*Transgenic Plant Journal* ©2009 Global Science Books

# Plant Regeneration and Transformation in the Rosaceae

**Herb Aldwinckle[1*] • Mickael Malnoy[2**]**

[1] Cornell University, 630 W. North Street, Geneva, NY 14456, USA
[2] FEM-IASMA Research Centre, Via E. Mach 1, 38010 San Michele all'Adige (TN) Italy
*Corresponding authors*: * **hsa1@cornell.edu** ** **mickael.malnoy@iasma.it**

## ABSTRACT

The plant family Rosaceae consists of over 100 genera and 3,000 species that include many important fruit, nut, ornamental, and lumber crops. Members of this family provide high-value nutritional foods and contribute desirable aesthetic and industrial products. A complete annotated genome sequence is expected momentarily for apple, and in the near future for peach and strawberry. These sequences will accelerate the next wave of research exploring and comparing the form and function of the many genomes that define the striking differences in morphology and physiology among the Rosaceae, e.g., a herbaceous plant vs. a tree, or a fleshy rather than a dehiscent fruit. Among the many dividends of this research will be the development of superior products for consumers, a better understanding of the genetic elements that contribute to horticultural traits of interest, and an enhanced vision of Rosaceae evolution. It will also answer some of the fundamental questions of plant biology, particularly around the regulation of plant architecture, which may be best answered by species within this family. This review serves as a synopsis of the genetic engineering resources available to study the function and production of new Rosaceae varieties of benefit to the consumer.

**Keywords:** *Fragaria*, *Malus*, *Prunus*, *Pyrus*, Rosaceae, rose, *Rubus*, transformation
**Abbreviations:** **ACC**, 1-aminocyclopropane carboxylic acid; **ACO**; 1-aminocyclopropane carboxylic acid oxidase; **ACS**, 1-aminocyclopropane-1-carboxylic acid synthase; **2,4-D**, 2,4-dichlorophenoxy acetic acid; **DIPM**, DspE Interacting Protein of Malus; **Dpo**, Depolymerase; **5-FC**, 5-fluorocytosine; **5-FU**, cytotoxic 5-fluorouracil; **BA**, N6 benzylaminopurine; **BSAA**, 3-benzo[b]selenienyl acetic acid; **CP**, coat protein; **CaMV**, *Cauliflower mosaic virus*; **Carb**, Carbenicillin; **Cef**, Cefotaxim; **GE**, genetic engineering or genetically engineered; **Gen**, Geneticin; **HIPM**, harpin $N_{Ea}$ Interacting Protein of Malus; **Hyg**, hygromycin; **IAA**, indol-3-acetic acid; **IBA**, indol-3-butyric acid; **Kan**, kanamycin; **LBAM**, Lightbrown apple moth; **MCP**, 1-methylcyclopropene; **MS**, Murashige and Skoog; **NAA**, 1-naphthalene acetic acid; **NPR1**, Nonexpressor of PR; ***nptII***, Neomycin Phosphotransferase; **"OSF"**, Okanagan Specialty Fruits; **PGR**, plant growth regulator; **pmi**, Phosphomannose isomerase; **PPO**, Polyphenoloxidase; **Ppt**, **PPV**, *Plum pox virus*; Phosphinothricin; **PTGS**, PostTranscriptional Gene Silencing; **sam-k**, S-adenosylmethionine hydrolase; **SAR**, systemic acquired resistance; **S6PDH**, Sorbitol 6 phospahte dehydrogenase genes; **TIB**, temporary immersion bioreactor; **Tic**, Ticarcilin; **TDZ**, thidiazuron

## CONTENTS

INTRODUCTION ..... 1
PLANT REGENERATION AND TRANSFORMATION ..... 2
*Fragaria* ..... 2
*Malus* ..... 8
*Pyrus* ..... 20
*Prunus* ..... 24
*Rosa* (rose) ..... 28
*Rubus* ..... 30
SIMILARITIES AND DIFFERENCES IN THE TRANSFORMATION SYSTEMS FOR DIFFERENT ROSACEAE GENERA ..... 30
FUTURE PERSPECTIVES ..... 32
REFERENCES ..... 33

