

Recombinant DNA Technology in Apple

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Abstract This review summarizes the achievements of almost 20 years of recombinant DNA technology applied to apple, grouping the research results into the sections: developing the technology, insect resistance, fungal disease resistance, self-incompatibility, herbicide resistance, fire blight resistance, fruit ripening, allergens, rooting ability, and acceptance and risk assessment. The diseases fire blight, caused by *Erwinia amylovora*, and scab, caused by *Venturia inaequalis*, were and still are the prime targets. Shelf life improvement and rooting ability of rootstocks are also relevant research areas. The tools to create genetically modified apples of added value to producers, consumers, and the environment are now available.

Keywords Disease · *Malus* · Pest · Transgenic

1

Introduction

The genus *Malus* includes a variety of species all closely related and easily crossable. Agriculturally relevant is the species *Malus* × *domestica*, the edible apple. Peculiarities that are relevant to its use are self-incompatibility, leading to high heterozygosity, and the difference between apple as a crop and *Malus* in natural conditions. Commercial orchards are uniform as they generally consist of few varieties (cultivars) with each individual tree being the result of a rootstock and a grafted scion, both obtained by vegetative propagation. These original selections, made possible centuries earlier, were intensively replicated, and are currently present worldwide. In contrast, in natural conditions each tree is a result of a meiotic event and assembles characteristics from the mother tree and the pollen donor tree, so that in nature no two trees will ever be genetically equal. Vegetative propagation by grafting has allowed the selection of high-quality apples through the centuries, by propagating a single tree developed from a seed which coincidentally had more appealing fruit and growth characteristics than other locally known varieties. Some of the popular cultivars, such as Golden Delicious, Granny Smith, or the more local Gravensteiner and Boskoop, are chance seedlings. Few cultivars are the result of an oriented breeding program. Although agronomically this genetic uniformity and the maintenance and distribution of a particular genotype is desirable, genetic uniformity is deadly for the resistance toward any natural enemy. In fact the most successful commercial apple cultivars have lost the efficacy of their resistance genes toward the most frequent fungal pathogens, first of all *Venturia inaequalis*, causal agent of scab, and secondly *Podosphaera leucotricha*, causal agent of apple mildew. Still efficacious resistance genes can be found in wild *Malus* species, but commercial apple cultivars need constant protection through fungicides, wherever the climatic conditions are favorable for either of these diseases. On average 10 to 15 fungicide applications are necessary during a season to produce scab-free fruits, with costs from US \$500 to 1500 per hectare per year. Common commercial cultivars are also susceptible to the sporadic, yet highly damaging, fire blight infections caused by the bacterium *Erwinia amylovora*. Fire blight is in some parts of the world a constant threat. Control measures against fire blight are relatively inefficient; only the antibiotic streptomycin is reliable, but is banned in several countries.

Not only the high costs of controlling diseases, but also the increasing concern from consumers and environmentalists over pesticide residues in organic (copper residues) and conventional production have fostered the breeding of disease resistant cultivars. Therefore, most current apple breeding programs are oriented toward reducing the need for pesticides, without losing the high plant and fruit quality.

Even though the advantage of a resistant cultivar is evident, resistant cultivars do not yet dominate the market. The reason is simple: after each cross

the seeds produced will lead to individuals differing from the mother, father, and each other; each seed will be genetically unique, and if selected will be a new cultivar with considerably different properties from those of the mother and father trees. This is a relevant limitation in apple breeding; each time we would like to introduce a new trait by breeding, a completely new genotype with new characteristics will be generated. Backcrossing with one of the ancestors is not possible (or only to a very limited extent) in apple breeding, and therefore the original cultivar with the incorporation of the trait of interest, mostly resistance to a pathogen, cannot be re-created. To eliminate unwanted genome segments a very time-consuming pseudo-backcrossing with different domestic cultivars is necessary. Even if modern DNA analysis methods, such as genetic maps, identification of DNA markers linked to traits of interest, and marker-assisted breeding, can help and accelerate the process, a new cultivar with fruits possessing a new taste, storage and conservation capacity, and a new tree form and growth patterns will be created. Last but not least this new cultivar also needs acceptance by the consumers, who often stick to a few favorite cultivars. Moreover, if not a single gene (single trait) but several have to be introduced, such as resistance to scab and mildew, to fire blight, or several genes for a single trait (e.g., several resistance genes against a single pathogen to achieve durable resistance), it becomes an almost impossible endeavor.

Under these premises, the introduction of specific gene(s) into a particular cultivar which already has all the necessary qualities, except the trait in question, is attractive. Recombinant DNA technology (gene technology) promises to do exactly this.

