GENE TRANSFER AS AN IMPORTANT APPROACH TO RESISTANCE BREEDING IN APPLE

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ABSTRACT

Apple trees were genetically engineered to increase resistance to diseases such as fire blight, apple scab and powdery mildew. To increase resistance to fire blight, genes coding for antibacterial proteins have previously been used, such as attacin E from *Hyalophora cecropia* and lysozyme from bacteriophage T4. We used the gene coding for EPS-depolymerase from an *Erwinia amylovora* bacteriophage. The transgenic plants were analyzed with molecular and biochemical techniques. Their susceptibility to fire blight was evaluated in the greenhouse.

To prevent the spread of transgenic seeds and pollen into the environment, we investigated introducing parthenocarpy and male sterility into transgenic apple plants. The plants obtained have been transferred to the greenhouse. Data on pollen development will be collected as soon as they form flower buds.

The stability of transgene integration and expression were studied after long-term *in vitro* cultivation of transgenic lines. Some irregularities were detected after analysis of the data gathered by PCR, RT-PCR, ELISA and Southern Blot.

Key words: *Agrobacterium tumefaciens*, transformation, resistance, male sterility, genetic stability

INTRODUCTION

The cultivated apple is the most important fruit crop in Europe. In several countries, extensive breeding programs have been established to develop new disease resistant varieties which reliably bear abundant crops of excellent quality fruit.

Genetic manipulation in fruit trees, especially the apple, has been the subject of intense research ever since the introduction of the non-commercial

transgenic apple cultivar 'Greensleeves' in 1989 (James et al., 1989). Since then, several transgenic plants have been produced by *Agrobacterium* mediated gene transfer.

Most studies on genetic transformation in apples have focused on improving agronomically important traits in commercial cultivars. A high priority has been resistance to bacterial and fungal pathogens, such as *Erwinia amylovora*, *Venturia inaequalis*, and *Podosphaera leucotricha.*

Before a genetically modified plant can be approved for general cultivation, it has to undergo stringent testing to ensure that it will not pose a risk for the environment any more than conventionally bred varieties. A major concern is that field experiments with transgenic plants carry the risk of gene transfer by seed or pollen. Parthenocarpy and male sterility have recently been examined as ways to prevent gene escape.

Whether transgenes are stably expressed in the host plant greatly depends on the successful physical insertion of the gene into the target genome in proximity to appropriate host regulatory sequences. In long-lived species such as fruit trees, little is yet known about the inactivation or loss of transgenes caused by gene silencing or somatic genome rearrangement.

Transgenic apples were produced by infecting leaf explants from proliferating shoot cultures with *Agrobacterium tumefaciens* (Norelli et al., 1996). Eight to twelve weeks after inoculation, meristem formation was induced in leaf explants originating from a single transformation event. The meristems were excised and propagated *in vitro,* giving rise to a line of genetically identical plants. These plants were then tested for successful incorporation and expression of the transgene using different molecular procedures.

Increasing resistance to bacterial and fungal pathogens

Fire blight is the most destructive disease in apples and pears. It is caused by the bacterium *Erwinia amylovora.* To improve resistance to fire blight, apples have been transformed with different genes, including the lysozyme gene from bacteriophage T4 and the attacin E gene from *Hyalophora cecropia* (Ko et al., 1997). Both genes code for products which kill a wide spectrum of gram-positive and gram-negative bacteria. We have also used the gene for extracellular polysaccharide (EPS)-depolymerase from *Ea1h,* a bacteriophage of *E. amylovora*.

Amylovoran is a major virulence factor in *E. amylovora*. This acidic extracellular polysaccharide (EPS) is a component of the cell capsule, where it binds water and nutrients, and also protects the bacterium against host defences. Some bacteriophages, including *Ea1h,* have EPS depolymerases in their coats which bind to and degrade capsular polysaccharides in the host bacteria. The phage can then binds to the outer membrane and inject its nucleic acid into the host.

The EPS-depolymerase gene under the control of the constitutive promoter CaMV35S was transferred by *Agrobacterium* mediated gene transfer into one apple rootstock and nine apple scion cultivars. The rootstock transformed was AU 56-83. The cultivars transformed were 'Elstar', 'Jonagold', 'Piflora', 'Pilot', 'Pinova', 'Pirol', 'Reka', 'Retina', 'Remo'. Three different strains of *Agrobacterium tumefaciens* were used: EHA105, KYRT1 and LBA4404 (Tab. 1). The aims of the experiment were to create transgenic plants which express the EPS-depolymerase gene, and to evaluate the effect of the gene product on susceptibility to fire blight.