## INTRODUCTION

Rosaceae constitutes the third most economically important family in temperate regions (Dirlewanger *et al.* 2004). The economic importance of rosaceous crops derives from the pleasurable eating quality, varied tastes, and the health benefits they provide. Fruit is important to the human diet as a major source of numerous photochemicals, such as flavonoids and other phenolic compounds. Numerous other phenolic compounds belonging to distinct chemical classes, many of which have also been shown to be potential antioxidants and anticancer agents (Eichholzer *et al.* 2001; Schieber *et al.* 2001), have also been isolated from rosaceous fruits (Macheix *et al.* 1991). *In vitro* and *in vivo* studies with animal models provide evidence that fruit and leaf extracts from many Rosaceae inhibit various forms of cancer or have pronounced antioxidant action (Yau *et al.* 2002).

For the past century, efforts in agricultural research have centered on increasing crop production. However, conventional breeding of temperate fruit trees is constrained by their long reproductive cycle with long juvenile periods, complex reproductive biology and high degree of heterozygosity. Advances over the past decade in genomics and bioinformatics of Rosaceae have provided new options to identify useful compounds, resistance genes and the genes responsible for their development. Therefore, there was great excitement and expectation when the first reports of genetic transformation of Rosaceae were made (James et al 1989;

Received: 9 June, 2009. Accepted: 13 November, 2009.

Invited Review

Graham *et al.* 1990; Mante *et al.* 1991; Firoozabady *et al.* 1994; El Mansouri *et al.* 1996; Mourgues *et al.* 1996). Suddenly it seemed possible that existing varieties with desirably altered characters could be produced within a shorter timeframe than by conventional breeding. Since these first reports, several members of the Rosaceae family have been transformed, with various efficiencies of transformation. In this review, we will synthesize the procedures of transformation and the application of this powerful tool for each member of the Rosaceae family, and we will compare the similarities and differences in the various transformation systems of Rosaceae species. In conclusion, we will propose one or two members of the Rosaceae family as translational genomics models.

## PLANT REGENERATION AND TRANSFORMATION

Development of an effective system for gene transfer in the different Rosaceae species depends largely on the availability of tissue culture techniques that permit regeneration of shoots, selection of transformants, and propagation of transgenic plants. Increasing leaf regeneration efficiency is critical for the development of a transformation system in the Rosaceae family using an *Agrobacterium tumefaciens* vector or by biolistic process. In many instances, the lack of efficient regeneration systems is the major limiting factor preventing the development of gene transfer technologies for perennial crops (Dandekar 1992). In the next paragraphs we will synthesize the regeneration and transformation systems for each Rosaceae species and identify the application of the gene transfer technology in each species.

### *Fragaria*

#### *1. Regeneration and transformation*

The first report of *in vitro* regeneration and genetic transformation systems for the cultivated strawberry, *Fragaria X ananassa*, by Nehra *et al.* (1990a, 1990b) and James *et al.* (1990), has opened up the opportunity for strawberry improvement through genetic engineering. Several later reports revealed critical factors affecting the frequency of regeneration in strawberry. These include plant growth regulators (PGRs), incubation conditions, explants source, antibiotic, and mode of infections (**Table 1**). This modification has been decribed in the reviews of Folta and Dhingra (2006) and Qin *et al.* (2008).

**Genetic lines:** *Fragaria X ananassa* 'Red Coat' (Nehra *et al.* 1990 a/b) and 'Rapella' (James *et al.* 1990) were the first strawberry cultivars to be transformed with efficiencies between 0.95 and 6.5% on a per explant basis. The genus *Fragaria* is comprised of over 20 species, representing many levels of ploidy (Folta and Dhingra 2006). The diploid species offer an opportunity for rapid forward genetic discovery, gene function studies, and reverse genetic analysis (Oosumi *et al.* 2006). Studies have been centered on development of transgenic procedures for various diploid (17) and octoploid (24) accessions (**Table 1**). However, the efficiency of transformation and regeneration capacity is highly dependent on the genetic background (Landi and Mezzeti 2006; Oosumi *et al.* 2006). This observation is consistent with the fact that octoploid strawberry accessions are very variable from genotype to genotype, and transformation and regeneration ability is as varied as the wide variation in morphology, photoperiod sensitivity and fruit quality.