2 Developing the Technology

Apple was an early target for the emerging recombinant DNA technology. *Agrobacterium tumefaciens*, a disarmed Ti binary vector, and leaf fragments or callus cultures used in the original experiments were and still are the materials of choice [1]. The first transgenic apple plants contained a cassette with the genes for nopaline synthase and neomycin phosphotransferase (*nptII*, conferring resistance to the antibiotic kanamycin). The selection of the transformed cells, regeneration, and rooting were made in the presence of the antibiotic. The incorporation of the genes into the genome was confirmed by Southern blot analysis. The nopaline synthase gene continued to be expressed in greenhouse-grown plants several months after removal from the *in vitro* growth conditions. The transgenic apple plants showed a normal phenotype, except for a somewhat reduced capacity to root, which is an important trait if the transformant is a rootstock but irrelevant if the target is a scion cultivar.

Other laboratories followed or paralleled the work of James et al. [2–11], all showing that apple can be genetically transformed and improved, step-wise, the transformation efficiency. Several papers summarize various aspects of the methodology (also reviewed in [12]). Technical problems such as stability of the expression were analyzed [13–15], often also using foreign genes such as the gene stilbene synthase [16]. As the various cultivars have different requirements for almost all steps, including regeneration [17] and rooting, several reports deal with special protocols for a particular cultivar [18–25]. Not only *A. tumefaciens* was used for transformation; some attempts were made with *A. rhizogenes* [26], mainly driven by the interest in the *rol* genes [27].

Most studies start from wounded leaf sections, following the customary technique ameliorated by crushing rather than by cutting [28]. Apical internodal explants from etiolated Royal Gala apple shoots had a high efficiency of producing transgenic shoots [29]. For transient expression studies protoplasts have been used [30].

For gene activation, in many cases researchers relied on the well-characterized constitutively expressing 35S promoter from cauliflower mosaic virus (CaMV 35S). Some attempts have been made to find and use other promoters. Although not mentioned specifically in conjunction with a target gene, promoters linked with targeted expression patterns have been identified, for example the 940 *extA* promoter which is active in young tissue [31], two promoters, RBCS3C and SRS1, suitable for the expression of transgenes in green photosynthetic tissues of apple described by Gittins et al. [32], and others with particular expression patterns which are under development and characterization [33].

A major technical problem for genetically modified (GM) apple is the use of the *nptII* selection gene which is still used exclusively. Recently, some groups started to develop alternatives. As described in the section on herbicide resistance, the herbicide resistance genes are not true alternatives for several reasons, including patent ownership restrictions. For obvious reasons the selection system has to be positive and, as stated in the Introduction, in apple removing it through backcrossing is not feasible. A selection system acceptable to the general public or an alternative post-selection elimination process should be developed.

Currently, the most promising alternative to antibiotic resistance and probably a more acceptable system is the use of the gene phosphomannose isomerase (PMI). PMI-transformed cells are able to use mannose as C source, which the untransformed apple cells cannot. First successes were reported [34, 35] even if the efficiency seems not as good as with the *nptII* [35]; however, by adapting various parameters, similar and even higher efficiency can be obtained (Iris Szankowski, personal communication).

Clearly a “clean vector technology for marker-free transgenic” apples is the ultimate aim. Plant Research International in The Netherlands recently

proposed such a technology [36], and the developers were able to obtain transgenic apple plants free of selection marker genes.

3

Insect Resistance

After establishing the technology, the time came to produce transformants with a potential value. The target had to be a trait which can be sufficiently enhanced through a single or relatively few genes and gives a potentially commercially interesting advantage to the transgenic apple plants. Essential targets were resistance to diseases or pests; however, the main constraint was the availability of functional genes. Resistance to the main insect pests is partial in apple and thought to be regulated by quantitative, additive genes and a few single, major genes. Only the rosy leaf curling apple aphid gene *sd-1* is well characterized [37, 38] and mapped [39]. However, neither *sd-1* nor any other resistance gene has been cloned. Therefore, insect pest resistance must rely on genes from other species. A gene encoding a cowpea trypsin inhibitor (*CpTI*) and the gene *cryIA(c)* from *Bacillus thuringiensis* encoding an intracrystalline protein [40, 41] were used to transform apple and tested against *Cydia pomonella* (codling moth) attack. *CryIA(c)* transgenic plants have been field grown since 1992 [42]. In the first obtained transformants only very low levels of *CryIA(c)* were expressed; however, using a synthetic version of the gene together with the promoter CaMV 35S, the problem was seemingly overcome. Currently, the further development of codling moth resistant GM apple is carried out by private nurseries and no published data on the pest resistance of the transgenic apple plants are available. Similar efforts were reported by Cheng et al. [43] who introgressed the Bt endotoxin; however, the intended resistance to *Adoxophyes orana* (tortrix moth) was not induced.

