination with pollen from equally treated plants as rye is self-incompatible. A more complicated variation on this technique is the micro-injection of DNA or the plasmid directly into the nucleus.

d) Pollen can also act as the vector in transferring exogenous DNA to pollinated plants as demonstrated with maize (21).

e) Lastly, there is the novel method developed at Cornell University in the USA (fig. 6). It entails shooting microbullets at high speed directly into cultivated cells (16). The "gun," which is in a protected chamber, was invented by Dr. Sanford, who has repeatedly tested the method by firing into epidermal cells of onion. The tungsten bullets, impregnated with nucleic acid (RNA or DNA), penetrate the walls of

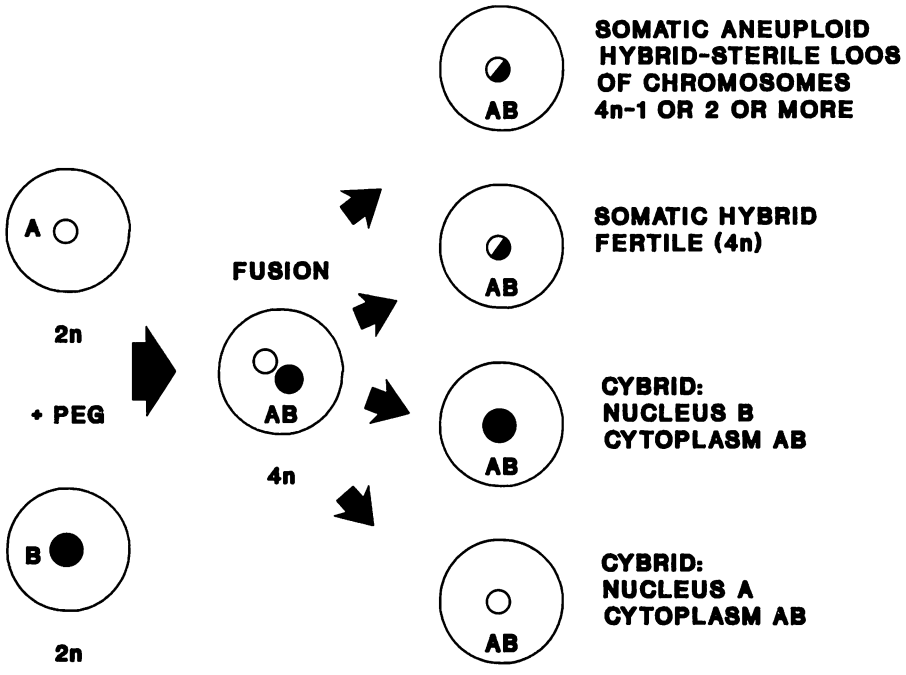

Figure 3. How fusion within protoplasts works.

the cells, which survive to express the acquired traits.

5) *Conclusions*

It has been stated that "the technology explosion is not a dream." We are living in a time of great expectations—expectations that daily seem to place the fruit growing industry on the verge of revolutionary events produced by biotechnology. This cursory review underscores the emphatic commitment of research, especially along the broadest interdisciplinary lines involving genetics, molecular biology, biochemistry, physiology. But it also discloses the obstacles that the models developed experimentally "in vitro" or on bacteria, viruses or grass plants encounter in being transferred or reproduced for the industry's economic ends. The nursery sector has certainly been the first to benefit from the many innovations that have been or are about to be introduced.

Yet it would be mistaken to think that conventional breeding techniques have been superseded. No one knows if and when the "transgenic" plants, the "engineered" organisms will enter the fruit growing industry. While it is true that in the United States plants (and even bacteria) with one or more engineered genes have been granted patents, it is also true that such authorization has been strongly contested by the general public. It is feared by some that the modification of ecosystems by the introduction of new, genetically manipulated organisms will entail risks which would only add to those already existing in current systems of cultivation.

The reservations and "cosmic" fears that have been reported mainly by the mass media (at times either poorly informed or even misinformed), have recently been exorcised by a Nobel laureate. Rita Levi-Montalcini polemically declared that she was more afraid

of 'cultural manipulation,' whether political, economic or informational, rather than the genetic kind. Nature, especially at the cellular and microorganism level, offers an enormous intrinsic potential of genetic variability. The potential induced by biotechnology is but an infinitesimal fraction of nature's, not to mention the fact that so far man has reaped only benefits from the former. There is nothing to fear in the advances of scientific research, even if in its free exercise it must serve the goals of humanitarian interest.

More doubtful is the expectation that biotechnology will become big business as a result of spin-offs, bringing with it an economic impact that will affect consumer and manufacturer alike. These uncertainties are clearly summarized by H. Hobbelink in an illuminating "booklet" entitled "Biotechnologies: New Hopes or False Promises?", published by the ICDA Seed Campaign. Although the author is referring to the Third World, it could be that the near future has a Fourth World, one even more extensive than the Third, in store for us: that of biotechnology-dependent countries. For the moment "hype" and "hope" still outweigh concrete results.

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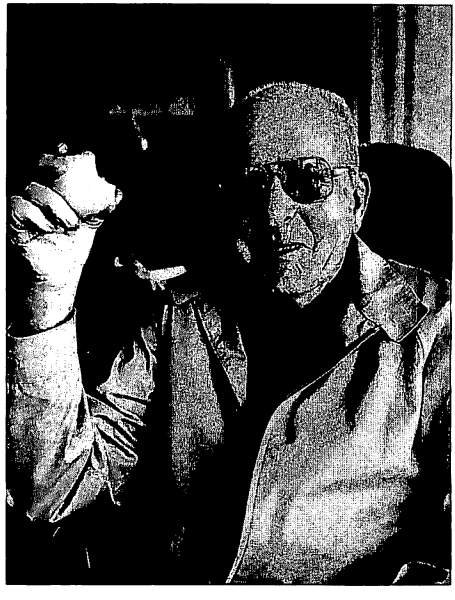
1988 Wilder Medal Recipients

In 1988 three distinguished pomologists were awarded the Wilder Medal for their distinguished careers and their contributions to pomology. Highlights from their careers are presented in the following summaries.

Grady Auvil



Following a short stay at Washington State University, Grady Auvil at age 19 purchased 22 acres of sagebrush land at the present Orondo ranch site. Since his first plantings in 1920, he has enlarged the orchard to include the current 600 acres. Although Grady was the initiator of the "Gee Whiz" orchard and ranch, he was joined in the venture by his brothers, Robert and David, as they graduated from high school. In March of 1988 Grady Auvil celebrated his 60th year as a tree fruit grower.

Grady Auvil has been and continues to be a pioneer in the tree fruit industry of Washington, the Pacific Northwest, and is recognized around the world as an innovative leader and very successful grower. In Washington he is recognized as the leader who gives all his efforts and ideas freely to other growers and supporting industries.