**Nature of explants:** Although genotype alone is an important variable in the regeneration capacity, the specific tissue chosen for regeneration is also an important consideration. Passey *et al.* (2003) observed that almost an entire plant can be used to generate shoots. In accordance with this finding, many strawberry cultivars have been successfully regenerated from many tissues, including leaves (Nehra *et al.* 1990b; Barcelo *et al.* 1998; Passey *et al.* 2003; Folta *et al.* 2006), stipules (Passey *et al.* 2003), petioles (Mezzetti *et al.* 2004), roots (Passey *et al.* 2003), unfertilized ovaries (Passey *et al.* 2003), and stems (Graham *et al.* 1995). For several *Fragaria X ananassa* genotypes, efficient regeneration procedures have been developed by using different types of somatic tissues (Liu and Sanford 1988; Rugini and Orlando 1992; Passey *et al.* 2003), although most of the studies relied almost entirely on leaf disks for transformation (**Table 1**). Recent studies have identified and optimized high-frequency adventitious shoot regeneration from petioles (Folta *et al.* 2006).

**Plant growth regulators:** The regeneration medium that has generally produced the greatest shoot regeneration was the Murashige and Skoog (MS) medium (1962) supplemented with 2-3% of sucrose in most of the procedures except for the report of Schaart *et al.* (2002) where glucose was used with standard vitamin complements. The major difference from procedure to procedure is in the combination and concentration of phytohormones (auxin and cytokinin) used to induce callus and adventitious shoots.

The cytokinin used in most of the *Fragaria* regeneration and transformation procedures is N6 benzylaminopurine (BA) at different concentrations (**Table 1**). In a few reports, the ability of thidiazuron (TDZ) to induce high shoot regeneration efficiency, in woody plant tissues in particular, has also been reported (Huettman and Preece 1993; Zhao *et al.* 2004; Folta *et al.* 2006a). The type and concentration of auxin is more variable between procedures (**Table 1**). For the regeneration of the diploids, indol-3-butyric acid (IBA) was used almost exclusively, whereas IBA, 2,4-dichlorophenoxy acetic acid (2,4-D), indole-3-acetic acid (IAA), and 1-naphthalene acetic acid (NAA) have been used in octoploid shoot regeneration (**Table 1**). Among the auxins, 3-benzo[b]selenienyl acetic acid (BSAA) is a new highly active molecule that has already been tested in some crops where it has shown to be highly effective for the induction of somatic embryogenesis. Although it has not yet been tested for its capability to control organogenesis in woody plants, Landi and Mezzeti (2006) have shown that BSAA promotes the highest number of shoots by explants.

**Antibiotics:** During the process of transformation, *Agrobacterium tumefaciens* tends to colonize the explants. To limit the development of this bacterium, explant tissues are commonly regenerated on a medium containing an antibiotic. Alsheikh *et al.* (2002) have carefully studied the effect of increasing concentrations of three antibiotics: Carbenicillin (Carb), Cefotaxim (Cef) and Cefoxitin. From this report, Carb had the least impact on regeneration, followed by Cefoxitin and Cef, and petioles were more sensitive to antibiotic toxicity than leaves. High concentrations of Cef, 100 to 200 mg/L, reduced the regeneration of shoots (Alsheikh *et al.* 2002; Hanhineva and Karenlampi 2007), and at 500 mg/L, caused severe necrosis and death of the leaf tissue (Hanhineva and Karenlampi 2007). Augmentin, Claforan, Mefoxin, and Tic (Timentin, Tic) were also used to reduce the growth of *A. tumefaciens* in some cultivars (**Table 1**).

**Inoculation procedure and *Agrobacterium* strain:** Many strawberry cultivars were transformed with several different genes, but most of the procedures relied on *Agrobacterium* mediated transformation, and are quite similar (**Table 1**). Due to the fact that all genotypes do not respond similarly to the same procedure of transformation, some modifications have been reported. The most common strain of *Agrobacterium* used in 50% of the transformation protocols is LBA4404. Apart from this strain of *A. tumefaciens*, other strains such as EHA105, EHA101, AGLO, GV3101, CBE31, MP90 and C58C1 were used. After wounding the explants, they are immersed in the *A. tumefaciens* inocula. This co-cultivation varies between 15 min (Nehra *et al.* 1990b) and 24h (Owens *et al.* 2002), and it is followed by a period of darkness on selective media for 2 days to 2 weeks.