Genes expressing the biotin binding proteins avidin and streptavidin were also used to infer resistance to transformants of the cultivar Royal Gala to *Epiphyas postvittana* (the light brown apple moth). In these experiments between 80 and 90% of the feeding larvae died in a period of 3 weeks, compared to 14% on the control plants [44]. The effect on larval weight in feeding experiments made in vitro on tissue-cultured apple shoots was inversely proportional to the expression of the two proteins with a threshold for activity.

4

Fungal Disease Resistance

Contrary to insect resistance, fungal disease resistance can be governed by single major genes and by quantitative additive genes. Since the 1980s great efforts have been made in Europe, the USA, and New Zealand to map the ma-

jor resistance genes against scab caused by *V. inaequalis* and mildew caused by *P. leucotrucha*. These two diseases absorb the majority, if not the entirety, of the fungicide treatments necessary to produce high-quality apples. However, in the early 1990s, once the apple transformation methodology was well established, no apple own resistance gene had been cloned. So the choice of the genes was directed toward foreign genes, with potentially toxic or inhibitory effects on *V. inaequalis*. Mildew, although a dominant problem in countries like England, was never a target. Suitable genes from other species were those encoding chitinases and glucanases from the fungus *Trichoderma*, a well-known biocontrol agent of fungal diseases, and those encoding lytic enzymes from *Lepidoptera* and antimicrobial peptides from phages (AMP). In 1998, several studies were presented by the research group at Cornell University. Wong et al. [45] reasoned that, since chitin is a major component of fungal cell walls and higher plants do not produce sufficient chitinases, the constitutive expression of chitinolytic enzymes like endochitinase and chitobiosidase from the biological control agent *Trichoderma harzianum*, which have been shown to have antifungal activity, may increase host resistance to scab. Two out of three endochitinase (*ech42*) gene transgenic lines of Royal Gala tested showed detectable levels of the enzymes, and were more resistant than the untransformed Royal Gala when micrografted shoots were spray inoculated with scab. Unfortunately no detailed values are reported. Similarly, Bolar et al. [46] reported the transformation of the cultivar McIntosh with the genes *ech42* and *Nag 70*, an exochitinase gene encoding *N*-acetyl- β -D-glucosaminidase, from the biocontrol agent *T. harzianum*. The expression of *ech42* varied considerably between lines, few reaching high levels. Plant growth was inversely correlated with its expression level. The number of scab lesions and extent of scab sporulation on micropropagated plants in greenhouse tests was relevantly reduced at high levels of *ech42* expression [47, 48]. *Nag 70* gave a lower level of scab resistance, but with no vigor reduction. The authors hypothesized that pyramiding both genes, each with a relatively low level of expression, may lead to high resistance whilst retaining good vigor. Transgenic lines with both genes (low *ech42* and high *Nag 70* expression) had a high level of scab resistance without significant growth reduction [49]. The authors reported that selected lines were evaluated in the field for resistance, growth, and fruit quality; however, no data are currently published.

Aldwinckle et al. [50] reported transfer of native (attacin E) and synthetic (SB-37) genes from the saturniid moth (*Hyalophora cecropia*), lysozyme genes from hen egg white, and T4 bacteriophage, all enhancing scab resistance to a variable degree. A similar program with the same genes was started in 1997 at the Institute for Fruit Breeding in Dresden Pillnitz, Germany, and resulted in several transgenic lines transformed with the lysozyme from the bacteriophage T4 and/or attacin E [51]; however, this program concentrated on subsequent effects on fire blight infections.

Concurrently, the research group from Leuven (Belgium) led by Keulemans attempted a different approach. As plants produce defense-related proteins, such as PR proteins and antimicrobial peptides, constitutive expression of these may enhance resistance. They introgressed, separately, the genes coding for Rs AFP2, an antifungal peptide from radish, and Ace AMP1, an antimicrobial peptide from onion, under the control of the CaMV 35S promoter into the cultivar Jonagold. Expression of Rs AFP2 reached high levels, up to 0.8% of the total protein fraction [52]. Ace AMP1 expression was much lower: up to 0.15% [53]. The proteins retained their antifungal activity after appropriate extraction against the test organism *Fusarium culmorum*, a wheat pathogen [52]. Transformants were micrografted and then chip budded in order to reach a sufficient number of replications for the resistance tests. Artificial inoculation with *V. inaequalis* conidia showed that some lines presented a reduced susceptibility expressed as a lower number of lesions with reduced and retarded sporulation [54]. Unfortunately this line of research was discontinued and no accessible publication reporting data on the disease resistance is available.