Table 1. Transformation of apple plants for disease resistance. Percentage of transformed shoots in one apple rootstock and eight apple scion cultivars

Cultivar	Number of leaf segments	Number of regenerated shoots	Percentage of putative transformed shoots
AU 56-83	2.848	187	6.6
Elstar	**712**	49	6.9
Jonagold	1.824	135	7.4
Pilot	1.360	24	1.8
Pinova	5.416	375	6.9
Pirol	1.104	22	2.0
Reka	1.488	42	2.8
Remo	1.224	148	12.1
Retina	1.048	0	0

Gene insertion was confirmed by PCR amplification of the marker gene *nptII*. 101 transgenic lines were identified among the 7,184 leaves inoculated in 100 different transformation experiments. Each was the product of a single transformation event.

Gene expression was confirmed by a sandwich ELISA assay for the NPTII protein, by Western blot analysis for the depolymerase, and by reverse transcription PCR for both genes. Enzymatic assay revealed that there were substantial differences in depolymerase activity among the different transgenic lines. The transgenic lines were analyzed by flow cytometry to determine ploidy and by Southern blot analysis to determine the number of gene insertions.

Susceptibility to fire blight was determined *in vitro* using an *E. amylovora* strain labeled with *gfp* (Hanke et al., 2002; Hanke and Geider, 2002). Susceptibility was also evaluated *ex vitro* by artificial inoculation of the transgenic plants in a greenhouse or growth chamber. Several lines had significantly milder symptoms after inoculation than the original cultivar. Susceptibility testing was carried out only on shoots, which does not provide

reliable data on susceptibility to flower infection. Field testing still needs to be performed in order to definitively confirm increased resistance to fire blight in the transgenic plants.

To increase resistance to fungal diseases such as scab and mildew, the plants were transformed using the endochitinase gene *ech42* and the exochitinase gene *nag70* from *Trichoderma harzianum* (Bolar et al., 2000; 2001).

Altogether, 170 apple lines were regenerated and selected. They can now be evaluated for pomological traits and disease resistance. Research on these lines will also increase our understanding of the stability of transgenes in genetically engineered trees.

Preventing gene escape by inducing pathenocarpy and male sterility

Gene flow from cultivated to wild plant populations has important evolutionary and ecological consequences. Transgenic plants need to be subjected to careful risk assessment to prevent the escape of transgenes into natural ecosystems. In the apple, there are two vehicles of gene flow; seeds and pollen. Bees and other insects can spread pollen from transgenic plants and seedlings to wild species. To prevent this, research had been carried out on inducing parthenocarpy and male sterility in transgenic plants. To induce parthenocarpy, the chimeric gene construct *DefH9iaaM* has been tried (Rotino et al., 1997). However, it has so far been impossible to obtain stable transformed plants.

To induce male sterility, six different constructs have been used. Two constructs express the barnase gene, a ribonuclease gene from *Bacillus amyloliquefaciens* which is toxic to plant cells. Two other constructs express the stilbene synthase gene *Vst1* from *Vitis vinifera* L. to block the flavonoid biosynthesis. Both genes (barnase and *Vst1*) are expressed under the control of the tapetum specific promoter *pTA29* from *Nicotiana tabaccum* L., and the pollen specific promoter *ClGPDHC* from *Cuphea lanceolata* L., respectively.

Two other constructs are based on the tapetum specific promoter of the *Nin88* gene from *Nicotiana tabaccum* L.. This gene plays an important role during the development of the anthers. The down regulation of the *Nin88* gene expression will be resulted in preventing anther development. Using the Nin88 constructs, we selected about 100 transgenic apple lines and evaluated them using molecular techniques. Based on molecular data about 30 lines (15 of each construct) have been selected, rooted and transferred to the greenhouse. Data on pollen development will be collected as soon as they form flower buds.

Finally, a FHT-anti-sense construct was used to block flavonoid biosynthesis. The flavonols quercetin and campferol play an essential role in pollen maturation. Interrupting flavonoid biosynthesis by blocking FHT (flavanon-3-hydroxylase) reduces the synthesis of quercetin and campferol.

Using the FHT-anti-sense construct, we selected eight transgenic apple lines and evaluated them using molecular and biochemical techniques. The

transgenic plants contained significantly more eriodictyol-7-glucoside than the original cultivar. These plants have been rooted and transferred to the greenhouse. Data on pollen development will be collected as soon as they form flower buds.

Stability of transgene expression

In order to effectively perform field trials on transgenic perennial fruit tree species like the apple, it is essential that the transgenes have been stably incorporated into the host genome. Physical loss of T-DNA sequences and gene silencing are problems commonly encountered in the laboratory, the greenhouse and the field.

In the laboratory, transgenic plants are usually propagated *in vitro* on media containing a selective agent such as an antibiotic. It is often difficult to estimate the degree of instability because those cells which do not express the transgene do not survive, and thus cannot be detected. When the plants are transferred to the greenhouse or the field, selection pressure is abruptly lifted. Instable transgene expression can have a major impact on tree characteristics.

To estimate the actual degree of genetic instability, 26 kanamycin resistant transgenic apple lines were grown without antibiotics for about four years. The lines were then checked for stable insertion, transcription and translation of the transferred genes (*nptII* and *gusA*).

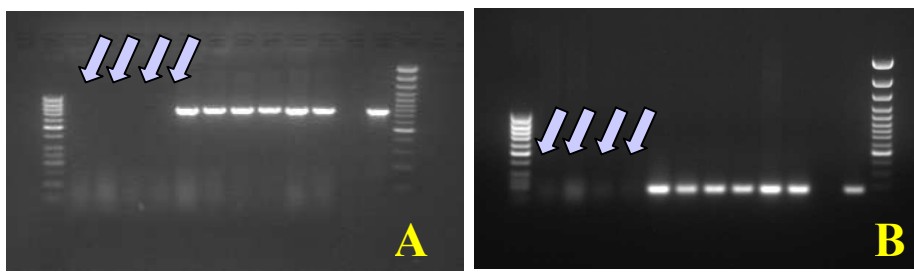

Figure 1. Transcription of transgenes in single shoots of the transgenic apple line T357. The graphic illustrates the transcription of the marker gene nptII (A) and the gene of interest AttE (B). Arrows highlight four shoots of the line T357 which did not survive on selective medium containing an antibiotic. The total RNA of the ten shoots was isolated. After reverse transcription, the cDNA was tested using transgene specific primers. No transcription was found in the highlighted shoots. All shoots which survived on selective medium showed correct fragments for both genes

For example, in line T357 (*AttE*), four out of ten shoots did not survive on medium containing kanamycin. Because all shoots had been cultivated in duplicate both with and without kanamycin, total RNA could be isolated and reverse transcribed into cDNA using gene specific primers. The shoots which did not survive did not express either the marker gene or the transferred genes (Fig. 1).

In some plants of those lines total or partial transcriptional and post-transcriptional silencing of the transferred genes was found. For several shoots of one line no T-DNA could be detected. In some lines, the tissue was found to be chimeric in nature with regard to *gusA* expression. Two separate genes at the same locus could be expressed to quite different degrees.

In order to gather information on the mechanism of *Agrobacterium*-mediated T-DNA insertion, the regions adjacent to the insertion were examined in several lines. Undesirable vector backbone sequences were frequently found among the expected plant DNA sequences. In one line, the T-DNA junction seemed to be a recombination with a bacterial transposon of a *Pseudomonas* subspecies.

Our results clearly show that current standard molecular techniques are of limited usefulness in evaluating transgenic plants.

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TRANSFER GENÓW JAKO WAŻNA STRATEGIA W HODOWLI ODPORNOŚCIOWEJ JABŁONI

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S T R E S Z C Z E N I E

Inżynieria genetyczna pozwala na uzyskanie odporności jabłoni na patogeny grzybowe i bakteryjne. Zastosowanie do transformacji roślin genów kodujących depolimerazę EPS z bakteriofaga *Ealh Ervinia amylovora* oraz genów endochitynazy *ech42* i egsochitynazy *nag70* z *Trichoderma harzianum* pod promotorem konstytutywnym 35S CaMV zaowocowało uzyskaniem 170 linii odpornych odpowiednio na zarazę ogniową oraz parcha i mączniaka jabłoni. Rośliny były transformowane przez Agrobacterium tumefaciens, a ich status roślin transgenicznych był potwierdzony w teście PCR, ELISA, Western i Southern blotting, a ploidia określona cytometrycznie. Dodatkowe badania poświęcono zapobieganiu przepływowi transgenów do naturalnych ekosystemów (indukcja męskiej sterylności i partenokarpii jabłoni poprzez wprowadzenie genów FHT i *DefH9iaaM* pod specyficznym promotorem z *Nicotiana tabacum*) oraz stabilności ekspresji wprowadzanych genów.

Słowa kluczowe: transformacja, *Agrobacterium tumefaciens*, odporność, męska sterylność, partenokarpia, stabilność genetyczna