Genetic Engineering of Apple for Resistance to Fire Blight

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Conventional breeding of apple is very long term and cannot reproduce the desirable qualities of our best commercial varieties and rootstocks. Genetic engineering offers an attractive alternative to conventional breeding for the creation of resistant varieties since it is faster, can use genes from many sources, and will preserve the desirable qualities of the transformed variety or rootstock.

Figure 1. Transgenic tree bore fruit within two years of initial gene transfer experiment. Transgenic buds were grafted onto M.9 at a cooperating nursery in California in April 1998, planted at Geneva in May 1999, after which they flowered, were successfully pollinated and developed mature fruit.

Other articles in this issue describe the efforts being made to control fire blight in orchards of susceptible apple varieties and rootstocks. Streptomycin is effective for control of blossom blight, when applied with the right timing. However, sometimes sprays are not applied and infection occurs, and sometimes sprays are applied unnecessarily. Every year losses are incurred and money lost. Some newer products look quite promising as alternatives for streptomycin, and Apogee may help with control of shoot blight. Nevertheless the apple industry is under great pressure from government and the public to reduce the use of chemicals in fruit production. The ultimate solution to fire blight, other diseases, and insect pests, would be resistant varieties and rootstocks. However, conventional breeding of apple is very long-term and cannot reproduce the desirable qualities of our best commercial varieties and rootstocks. Genetic engineering offers an attractive alternative to conventional breeding for the creation of resistant varieties since it is faster, can use genes from many sources, and will preserve the desirable qualities of the transformed 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 in the United States in 1999. Similar technology should solve many of our apple problems. It will allow us to 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 thousands of 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 make it possible to combine genes to control several different problems in the same variety.

Several researchers, particularly David James at East Malling, United Kingdom, pioneered methods to transfer genes into apple. We drew upon 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 resistant gene to select the transformed cells, and have added other techniques 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 eight

TABLE 1

Disease evaluation in the field of two-year-old plants of Royal Gala lines transformed with lytic protein genes.

Vigorously growing shoot-tips were inoculated with the fire blight pathogen and eight weeks after inoculation the percent of the current season's shoot length blighted was used as a measure of resistance ("% shoot blighted" in table below). Three to five shoots were inoculated per plant on one to nine plants of each transgenic line and the total number of individual inoculated shoots is indicated as "N" in the table below. Waller Group: cultivars followed by the same letter did not differ in their fire blight resistance.

Cultivar	Lytic Protein	N	% shoot blighted	Waller Group	
TG149	cecropin	21	81	a	
TG267	vector	3	80	ab	
TG243	cecropin	40	78	abc	
TG163	attacin	26	75	abc	
TG204	cecropin	29	69	abcd	
TG242	cecropin	16	67	bcde	
TG182	vector	30	65	cdef	
TG550	egg lysozyme	25	62	defg	
TG192	cecropin	14	61	defg	
TG224	attacin	23	60	defgh	
TG145	cecropin	20	60	defgh	
TG226	attacin	19	58	defghi	
Royal Gala	parent	12	56	defghij	
TG160	cecropin	33	55	efghijk	
TG244	egg lysozyme	28	54	efghijkl	
TG135	attacin	25	54	efghijkl	
TG142	cecropin	28	54	efghijkl	
TG254	cecropin	19	54	efghijkl	
TG248	cecropin	29	53	fghijkl	
TG223	egg lysozyme	35	52	fghijkl	
TG262	cecropin	22	52	fghijkl	
TG180	attacin	37	51	fghijkl	
TG468	cecropin	29	51	fghijklm	
TG181	cecropin	29	51	ghijklmn	
TG125	cecropin	14	49	ghijklmno	
TG251	cecropin	12	48	ghijklmno	
TG126	cecropin	34	47	hijklmnop	
TG545	cecropin	22	47	hijklmnop	
TG141	attacin	36	45	ijklmnopq	TG172
vector	25	45	ijklmnopqr		
TG179	cecropin	17	44	jklmnopqr	
TG208	cecropin	32	44	jklmnopqr	
TG466	egg lysozyme	34	44	jklmnopqr	
TG207	attacin	20	43	jklmnopqr	
TG247	cecropin	25	42	klmnopqr	
TG193	cecropin	39	42	klmnopqr	
TG171	vector	44	42	klmnopqr	
TG221	cecropin	20	41	lmnopqrs	
TG225	cecropin	39	41	lmnopqrs	
TG272	cecropin	24	40	lmnopqrst	
TG549	cecropin	20	38	mnopqrstu	
Liberty	resistant	22	37	nopqrstu	
TG546	cecropin	10	37	nopqrstu	
TG154	cecropin	30	36	opqrstu	
TG228	cecropin	25	36	opqrstu	
TG161	attacin	17	34	pqrstu	
TG201	cecropin	4	33	qrstu	
TG203	attacin	25	33	qrstu	
TG222	egg lysozyme	23	31	rstu	
TG159	vector	29	28	stu	
TG253	cecropin	5	27	tu	
TG547	cecropin	5	27	tu	
TG202	attacin	44	26	u	
TG250	cecropin	28	26	u	
TG205	attacin	22	26	u	TG138
attacin	26	5	v		