The fourth group involved in producing GM apples is the French group at the INRA Angers led by Chevreau. The Angers station is well known for its apple breeding program, so transgenic apples are considered an additional improvement to the traditional breeding program. One line of research is similar to the one mentioned above, i.e., introgression of endochitinase *ech42* and exochitinase *Nag 70* genes into Galaxy, a scab-susceptible cultivar, and Ariane, a cultivar carrying the *Vf* resistance gene. The theory is that in new commercial cultivars the *Vf* resistance gene requires supplementation by additional resistance factors in order to maintain resistance durability. The transformed lines obtained were tested with the common genotypes of *V. inaequalis*, against which *Vf* is still efficacious, and with race 6 which can overcome *Vf*. Half of the lines showed very limited growth in the greenhouse. The others were tested for scab resistance and two Galaxy lines (with a wild-type growth) were found to be less susceptible. Both lines had an increased endochitinase activity and equal, or slightly higher, exochitinase activity compared to the wild type [55]. The Ariane lines, including the greenhouse acclimatized plants, had a reduced growth rate. One line, which only had a shoot height reduction of 35%, showed a scab reduction of 25%, whereas another line with 50% shoot reduction achieved an 80% reduction of scab infection. High exochitinase activity was in all cases correlated to plant vigor reduction [56]. The endochitinase activity in the plants leads to changes such as increase in peroxidase and glucanase activity and higher lignification. As the authors [55, 56] discuss in detail, these changes have a relevant impact on growth, and therefore it is difficult to evaluate any direct effect on the fungus.

Antimicrobial peptides from plants may not create the side effect caused by fungal chitinases. Puroindolines [*PinA* and *PinB*] are antifungal cysteine-rich

proteins present in wheat seeds. Both genes are characterized and have already been used to transform rice. Transformant lines expressing these genes showed an increased resistance to major fungal pathogens of rice [57]. A similar experimental setup to that for the chitinase experiments was used to test the potential of *PinB* in apple. The introduction by genetic engineering of the gene into two cultivars had no effect on growth [58], regardless of the expression level, which reached up to 0.24% of the total soluble proteins [59]. The results of the scab susceptibility test are rather curious. Strain 104, race 1, which represents the common *V. inaequalis* population on the commercial cultivars, is not affected by the *PinB* at any expression level; strain EU D 42, race 6, is inhibited progressively with the increasing amount of *PinB*. So the two strains must exhibit differential tolerance to *PinB* [59]. If the gene is to be used to render *Vf* resistance more durable, it would be of interest to check other strains to know if this effect is correlated to the race or, more likely, to another independent factor.

A different approach to obtaining scab resistant plants was attempted by the joint team of the Department of Fruit Tree and Woody Plant Sciences of the University of Bologna, Italy, and the Plant Pathology group at ETH Zurich, Switzerland [60]. Starting from an EU project which offered an excellent linkage map of apple and molecular markers mapped in the region of the scab resistance *Vf*, the positional cloning of *Vf* was initiated. A contig of BAC clones spanning the region between the two *Vf* molecular markers M18 and AM19, one on each side of *Vf*, was constructed and allowed the identification of four genes, named *HcrVf1* to *HcrVf4*, coding for receptor-like proteins with a high homology to the *Cladosporium fulvum* (*Cf*) resistance gene family of tomato. The genes have an extracellular leucine-rich repeat domain and a transmembrane domain [61]. The gene *HcrVf2* under the control of the CaMV 35S promoter was introduced into the susceptible cultivar Gala using the *nptII* gene for selection. First in vitro tests evaluating the progression of the scab infection (penetration and stroma formation by the fungus) [62, 63], and later greenhouse scab inoculations of lines containing a single functional copy of *HcrVf2*, unambiguously demonstrated that the four lines carrying *HcrVf2* were at least as resistant to scab [64, 65] as the conventionally bred *Vf* cultivar Florina. As the resistance *Vf* is known to be overcome by race 6 and 7, it was of interest to see if the introgressed gene really conferred the same type of resistance, i.e., recognition of the avirulent *V. inaequalis* genotypes and induction of the defense cascade, or the plants were resistant through some artifact. Plants transformed with both *HcrVf2* and *nptII*, those transformed with *nptII* only, and wild-type Gala and Florina (*Vf*) were challenged with a field inoculum (mixture of genotypes) known to have the ability to cause scab on Gala but not on Florina and with an inoculum derived from *Malus floribunda* 821, the original donor of *Vf*. The *HcrVf2* line was, as expected, resistant to the field inoculum similarly to Florina, whereas all the other plants showed typical scab lesions with abundant sporulation. Inocu-

lation with race 7 from *M. floribunda* 821 resulted in sporulating lesions on all plants; however, the inoculum was less aggressive and the sporulation was less abundant than the field inoculum. Moreover, the *HcrVf2* line still retained some resistance, being slightly more resistant than the line transformed with only *nptII* and the original Gala [66]. This experiment demonstrated that resistance in *HcrVf2* transformed lines reflects that of a *Vf* carrying plant. A further step has been made by identifying the promoter sequence of the *HcrVf1*, 2, and 4 and demonstrating their functionality [67]. Current work is focused on testing *HcrVf1*, 2, and 4 under the control of their own promoter and *nptII*, and on the testing of an alternative selection system (unpublished results).