Aside from *Agrobacterium*-mediated transformation,

**Table 1** *Fragaria* variety transformed genetically.

| Cultivars | Explants used | PGRs used for regeneration, nutrients | Antibiotics | Inoculation procedure | *Agrobacterium* strains | Selectable marker | Efficiency of transformation | References |
|---|---|---|---|---|---|---|---|---|
| **Stable transformation** | | | | | | | | |
| ***Fragaria vesca*** | | | | | | | | |
| 1) semperflorens | Leaf discs and petiole | IBA, BA | Carb 500 mg/l then 250 mg/L | Immersion 20 min in *Agrobacterium* inoculum | LBA4404 | Kan 10 increasing to 25 mg/L | 15% | Alsheikh *et al*. 2002 |
| | Leaf | BA, IBA | Carb 500 mg/L then 250 mg/L | -Immersion 20 min in *Agrobacterium* inoculum | LBA4404 | Kan 25 mg/L | 7.7% | El Mansouri *et al*. 1996 |
| 2) Alpina W. Original | Leaf and petiole | BA, 2,4D | Cefotaxime 100 mg/L | Immersion 20-30 min in *Agrobacterium* inoculum | LBA4404 | Kan 50 mg/L | | Mezzetti *et al*. 2004 |
| 3) Alpine | Leaf and petiole | BA, NAA | Mefoxin 250 mg/L | Immersion 20 min in *Agrobacterium* inoculum | LBA4404 | Kan 50 mg/L then 25 mg/L | nd | Haymes and Davis 1998 |
| | | TDZ, IBA | Carb 100 mg/L Cefoxitin 100 mg/L | Immersion 30 min in *Agrobacterium* inoculum | EHA105 | Kan 50 mg/L | 64-68% | Zhao *et al*. 2004 |
| Alpine<br>4) PI 551552 ssp. bracteata<br>5) PI 551572 Hawaii-4<br>6) PI 551782<br>7) PI 551791<br>8) PI 551792 ssp. vesca<br>9) PI 551833<br>10) PI 551834 ssp. vesca f. semperflorens, cv. Reugen<br>11) PI 551890 ssp. vesca<br>12) PI 551892<br>13) PI 602578 ssp. vesca f. alba<br>14) PI 602923 ssp. vesca, cv. Alexandria o<br>15) PI 602924 ssp. vesca<br>16) PI 602931<br>17) PI 616513 | Leaf | IBA, BA | Carb 500 mg/L | Immersion 20 min in *Agrobacterium* inoculum | GV3101 | Hygromycin 4 mg/L | 0-100% | Oossumi *et al*. 2006 |
| ***Fragaria ananassa*** | | | | | | | | |
| 1) AN93.231.53 | Leaf, petiole | BA, 2,4D | Cefotaxime 100 mg/L | Immersion 20-30 min in *Agrobacterium* inoculum | LBA4404 | Kan 50 mg/L | | Mezzetti *et al*. 2004 |
| 2) Amther | Leaf | 2,4D, BAP | Augmentin 375 mg/L | Immersion 20 min in *Agrobacterium* inoculum | LBA4404 | Kan 50 mg | nd | Park *et al*. 2006 |
| 3) Calypso | Leaf | TDZ, NAA | Claforan 2.5 g/L | Leaf immersed in *Agrobacterium* inoculum | EHA101 | Kan 50 mg/L | nd | Vaughan *et al*. 2006 |
| | | 2,4D, INA TDZ | Cefotaxime 250 mg/L | Leaf cut and floating in inoculum for 15-30 min. | AGLO | Kan 50 mg/L | | Lunkenbein *et al*. 2006a |
| 4) Chambly | Leaf | BA, IBA, GA | nd | 2-3 mm explants immersed 90 min in *Agrobacterium* inoculum | GV3101 | Kan 50 then 450 mg/L | nd | Houde *et al*. 2004 |
| 5) Chandler | Leaf disks | IBA, BA | Carb 500 mg/l | Immersion 20 min in *Agrobacterium* inoculum | LBA4404 | Kan 25 mg/L | 4.2% | Barcelo *et al*. 1998; Jimenez-Bermudez *et al*. 2002 |
| | Leaf | IBA, BA | Cefotaxime 250 mg/L | Bombardment of leaf with *Agrobacterium* coated into gold particle | | | 20.7% | Cordero de Mesa *et al*. 2000 |
| 6) Elista | Leaf | IBA, BA | Carb 200 mg/L | Immersion 90 min in *Agrobacterium* inoculum | LBA4404 | Kan 25 mg/L | 8-9.5% | Gruchala *et al*. 2004 |
| 7) Firework | Leaf | TDZ, IBA | Cefotaxime 250 mg/L | Immersion 30 min in *Agrobacterium* inoculum | CBE31 | Kan 50 mg/L | 11% | Schestibratov and Dolgov 2005 |
| 8) Gariguette | Young folded leaf | 2,4D, INA TDZ Glucose | Cefotaxime 250 mg/L | Leaf cut and floating in inoculum for 15-30 min. | AGLO | Kan 50-75 mg/L | nd | Schaart *et al*.. 2002 |
| 9) Hecker | Leaf and petiole | TDZ, IBA | Carb 100 mg/L Cefoxitin 100 mg/L | Immersion 30 min in *Agrobacterium* inoculum | EHA105 | Kan 50 mg/L | 10.4% | Zhao *et al*. 2004 |
| 10) Honeoye | Leaf | 2,4D, BA | Ticarcillin 250 mg/L | Immersion 24 h in *Agrobacterium* inoculum | LBA4404 | Kan 250 mg/L | | Owens *et al*. 2002 |
| 11) Induka | Leaf lamina | IBA, BA | Carb 200 mg/L | Immersion 90 min in *Agrobacterium* inoculum | LBA4404 | Kan 30 mg/L | 4.2% | Gruchala *et al*. 2004 |
| 12) Jonsok | Leaf | TDZ, IBA | Cefotaxime | Immersion 30 min in *Agrobacterium* inoculum and transfer leaf onto temporary immersion bioreactor | LBA4404 | Hygromycin 10-15 mg/l | 38% | Hanhineva and Karenlampi 2007 |
| 13) Joliette | Stipule | IBA, BAP, $GA_3$, | Carb 500 mg/L | Immersion 30 min in *Agrobacterium* inoculum | LBA4404 | Kan 50 mg/L | 0.2% | Chalavi and Tabaeizadeh 2003 |

