

Making Apple Varieties More Resistant to Diseases by Use of Apple Genes

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For a number of years we have been using biotechnology to increase the resistance of apple varieties to disease, especially fire blight and apple scab. Initially we showed that we could transfer genes from other organisms into disease-susceptible apple varieties by gene transfer techniques that had been developed for other crops. Although these early experiments were technically successful in that resistance was substantially increased and the quality of the fruits were not affected in any way, we decided not to undertake the costly process of obtaining government approval for their use in horticulture, because of the origin of the genes. Also the genetically engineered apple strains contained an antibiotic resistance gene used in the gene transfer process.

We were excited several years ago when genes from plants, and recently from the apple plant itself, began to become available through the new science of Genomics, the study of all the genes in an organism. This has resulted in identification of a gene that occurs naturally in apple and which, when present as an extra copy, significantly improves disease resistance. We also used a promoter (switch to turn a gene on) from plants, instead of the commonly used virus promoter. At the same time we have developed a new technique for transferring genes into apple without using any antibiotic resistance gene (this technique will be described in a future article in *Fruit Quarterly*). Together we refer to these technologies as Clean Genetics for apple

improvement. We believe that they are the most promising method to improve apple varieties for growers' use by biotechnology.

Background

The fact that most plants are resistant to most diseases may sound surprising to apple growers, who are struggling to control diseases like fire blight, scab, mildew and rusts on their trees. Nevertheless, those diseases are the exceptions: apple trees are resistant to all the diseases that affect wheat, cabbages, potatoes, and other crops. This is due to the plant's natural immunity. Plant immunity is based on a complex response that is very flexible in its ability to recognize and attack different invaders. When a pathogen is detected, the plant activates several early responses that lead to the production of a wide array of defensive molecules. These induced defenses are often expressed not just locally but also in parts of the plant distant from the site of primary infection, thereby protecting the plant systemically against subsequent pathogen attacks. One of the best understood of these responses is systemic acquired resistance (SAR), in which several so-called pathogen resistance (PR) Genes, which inhibit pathogens, are activated. The gene, *NPR1*, is a key factor for triggering SAR. When an additional copy of *NPR1* was transferred to rice, tomato and wheat plants, it led to increased fungal and bacterial resistance. Therefore we decided to add a copy of *NPR1* to apple plants with the goal of obtaining broad

The transfer of genes from other species into apple has been successfully done in our lab since the mid-1990's. However, we recently were able to transfer an apple gene into Galaxy Gala apple which resulted in transformed lines with increased resistance to fire blight, apple scab and Cedar apple rust. We refer to these technologies as Clean Genetics for apple improvement. We believe that this is the most promising way to improve apple varieties for growers' use by biotechnology.

disease resistance, in particular to fire blight.

Procedures

We obtained the apple *NPR1* gene (*MpNPR1*) from our collaborators, Sheng Yang He and Qiaoling Jin, at Michigan State University. Leaf pieces were cut from tissue-cultured shoots of the Galaxy apple variety and inoculated with the natural gene transfer agent, *Agrobacterium*, carrying the binary vector pKYLX-PINM $pNPR1$, which contains the *MpNPR1* gene. Regenerated shoots were tested by PCR for the presence of the new gene and by Southern analysis to determine how many copies of *MpNPR1* were added. Lines testing positive for the additional copy of *MpNPR1* were micro-propagated, and then acclimated to soil and ambient conditions in a growth chamber. When plants were 9-12 inches tall, their shoot tips were inoculated with a virulent strain of *Erwinia amylovora* to test for fire blight resistance in the growth chamber. Selected lines were grafted on seedling rootstocks and also screened for fire blight resistance in the greenhouse.

Other plants of the positive lines were also tested for resistance to apple scab and cedar apple rust by inoculation of young leaves with spores of *Venturia inaequalis* and *Gymnosporangium juniperi-virginianae*, respectively.

Results

Nine lines of Galaxy apple containing an extra copy of the apple gene *MpNPR1* (*MpNPR1*-plus) were obtained and confirmed by PCR. Each of the lines contained only a single extra copy of the *MpNPR1* gene as shown by Southern analysis.

Resistance to Fire Blight. To determine the response of the lines to fire blight, ten to 20 acclimated plants per line, including check Galaxy (susceptible control) and M.7 (resistant control), were inoculated with *E. amylovora* strain Ea 273 at 5×10^7 CFU ml⁻¹ in two to three independent experiments. The responses were statistically similar in the replicated experiments. Three weeks after inoculation, 83% of the shoot length of the Galaxy check showed necrosis, whereas 22% of the shoot length of M.7 was necrotic (Table 1, and figure on back cover). All of the *MpNPR1*-plus Galaxy lines showed a reduction in susceptibility to *E. amylovora*.

We further analyzed the *MpNPR1*-plus apple plants in the greenhouse. Selected Galaxy lines were grafted onto seedling rootstocks, and inoculated with *E. amylovora* strain Ea 273 at 5×10^7 CFU ml⁻¹. Three weeks after inoculation, 79% of the shoot length of the Galaxy check showed necrosis, whereas 17% of the shoot length of M.7 was necrotic (Table 1). All the *MpNPR1*-plus Galaxy lines showed a reduction in susceptibility to *E. amylovora*, which was similar to the reduction of susceptibility observed in the growth chamber.

Resistance to Scab and Cedar Apple Rust. Symptoms were evaluated 21 days after *V. inaequalis* (scab) inoculation of plants in a growth chamber, and the percentage of leaves with sporulation was determined. Interestingly, there was a reduction of symptoms on leaf surfaces of *MpNPR1*-plus plants, as well as a reduction in the number of leaves infected compared to the Galaxy check (Table 2).

Inoculation with cedar apple rust (*G. juniperi-virginianae*) caused the formation of lesions (pycnidia) on the leaf surface (Figure 1). One month after inoculation an average 66 lesions were counted on leaf surfaces of the Galaxy check. Plants of the *MpNPR1*-plus lines tested showed

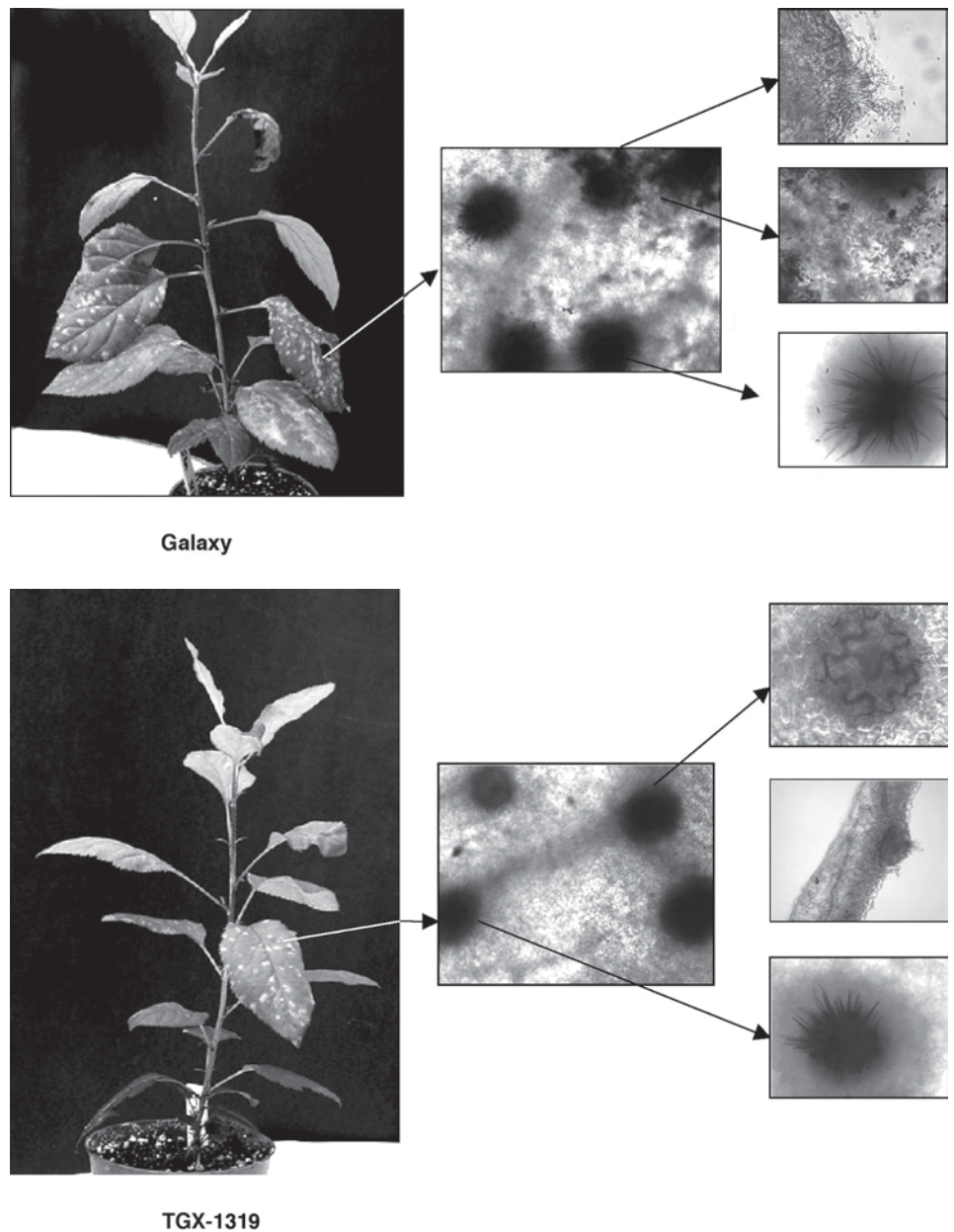


Figure 1. Microscopic analysis of Galaxy apple line TGx-1319 containing an extra copy of the apple gene *MpNPR1*, and check Galaxy three weeks after inoculation with cedar apple rust (*Gymnosporangium juniperi-virginianae*).

TABLE 1

Evaluation of fire blight resistance of own-rooted and grafted Galaxy plants containing an extra copy of the apple gene *MpNPR1* by shoot inoculation with *E. amylovora* strain Ea 273 at 5×10^7 CFU/ml in a growth chamber.

Lines	% shoot length necrotic of own rooted plants in growth chamber	Waller group	% shoot length necrotic of grafted plants in greenhouse	Waller group
Galaxy	83.0	A	78.9	A
TGx-1320	52.9	B		
TGx-1491	47.3	BC	49.2	B
TGx-1321	46.9	BC	49.0	B
TGx-1322	40.3	CDE	37.5	C
TGx-1323	35.0	CDEF	35.0	C
TGx-1319	31.0	EF	34.0	C
TGx-1509	28.9	F		
M.7	22.3	F	17.0	D

TABLE 2

Resistance to apple scab (*Venturia inaequalis*) of own-rooted Galaxy lines containing an extra copy of the apple gene *MpNPR1* as determined in a growth chamber.

Line	Infection rating ^a	# leaves with sporulation
Galaxy	2.48	4.86 ± 0.5
TGx-1319	1.12	2.95 ± 0.3
TGx-1320	1.35	3.02 ± 0.4
TGx-1323	1.01	2.62 ± 0.2
TGx1509	1.09	2.83 ± 0.3

^a 0: <5% leaf surface with sporulation,

1: 5 to 25% leaf surface with sporulation,

2: 25 to 50% leaf surface with sporulation,

3: >50% leaf surface with sporulation.

an average reduction of 40% in the number of necrotic lesions on the upper surface of the leaves, and a reduction in formation of fruiting lesions (aecia) on the lower surfaces. After staining of three-week-old rust-inoculated leaves, microscopic analysis revealed that most of the lesions observed on the *MpNPR1*-plus lines had necrosis or mycelia without sporulation compared to the Galaxy check, on which all lesions had mycelial growth with abundant sporulation.

Conclusions

Our results showed that “over-expression” of the *MpNPR1* gene increased the resistance of apple, to varying degrees, to three important pathogens: fire blight bacteria, the apple scab fungus, and the cedar apple rust fungus. The broad-spectrum resistance to both bacterial and fungal diseases observed is consistent with previous reports in rice and wheat. The fact that over-expression of *NPR1* proteins causes similar effects in different plants suggest the presence of similar defense pathways in apple, rice and wheat. In contrast to rice and wheat, however, the over-expression of *MpNPR1* did not result in any unexpected changes in apple.

Over-expression of the *NPR1* genes in tomato and rice leads to enhanced non-specific disease resistance in a dosage-dependent fashion. Consistent with these observations, *MpNPR1*-plus Galaxy apple lines that accumulated higher levels of *MpNPR1* mRNA and protein exhibited broad-spectrum resistance to several important and different diseases tested (fire blight, apple scab and cedar apple rust). It will be of interest to determine in the future whether such plants have enhanced resistance to other apple diseases.

An important question is whether resistance due to over-expression of *MpNPR1* will be durable, or whether it will break down in face of virulent races of the pathogens. This is impossible to predict. However, *MpNPR1* protein is not a “gatekeeper” molecule, and does not interact directly with pathogens, but is instead a signaling molecule, passing on a message from receptor molecules to molecules turning on the plant’s defenses. In its signaling role, *MpNPR1* is less likely to induce changes in the pathogen that would interfere with its role, than are true Resistance (R) genes. Thus it is possible that over-expression of *MpNPR1* will be a durable type of resistance.

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