5

Self-Incompatibility

A further area of interest is the fertility of *Malus*; each genotype, i.e., cultivar is self-infertile. Fruit setting depends, therefore, on insects which have to bring foreign pollen to the flower. For that reason orchard planning must include plants of another cultivar or wild *Malus* at regular intervals (pollinators). Under adverse climatic conditions pollination may be hampered and can lead to irregular fruit production. Self-incompatibility (SI) is controlled by a single multiallelic locus (S). When one of the S alleles matches one from the other partner, pollen tube growth is blocked. Several of the S alleles have been cloned and sequenced. Silencing this gene should allow self-pollination. The research group in Leuven transformed the cultivar Elstar with one of its own S alleles (S3) with sense, antisense, and incomplete antisense sequences. Some lines with the sense and the incomplete antisense sequences showed good self-pollination [68]. Three years of field trials showed that the production of fruits from two transgenic lines produced by selfing was comparable to that reached with cross-pollination. The inactivation of the SI mechanisms was also achieved by suppressing the S RNA expression in Elstar, rendering both alleles S3 and S5 dysfunctional. The two lines had no negative feature and were identical to the wild-type Elstar [69]. A self-fertile cultivar can improve and facilitate apple production and breeding, so this is a trait of great relevance. However, the use of the selection gene *nptII* and the CaMV 35S promoter may make the two lines unacceptable for practical use.

6

Herbicide Resistance

Herbicide resistance may have two applications: it could be used as an alternative selection system to the overwhelmingly used *nptII* and as a practical

application in nurseries during rootstock production. In fruit production there is little advantage, as herbicides damage trees only in exceptional situations and when normal safety precautions are not observed. The gene for resistance to the herbicide bialaphos (bisanafos) (*bar*, encoding phosphinothricin acetyltransferase) was introduced into *M. prunifolia* var. *ringo*. The herbicide resistance also served as the selectable marker. Transgenic plants were fully resistant to bialaphos [70]. The transformation and selection of the phosphinothricine (PPT, Basta) resistant apple clonal rootstock No. 545 offered some interesting information. PPT compared to *nptII* was inefficient as a selectable marker, giving a high frequency of escapes and a transformation frequency of 1.3% compared to 3.5–14% obtained with *nptII* and the same rootstock. However, once an appropriate line was selected, the plants were resistant to the commercially applied dosages of Basta [71].

7

Fire Blight Resistance

Probably the first and most important target of transgenic apple was fire blight resistance, which was pioneered by the Cornell University group led by Aldwinckle [72, 73]. As for the experiments performed to infer resistance against fungi and insects, several genes with different characteristics were used to transform apple cultivars and rootstocks with the aim of increasing their resistance to fire blight. Attacin A [74] and E genes were used to transform the rootstock cultivar Malling 26. In both in vitro and greenhouse trials the plants from the transformed line were more resistant. The GM plants supported a tenfold higher inoculum dosage before reaching the 50% lethal factor [75]. GM lines of the cultivar Galaxy, with the genes for attacin E and T4 lysozyme, have increased resistance to fire blight [76]. A set of German cultivars (Elstar, Pinova, Pilot, Pingo, Pirol, and Remo) have been similarly transformed with T4 lysozyme gene [77]. Unfortunately, we found no further report on the outcome of the mentioned fire blight resistance tests. Norelli et al. [78] overviewed the ongoing work and additionally reported transformation of Gala, Royal Gala, and Galaxy with the cecropin analogs SB 37 and Shiva 1, and a hen egg white lysozyme (HEWL). However, the best results were obtained with the attacin E. One particular line displayed only 5% shoot blight compared with approximately 60% of the untransformed Royal Gala control plants in 1998 field trials [79]. A short overview of the field trials with the various lytic protein transgenic lines was presented by Aldwinckle [72, 73].

Instead of using the constitutive promoter CaMV 35S, Liu et al. [80] genetically engineered into Royal Gala a modified cecropin SB37 gene (MB39), fused to a secretory coding sequence from barley alpha amylase and placed under the control of a wound-inducible osmotin promoter from tobacco.

Seven diploid transgenic lines were produced (transformation efficiency of 1.7%) of which three lines, all having multiple insertions, had a higher proportion of shoots with low infection scores than Royal Gala.

A similar approach was attempted by the German team of the Max Planck Institute in Lenburg and the Institute for Fruit Breeding in Dresden. The extracellular polysaccharide (EPS) amylovoran produced by *E. amylovora* is an essential pathogenicity and survival factor, building a capsule around the bacterium. *E. amylovora* bacteriophages produce an EPS depolymerase to degrade this capsule to finally reach the bacterium. The EPS depolymerase gene was inserted into an apple rootstock JTE H by *Agrobacterium*-mediated transformation and *nptII* as selection gene. In the first pathogenicity tests, four out of five transgenic plants were completely resistant to fire blight [81]. A further extension of the work used the cultivar Pinova as the host plant. In vitro, 61 out of 83 transgenic lines were significantly more resistant to fire blight. Some transgenic lines were transferred to greenhouse conditions and tested for increased resistance. The authors do not report any data, but state that the correlation between the in vitro data and the greenhouse data is a mere $r = 0.5$ [82]. The same gene has been used in pear and only two out of 15 lines showed consistent increase of fire blight resistance in vitro and in greenhouse tests. The two lines were also those with the highest EPS depolymerase expression [83].

The pathogen-induced plant resistance approach starts from the theory that the pathogen secretes substances which are recognized by the host and may initiate the defense cascade. It has been shown that by applying these substances prior to inoculation with a pathogen, an incompatible reaction can be induced. The *E. amylovora* effector protein, harpin, when sprayed on flowers protects against subsequent *E. amylovora* infection, probably by inducing the systemic acquired resistance responses. However, high dosages lead to cell death. The gene encoding for harpin N (*hrpN*) under the control of the promoter *Pgst1*, which is induced in *E. amylovora* challenged leaves [84, 85] and under the weak constitutive promoter *nos* with and without a signal peptide (SP) which should direct the harpin N to the intercellular space, were introduced into the rootstock M.26 [86]. Transgenic plants expressing *hrpN* under the control of the *nos* promoter have been obtained [86] as well as a few lines under the control of *Pgst1* [72]. In an overview Aldwinckle [73] reports that some lines showed an increase of resistance in the field. Also, the cultivar Galaxy has been transformed with the constructs *Pgst1*-SP-*hrpN*. Field trials for fire blight resistance are currently under way [73] and it will be highly interesting to know the outcome.

In all the above reported approaches to create a fire blight resistant plant, the resistance induction was entrusted to a non-*Malus* gene. Besides the harpin N, *E. amylovora* also produces the pathogenicity effector protein *dspE*, which interacts directly with four leucine-rich repeat (LRR) receptor-like serine/threonine kinases of apple (DIPM). If this interaction does not take

place *E. amylovora* will not be able to infect that host, i.e., in the absence of the host DIPM receptor proteins the specific plant is a nonhost. Constructs with complete or partial hairpin *DIPM* gene sequences controlled by the CaMV 35S promoter were made. Several GM lines were recovered and in most the corresponding *DIPM* genes were silenced. However, no line with all four *DIPM* genes silenced was recovered. Preliminary fire blight resistance tests in growth chambers indicate that some lines are more resistant to artificial infection of the shoot tips [87]. As the authors point out, “resistance due to silencing of a native apple gene(s) is likely to be more acceptable to regulators, growers, and consumers than the addition of any foreign genes.”

Overexpression of an apple own gene, linked to defense, was proposed by Aldwinckle. The *NPR1* protein in *Arabidopsis* is linked to the defense reaction and when overexpressed enhances resistance to *Pseudomonas syringae* and *Peronospora parasitica* [88]. Aldwinckle’s group incorporated the apple ortholog *MpNPR1* with the promoter *Ppin2* (*E. amylovora* induced) into Galaxy and M.26. Two transgenic lines of Galaxy were clearly more resistant to fire blight, with 36% compared to 93% (controls) of the shoots blighted in a growth chamber test with potted plants [72].

8

Fruit Ripening

When discussing the pros and cons of replacing popular cultivars with high susceptibility to diseases with new cultivars possessing improved disease resistance, one of the principal arguments against any change is the lack of adequate storage and shelf life [89]. A number of genes have been identified which play a determinate role in the ripening and softening of fruit; however, ethylene remains a key factor. Ethylene is synthesized in plants from *S*-adenosyl methionine with the aid of two enzymes: 1-aminocyclopropane-1-carboxylic acid synthase (ACS), which converts *S*-adenosyl methionine to 1-aminocyclopropane carboxylic acid (ACC), and 1-aminocyclopropane carboxylic acid oxidase (ACO), which oxidizes ACC to ethylene.

Hrazdina et al. [90] transformed Royal Gala with an antisense sequence of one of the ACS genes. Transgenic lines were planted in the field and evaluated. GM plants developed normally and the fruits were, in most lines, similar to the nontransgenic controls. There was no significant decrease in ethylene production per fruit, nor in the other relevant parameters (soluble solids, pH, and penetration force) when the average value of the various parameters of all plants of a line was considered. However, the authors write that “on the percent basis ethylene production in apples from the individual trees from different transgenic lines was downregulated after 69 days of storage by 11 to 97% with the majority of fruits falling in the 60–80% range.” Apparently the time course of ethylene presence was changed so that, at the last evalua-

tion date, the fruit from the transgenic lines produced only half the amount of ethylene compared to the control fruits. The authors infer that the data indicate that the structural integrity of the fruit may be maintained simply under refrigerated storage and controlled humidity. Similar results were achieved by Galli et al. [91] with fruit from lines of transgenic Royal Gala with down-regulated ethylene synthesis. The fruits remained firm and had an increased resistance to shriveling, splitting, and spoilage following extended storage at room temperature.

In a very careful and accurate study the research group of the Department of Pomology, University of California, Davis, evaluated the impact of the suppression of ethylene on the fruit quality [92–94] using transgenic apple trees with ACC synthase or ACC oxidase enzyme activity suppression. Sucrose and fructose levels were lowered, malic acid degradation was reduced, and the volatile aroma ester and alcohol fractions were similarly reduced. In a collaborative work with Horticulture Research International, East Malling, England, some incongruence still remains, as the results suggest that sugar and acid composition are not under the direct control of ethylene and alcohol volatiles seem not to be influenced. However, for practical application, the firmness of the fruits from transgenic lines with suppressed ethylene biosynthesis remained almost constant after storage (shelf life), whereas in the control Greensleeves fruits it decreased dramatically [95]. These studies can be viewed from an application point of view but they also contribute highly to our understanding of the ripening processes in apple. Others do not have such an evident potential of application, but merit being mentioned as they contribute to basic knowledge. Cheng et al. [96] demonstrated the plasticity of the apple photosynthesis system by using antisense inhibition of sorbitol synthesis in GM apple; Kanamuaru et al. [97] were able to determine that S6PDH is a key enzyme regulating partitioning between sorbitol and sucrose in apple leaves.

Atkinson et al. [98] overexpressed polygalacturonase and obtained a range of new phenotypes, altering leaf morphology, plant water relation, stomata structure and function, as well as leaf attachment. Underexpression of polyphenol oxidase (PPO) (catechol oxidase), the enzyme responsible for enzymatic browning of apples, by use of an antisense PPO gene clearly led to reduced calli browning [99] and shoots had a similarly lower tendency of browning through the PPO activity [100].

9

Allergens

Malus has four allergenic proteins, Mald 1 to 4. They cross-react with the birch pollen Bet v 1 specific IgE antibodies, so that an apple allergy is common in patients with a birch pollen allergy. Apple cultivars have different

allergenic potential, Golden Delicious being a cultivar with a high potential. A hairpin construct from Mald 1 was introgressed by *Agrobacterium*-mediated transformation with the selection gene *nptII* in the cultivar Elstar. Six plantlets displayed a significant reduction of Mald 1 production (at least tenfold) and induced significantly less reaction in patients than the control plantlets [101]. Specific RNAi silencing can therefore solve a diet problem for birch pollen allergic patients.

10

Rooting Ability

Vegetative propagated plants depend on a high rooting capacity or have to be grafted on rooted “rootstocks”. Clearly such a rootstock cultivar has to have, besides its specific growth characteristics (mostly vigor leading to dwarfing or allowing a high tree), a good rooting ability. Apple rootstocks are propagated by stool layering, seldom by rooting of cuttings as some are recalcitrant to root from cuttings even with the use of auxin. Root-inducing genes have been characterized in *Agrobacterium rhizogenes* (*rolA*, *rolB*, and *rolC*), and contribute to causing “hairy root” disease in the host. The bacterium introduces parts of its DNA containing the *rol* genes into the host plant, which reacts by producing additional roots and often assuming a dwarfing stance. Almost all transgenic works with the *rol* genes have their origin at the Department of Horticulture at the Swedish University of Agricultural Science in Alnarp. Transforming the apple rootstock M.26 with *rolA* controlled by its own promoter resulted in four lines with variable growth reduction and wrinkled leaves [102]. Incorporation of the *rolB* gene, also under the control of its own promoter, into the rootstock M.26 increased auxin sensitivity and rooting ability [103]. To think of commercialization of a rootstock with increased ability to root, it is necessary to demonstrate that no negative effect will be transmitted to the graft scion. Zhu and Welander [104] used the cultivar Gravenstein as a test scion. Its growth characteristics were not influenced under unlimited nutrient conditions. The specific root length of the M.26 rootstock was significantly reduced, from which it can be speculated that under limited nutrient supply, as in orchard conditions, it may induce tree dwarfing.

M9 is another very popular rootstock with excellent dwarfing characteristics; however, it roots badly from cuttings. GM M9 with the *rolB* gene roots extremely well. In vitro rooting ability went from almost nil to almost 100% with 3.5 to 9.5 roots per cutting [105]. Also, the rootstock Jork 9 does not root readily from cuttings. Incorporation of *rolB* dramatically increased the rooting of shoots in the absence of externally added auxin. The control untransformed shoots were able to reach almost the same rooting ability with the addition of indole-3-butyric acid (IBA) [106]. The rootstock Jork 9 and

the various transgenic lines have been used to study plant response to the rooting process, natural or auxin induced [107]. Welander et al. [108] report that “the permission for field trial on the transformed rootstocks has been obtained from the Board of Agriculture in Sweden.” Plants of different transformed clones and the untransformed controls of M.26 and M9/29 have been produced and grown in the field for 2 years. Five apple cultivars commonly used in Sweden and Europe have been budded onto the rootstocks to evaluate the influence of transgenes on the growth and development of the grafted cultivars.

The *rolC* gene has been introduced into a Japanese rootstock (*M. prunifolia* var. *ringo* Asami Mo 84 A), which roots well but has not the desired dwarfing ability. The aim was to determine whether *rolC* can reduce growth characteristics without altering the rooting characteristics. Some transformants had shortened internodes, some reduced height, some both and some were normal; rooting was intensive in some transformants. The authors state that a few lines may be suitable candidates for dwarfing rootstocks [109]. Radchuk and Korkhovoy [110] transformed the scion cultivar Florina with *rolB* with the theory that multiplying and growing it on its own roots would reduce costs and accelerate production of plant material. They obtained various lines with enhanced rooting ability. From the data for 2 years of greenhouse experiments they report no change to the above-ground growing characteristics compared to those of the original untransformed Florina.

11

Acceptance and Risk Assessment

Apple is vegetatively propagated and seeds only have a role in breeding. Transformation of the apple into a weed through a selective advantage gained from a foreign gene is not plausible under any imagination. Environmental risk is restricted to the gene products and is not inherent to apple. Apple is a fresh product often consumed raw, and therefore consumers are particularly attentive to any manipulation. Currently it is improbable that foreign genes will be acceptable under European law where transgenics are highly regulated and have to be declared as such. So the arguments listed in 1996 by Koller et al. [111, 112], leading to the opinion that transgenic apple carrying foreign DNA will not be commercialized in the near future, are still valid.

Almost all work cited in this review, excluding the few specially mentioned exceptions, relied on the selection gene *nptII*, on non-apple gene promoters (CaMV 35S amongst others), and on *Agrobacterium tumefaciens*-mediated transformation. Often, for experimental purposes, genes not influencing the target trait were used, such as the gene producing β -glucuronidase (GUS). To our knowledge no environmental risk studies specific to apple have been published. The only argument studied was the possibility that particular insects

may become resistant to the Bt toxin [113]. The development of Bt resistance was analyzed using simulation experiments in the agrosystems apple and clover, both hosts for various leafrollers [114]. Probably the researchers are still concerned with producing acceptable GM apple cultivars with a commercial interest [115] and possibly having environmental benefits, such as reduction of pesticide use.

However, an in-depth discussion should be conducted on any gene used. For example, genes expressing the biotin binding proteins avidin and streptavidin, as indicated above, and used to infer insect resistance [44] in fruits would also bind biotin for the consumer and may lead to reduced vitamin B absorption similar to a surplus of egg consumption.

What is acceptable, however, is debated and some argue that only apple-derived genes will have the possibility of encountering the favor of the producer and consumer. In this context the research by Kassardjian et al. [116] on purchase behavior is interesting, mainly as a guideline that includes innovative ideas on how to elicit consumers' willingness to purchase GM apples. The goal of their research was to try a new methodology (thought-listing technique and questionnaires) to elicit consumer willingness to pay for GM food in New Zealand. However, it is not that the researchers are inactive; in popular journals [117–119], interviews, and web sites arguments for and against the use of GM apples are presented and debated.

12

Conclusion

In the next few years many “apple own” resistance genes will be sequenced and transferred into some test cultivars, probably under the control of their own promoters. RNA interference technology will be able to block some unwanted traits. Pathogen-derived genes inducing host resistance will be available. Apple own promoters expressing genes only where and when desired will no longer be a mirage but will appear slowly on our horizon. With the “clean vector technology” [36] allowing the removal of the selection marker, it will be possible to produce cisgenic apples, i.e., apple plants which will be modified exclusively with *Malus* genes and controlled by *Malus* promoters. Many of the discussions about risks, phantom or truly demonstrated, will be obsolete. However, the insertion site will not correspond to the original site of the gene and this could lead to epigenetic effects. Major effects are readily discovered and such lines are discharged; subtle effects are more difficult to discover, so we have to strive to devise smart experiments allowing for selection of lines which harbor no surprises.

The apple cropping system based on artificial vegetative multiplication of the particular genotype and its planting over large areas has rendered this crop susceptible [120]. The possibility offered by DNA recombinant technol-

ogy can be used to replace nonfunctional resistance alleles by not-overcome alleles (gene therapy). The benefits of GM apple resistant to various diseases will be real, not only for the owner of a patent but also for the producer, environment, and consumer. The reduction of fungicide and, in some parts of the world, antibiotic use alone justifies all the efforts [121]. It remains to be seen how long it will take until a broad acceptance by the public is achieved.

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