Figure 2. To contain the pollen of experimental transgenic trees in order to prevent it pollinating bearing trees in the plantings around our field trial, a large netting structure supported on steel hoops was erected to cover flowering transgenic trees. Netting was removed from structure after flowering.

months after the start of a transformation experiment, we can ship buds from transgenic plants raised in the greenhouse to California for budding on to plants there in early spring. During 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 two years of the initial transformation experiments (Figure 1). This improvement in our ability to obtain transgenic fruiting trees quickly will allow us to insert new, better gene constructs much more quickly than in the past.

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. Therefore, using Agrobacterium-mediated transformation, we introduced genes for several lytic proteins, which are known to inhibit plant 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 in-

Figure 3. Fruit on experimental transgenic trees appeared indistinguishable from normal Royal Gala. All transgenic fruit was evaluated for size, color, firmness, soluble solids and acidity.

creased 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 two- and three-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 transgenics containing the attacin protein. One attacin-transgenic line had only 5 percent shoot blight compared with approximately 60 percent in non-transgenic Royal Gala controls and approximately 40 percent in the moderately resistant Liberty controls (Table 1). In the case of transgenics containing the cecropin and the lysozyme protein, several lines were identified that are significantly more resistant than the Royal Gala parent, but 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 two- to four-year-old trees of Royal Gala transgenic lines containing lytic proteins (attacin, cecropins, and avian lysozyme). 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, and prevent it from pollinating bearing trees in the plantings around our field trial, a large netting structure supported on steel hoops, and covering the two rows containing most of the flowering transgenic trees was erected. 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 (Figure 2). 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 (Figure 3). 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, other plants, and also the fire blight bacterium itself are being tested for their ability to make apple plants more resistant to fire blight. These new genes should act to enhance apple's own natural defenses against pathogens, rather than acting directly against the fire blight bacterium by producing proteins that are antimicrobial. The natural protection of plants against pathogens is partly based on a variety of barriers already present in the plant before pathogen invasion. Plants can activate protective mechanisms upon detection of invading pathogens. If this protection is expressed locally at the site of pathogen invasion and also systemically in parts of the plant remote from the initial invasion, it is called systemic acquired resistance (SAR). SAR has now been demonstrated in many different plants, with many different pathogens. Often SAR is active against a broad range of pathogens, including fungi, bacteria, and viruses.

Commercial products, such as benzothiadiazole (Actigard, Novartis) have now been registered for use as an inducer of SAR against wheat powdery mildew and is effective against certain diseases of rice and tobacco. Similarly, Harpin, a protein (discivered by Dr. Steve Beer, Cornell University, Ithaca) produced by the fire blight bacteria, has been shown to induce host resistance in tomato and is commercially available as Messenger (Eden Bioscience). Orchard trials conducted by our group have shown that when apple trees are sprayed with Actigard or Harpin, significant reductions (40-50 percent) in the amount of blossom blight of apple can result. By expressing Harpin transgenically in apple we hope to either pre-activate its natural defenses against fire blight and apple scab, or activate them earlier in the infection process to render apple plants more resistant to these diseases. The Harpin gene has been transferred to M.26 apple rootstock and is currently being evaluated for its effect on fire blight resistance.

Basic research in the Arabidopsis model system has identified a gene that is necessary for that plant to be able to detect pathogen invasion and activate SAR resistance. When this "signaling" gene was over expressed in Arabidopsis it resulted in significantly enhanced resistance to bacterial and fungal pathogens. Researchers in the laboratory of Dr. Sheng Yang He, Michigan State University, have identified and cloned this same signaling gene from apple. We will be cooperating with Dr. He to enhance the expression of the apple signaling gene and determine its effect on fire blight resistance. Arabidopsis is also being used as a source of plant resistance genes with potential application to confer resistance to fire blight in transgenic apples.

The transgenic lines reported in this paper are experimental. Transgenic lines designed for use in commercial apple growing will likely 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 complete safety for consumers, the environment, and agriculture.

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