**Table 1** (Cont.)

| Cultivars | Explants used | PGRs used for regeneration, nutrients | Antibiotics | Inoculation procedure | *Agrobacterium* strains | Selectable marker | Efficiency of transformation | References |
|---|---|---|---|---|---|---|---|---|
| **Stable transformation** | | | | | | | | |
| ***Fragaria vesca*** | | | | | | | | |
| 14) Kaster | Leaf | IBA, BA, $GA_3$, | Carb 500 mg/L | Leaf dipped in *Agrobacterium* inoculum | LBA4404 | Kan 50 mg/L | nd | Wawrzynczak *et al*. 2003, 2005 |
| 15) La Sans Rivale | Leaf and petiole | TDZ, IBA | Carb 100 mg/L Cefoxitin 100 mg/L | Immersion 30 min in *Agrobacterium* inoculum | EHA105 | Kan 50 mg/L | 7.4% | Zhao *et al*. 2004 |
| 16) LF9 | Petiol and segment | TDZ, 2,4D, BA | Timentin 200 mg/L | Immersion 60 min in *Agrobacterium* inoculum | nd | Kan 2.5-12.5 mg/L | 100% | Folta *et al*. 2006a |
| 17) Melody | Stem | | | Immersion | | | | Graham *et al*. 1995 |
| 18) Pajaro | Leaf disks | BA, 2,4D | Cefotaxime 500 mg/L | Immersion 15 min in *Agrobacterium* inoculum | LBA4404 | Kan 25 mg/L | 0.5-6.6% | Ricardo *et al*. 2003 Vellicce *et al*. 2003, 2006 |
| 19) Polka | Young folded leaf | 2,4D, INA TDZ | Cefotaxime 250 mg/L | Leaf cut and floating in inoculum for 15-30 min. | AGLO | Kan 50-75 mg/L | nd | Schaart *et al*.. 2002 |
| 20) Red Coat | Leaf disks | BA, INA | Carb 500 mg/L Cefotaxime 500 mg/L | Leaf wounded and incubated 15 min in *Agrobacterium* inoculum | MP90 | Kan 50 mg/L | 6.5% | Nehra *et al*. 1990 |
| 21) Rapella | Leaf and petiole | BA, 2,4D | Cefotaxime 100 mg/L | Immersion 20-30 min in *Agrobacterium* inoculum | LBA4404 | Kan 50 mg/L | 0.95% | James *et al*. 1990 |
| 22) Selekta | Stem | TDZ, NAA | Cefotaxime 100 mg/L | Leaf wounded and incubated 20 min in *Agrobacterium* inoculum | C58C1 | Kan 50 mg/L | 10% | du Plessis and Brand 1997 |
| 23) Symphony | Stem | NAA | | Immersion | | | | Graham *et al*. 1995 |
| 24) Toyanaka | Anthers | BA, NAA | none | Bombardment | | Phosphinothricin 10 mg/L | 14-17% | Wang *et al*. 2004 |
| **Transient expression** | | | | | | | | |
| 1) Chandler | Fruit and leaf | | none | Bombardment | | | | Agius *et al*. 2003 |
| 2) Elsanta | Fruit | | none | Agroinfiltration with an hypodermic syringe | AGLO | | | Spolaore *et al*. 2001; Hoffmann *et al*. 2006 |
| 3) Line 77101 | Protoplasts | NAA, TDZ | none | Electroporation in protoplasts | | Hygromycin 10 mg/l | | Nyman and Wallin 1992 |

other inoculation procedures have been described, such as protoplast electroporation (Nyman and Wallin 1992). The authors reported only a transient expression of the reporter gene *gus* in the cell transformed, but no regeneration of transformed lines from the transformed protoplast. Transient expression was also reported in strawberry tissues after bombardment of leaves (Aigus *et al.* 2003) or by agroinfiltration of fruit with a hypodermic syringe (Spolaore *et al.* 2001; Hoffmann *et al.* 2006). Stable transformation was also achieved via particle bombardment (de Mesa *et al*. 2000; Wang *et al.* 2004).

**Selectable marker:** Regeneration of transgenic plants is dependent upon efficient incorporation of the transgenes and the ability to grow in the presence of the selection agent. In most cases, Kanamycin (Kan) has been reported as the selection agent. Hygromycin (Hyg) (Nyman and Wallin 1992; Mathews *et al.* 1995a; Oosumi *et al.* 2006), geneticin (Gen) (Mathews *et al.* 1995a) and phosphinothricin (Ppt) (Wang *et al.* 2004) were also used as a selection agent. Strawberry explants seem to have a different sensitivity to Kan. Indeed, Owens *et al.* (2002) used 250 mg/L Kan to select transgenic plants from the octoploid strawberry 'Honeoye'. However, several reports indicated also that strawberry tissue maintains a high sensitivity to this antibiotic when its concentration is increased (El Mansouri *et al.* 1996; Haymes and Davis 1998; Alsheikh *et al.* 2002; Folta *et al.* 2006). Based on this sensitivity, selection has been generally performed with lower concentrations of Kan after co-cultivation, followed by an increased concentration in subsequent subculture (Mathews *et al.* 1995a; Alsheikh *et al.* 2002; Folta *et al.* 2006). The lower concentration of antibiotic permits a rapid growth of callus and early shoot initiation, whereas the eventual higher concentration eliminates non-transgenic shoots from survival. Increasing the concentration of Kan from 40 to 120 mg/L during subculture, compared to the use of a constant concentration of Kan limits the phenomenon of transgenic/non-transgenic chimeras (Mathews *et al.* 1998). For the cultivar, 'Chambly' Houde *et al.* (2004) recommended more extreme parameters, where the concentration of Kan starts at 50 through 450 mg/L. The opposite approach has been also reported; with Haymes and Davis (1998) initiating the regeneration with a high concentration of Kan, and after several weeks decreased the concentration of Kan to 25 mg/L.

Effective production of marker-free transgenic strawberry plants was reported using a plant-adapted inducible R recombinase gene and a bifunctional, positive/negative selectable marker to reduce the appearance of chimeras due to incomplete DNA excision (Schaart *et al.* 2004). The positive selection was provided by the neomycin phosphotransferase gene (*nptII*), whereas the negative selectable marker was the *codA*, a conditionally lethal dominant gene encoding an enzyme that converts the non-toxic 5-fluorocytosine (5-FC) to cytotoxic 5-fluorouracil (5-FU). With this procedure, 22 to 62% of the strawberry plants regenerated were markerless, depending on the timing of applying the negative selection (Schaart *et al.* 2004). However, a downside to these procedures is that in some lines the selectable marker is not excised and is still present in the plant

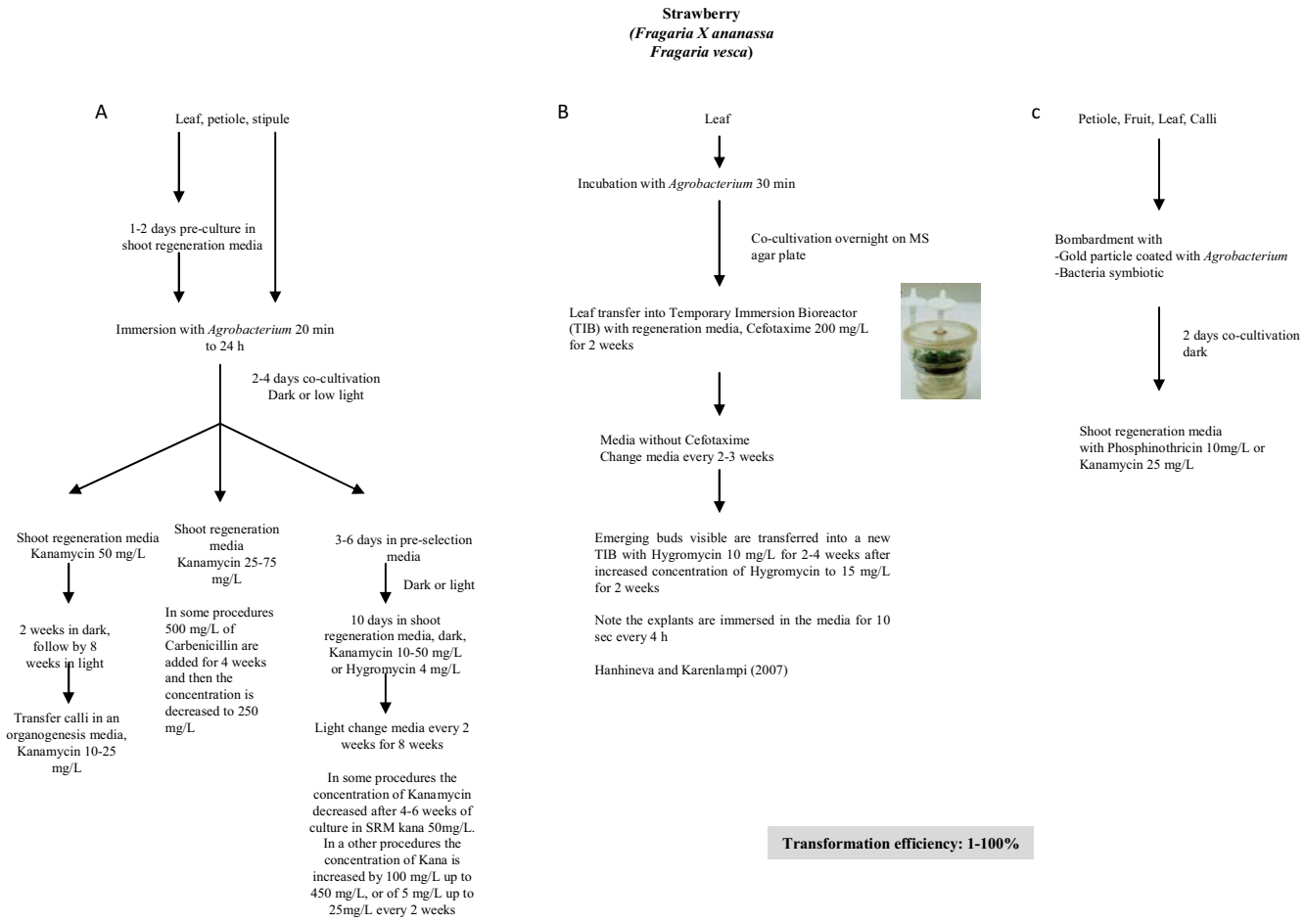


**Fig. 1 Representation of the strawberry (*Fragaria vesca*, *F. X ananassa*) transformation procedure.**

genome (Schaart *et al.* 2004; Kondart *et al.* 2006).

**Transformation efficiency:** Transformation efficiency is typically described as the percentage of explants that produce a transgenic shoot. Earlier methods of transformation reported an efficiency of transformation of 0.2 to 20% (**Table 1**). After improvement in the procedure, the rate of transformation can be as great as 100% (Folta *et al.* 2006; Oosumi *et al.* 2006).

**Other modifications:** Hanhineva and Karenlampi (2007) used the technique of temporary immersion bioreactor (TIB) as a part of regeneration process after *Agrobacterium*-mediated transformation of strawberry (**Fig. 1**). In TIB, the liquid medium is applied in intervals to the plant material which is located in a separate compartment from the medium. The advantages of TIB have been well documented, and benefits have been shown both for reducing workload and thus the cost, and for better plant performance by allowing direct contact of the medium throughout the plant material, and by renewing the culture environment after each immersion (Etienne and Berthouly 2002).

The different procedures for stable transformation of strawberry (*Agrobacterium*-mediated transformation and bombardment) are summarized in **Fig. 1**.

### *2. Application of transformation procedures for strawberry improvement*

The first report of transformed strawberry plants in 1990 raised expectations for new strawberry cultivars that would be better tasting, healthier and easier to grow. More recently the focus has moved on to functional testing with traits of potential commercial interest. Although many different traits have now been been introduced successfully into *Fragaria* (**Table 2**), no transformed cultivars have yet made it to commercial production. Most early reports on transformed strawberry described 'proof of concept' experiments. 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 (**Table 2**). Although not mentioned specifically in conjunction with a target gene, promoters linked with targeted expression patterns have been identified: *AtSUC2*, which has a restricted activity in the phloem (Zhao *et al.* 2004), a promoter of *Fragaria faRB7*, shown to be specifically induced near the root (Vaughan *et al.* 2006), and some other promoters, from *Fragaria* as well as from other crops that present a fruit specific activity (Schaart *et al.* 2002; Rosin *et al.* 2003; Agius *et al.* 2005).

Strawberry is an economically important crop but displays only a short postharvest shelf life due to its rapid softening. In addition to breeding, genetic engineering (GE) constitutes a suitable new tool for improvements to the fruit. The genetic transformations of the cultivated strawberry (*Fragaria X ananassa* Duch.) and wild strawberry (*F. vesca*) have already been reported (**Tables 1, 2**). Although they are considered to be relatively amenable to transformation using *Agrobacterium*, transformation frequencies are greatly influenced by the cultivar and the procedure used. The technique is being used successfully to genetically engineer broad spectrum fungal resistance through the use of chitinase and thaumatin II gene (Chalaci and Tabaeizadeth 2003; Schestibratov and Dolgov 2005; Vellice *et al.* 2006), soft rot resistance (Mehli *et al.* 2004), freezing tolerance (Owens *et al.* 2002; Houde *et al.* 2004), salt tolerance (Wang *et al.* 2004), dwarfing (Wawrzynczal *et al.* 2003, 2005), and the manipulation of fruit softening, aroma, and ripening (**Table 2**). The traits related to the ripening and production of strawberry were the most studied and we will describe some of the applications of GE to improve straw-

berry ripening.

Fruit softening is a complex process that involves three sequential steps: loosening of the cell wall mediated by expansins, depolymerization of hemicelluloses, and finally polyuronide depolymerization by polygalacturonase or other hydrolytic enzymes. Isolation of a pectate lyase gene

**Table 2** Traits expressed in transformed *Fragaria*.

| Trait | Cultivars | