Improvement of Apple Varieties and Rootstocks by Biotechnology

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Before being commercialized, transgenic apple varieties will go through rigorous deregulation requirements to demonstrate their safety for consumers, the environment and agriculture.

Apple growers need improved varieties that have the advantages of, for example, Gala and McIntosh, but without their problems, such as excessive susceptibility to fire blight (Gala), to scab (both varieties) and poor storage (especially McIntosh). Similarly they need good dwarfing rootstocks like M.26 and M.9 but without their susceptibility to fire blight. The industry is under great pressure from government and the public to reduce the use of chemicals in fruit production.

Bacterial and fungal diseases are constant threats to apple production in all production areas worldwide. Some diseases like fire blight (caused by the bacterium *Erwinia amylovora*) are not adequately controlled and result in considerable losses annually. The best available control measure, used in certain countries, is application of the antibiotic streptomycin to the blossoms. In many areas this has resulted in development of resistance to streptomycin in *E. amylovora*. Fungal diseases, especially the most prevalent and damaging disease worldwide (apple scab caused by *Venturia inaequalis*), are controlled with multiple applications of chemical fungicides which are costly to producers and have raised environmental and health concerns. A desirable solution to these problems is the creation of resistant varieties. However, conventional breeding of apple is very long term and cannot reproduce the desirable qualities of our best commercial varieties and rootstocks. Furthermore resistance based on resistance genes from crabapples tends to be unstable due to presence of matching virulence genes in pathogen populations. Genetic engineering offers an attractive alternative to conventional breeding for the creation of resistant varieties since it is faster, it can use genes from many sources and it will preserve the desirable qualities of the variety or rootstock.

Genetic engineering has been used very successfully with other crops, including corn, cotton, soybean, potato, tomato and papaya, to produce disease-, insect- and herbicide-resistant varieties that were grown on over 75 million acres worldwide in 1999. Similar technology should solve many of our apple problems. It will let us improve the shortcomings of our present varieties and rootstocks without altering their desirable features, especially familiarity to nurseries and growers and recognition in the market by brokers, supermarkets and consumers. Genetic engineering leaves the genes of the popular variety or rootstock intact, except for one or a few genes to remedy the problem character such as susceptibility to diseases or insects, or premature fruit drop and softening. It will also be possible to combine genes to control several different problems in the same variety.

Several researchers, particularly David James at East Malling, UK, pioneered methods to transfer genes into apple. We drew on their work and our own early work to develop the techniques we now use for efficient genetic transformation of several varieties. We use modified strains of the common soil bacterium *Agrobacterium tumefaciens* which transfers genes into plants in nature as the gene delivery system. We use a kanamycin resistance gene to select the transformed cells. We have added other techniques and genetic material to improve the efficiency and speed of the process. The cooperation of a nursery in California has allowed us to accelerate growth of grafted plants of transformed ("transgenic") fruit varieties. About 8 months after the start of a transformation experiment, we can ship buds from transgenic plants raised in the greenhouse to California for budding onto plants there in early spring. Under the very long growing season in California, the budded trees make excellent growth (6 ft) and are then shipped back to Geneva for planting the following spring. Some of these trees have flowered in their first year in the field at Geneva, allowing us to examine fruit of a transgenic line within 2 years of the initial transformation experiment. This improvement in our ability to obtain transgenic fruiting trees will allow us to insert new, better gene constructs much more quickly than in the past.

RESISTANCE TO FIRE BLIGHT

We hypothesized that, by transferring genes for antimicrobial proteins into apple, we might be able to make the apple plants more resistant to the bacteria that

cause fire blight. Using *Agrobacterium*-mediated transformation, therefore, we introduced genes for several lytic proteins, which are known to inhibit pathogenic bacteria, into several apple varieties. Using molecular techniques, we confirmed the presence of the genes in the transformed plants and showed that the proteins were actually being produced in the plants. We did preliminary tests in the growth chamber and greenhouse and found that some lines did in fact have increased resistance to fire blight. However, we wanted to make sure the plants remained resistant under field conditions and also produced normal trees and fruits.

In 1998, tests of the fire blight resistance of 2- and 3-year-old trees in the field of Royal Gala transgenic lines, containing lytic proteins (attacin, cecropins, or avian lysozyme), showed that several lines had significantly increased resistance. This was the first demonstration in a well-replicated test of increased shoot resistance of transgenics in the field. The greatest level of fire blight resistance was observed with attacin-transgenics. One attacin-line had only 5% shoot blight compared with approximately 60% in non-transgenic Royal Gala controls and approximately 40% in the moderately resistant Liberty controls. In the case of cecropin and the lysozyme transgenics, several lines were identified that are significantly more resistant than the Royal Gala parent; however, the observed resistance was generally at a lower level than that observed with attacin.

In 1999 we again carried out several field trials of the resistance to fire blight of 2- to 4-year-old trees of Royal Gala transgenic lines containing lytic proteins. Many lines had significantly increased resistance. It was particularly noteworthy that many of the lines that had been identified as resistant in 1998 tests also were resistant in 1999 tests. This was especially true for line TG138, transgenic for the attacin gene, which was most resistant of all lines tested in 1998 and was again most resistant in 1999.

The first flowering of transgenic trees occurred in 1998 and, as expected, many more trees flowered in 1999. These included Royal Gala lines transgenic for attacin and avian lysozyme. To contain the pollen of the transgenic trees in order to prevent it from pollinating bearing trees in the plantings around our field trial, a large netting structure was erected, supported on steel hoops and covering the two rows containing most of the flowering transgenic trees. Flowers on trees in rows outside the netting structure were bagged to contain pollen. Flowers were manually pollinated under the netting, and a good crop of fruit was obtained. Transgenic fruits appeared indistinguishable from normal Royal Gala. All transgenic fruit, along with fruit of normal Royal Gala from the same rows, has been graded for size and color, pressure tested for firmness with and without skin, and assayed for soluble solids and titratable acidity. Data are now being analyzed.

The results show the potential for using lytic protein genes in apple to increase resistance to fire blight while retaining normal fruit characteristics. More information is needed on field resistance and tree performance of transgenic apples. Now that transgenic lines are flowering, progeny analysis from crosses will allow conclusive determination of the role of the transgenes in resistance. Besides the lytic protein genes, other genes derived from apple and also from the fire blight bacterium itself are being tested for their ability to make apple plants more resistant to fire blight.

RESISTANCE TO SCAB

The cell walls of most fungi, including the apple scab fungus *Venturia inaequalis*, contain the polymer chitin. The biological control organism *Trichoderma harzianum* produces chitinolytic enzymes that attack chitin and play a role in its activity against pathogenic fungi, including *V. inaequalis*. We hypothesized that introducing genes for chitinolytic enzymes from *T. harzianum* into apple would make the plants more resistant to scab.

We transferred an endochitinase gene, an exochitinase gene, and both genes together into Marshall McIntosh apple. The transformed plants were checked for the presence of the genes and for production of the enzymes. Own-rooted transgenic plants were inoculated with a suspension of *V. inaequalis* conidia, incubated in a mist chamber (18 ± 1°C and 100% relative humidity) for 48 hours and later moved to a growth chamber or greenhouse. Scab resistance was evaluated based on the number of sporulating lesions per leaf, the percentage of leaf area infected and the number of conidia rinsed off per leaf.

The level of endochitinase expression in transgenic plants was significantly correlated with scab resistance level. However transgenic plants producing high levels of endochitinase also had reduced growth, and the degree of growth reduction was correlated with the amount of endochitinase. The level of exochitinase expression was also significantly correlated with scab resistance level, although resistance of exochitinase-transgenic lines was less than of endochitinase-transgenic lines. However, exochitinase had no effect on plant growth. When both enzymes were expressed together in transgenic plants, they acted synergistically to reduce scab infection. Certain Marshall McIntosh lines transgenic for both genes that were selected for a low level of endochitinase expression and a high level of exochitinase expression were highly resistant to scab and had negligible reduction in growth in a greenhouse trial. These lines are now being grown in the field as own-rooted plants for evaluation of scab resistance and are being propagated on M.9 rootstock for evaluation of tree performance, fruit quality and yield.

The results reported here indicate the potential for use of genes for chitinolytic enzymes to enhance resistance to apple scab. To avoid the negative effects of endochitinase expression on apple vigor, we are exploring the utility of other cell wall degrading enzymes and the development of pathogen-specific expression of the endochitinase. The glucanase gene of *T. harzianum* is being cloned and will be evaluated for its effect on apple scab resistance, both singly and in combination with exochitinase. Various promoters are being evaluated in apple for induction by *V. inaequalis* to allow for expression of endochitinase only under conditions of pathogen.

The transgenic lines reported in this paper are experimental. Transgenic lines designed for use in commercial apple growing likely will differ in genes, promoters, and regulatory sequences from those described here. Before being commercialized, transgenic apple varieties will go through rigorous deregulation requirements to demonstrate their safety for consumers, the environment and agriculture.

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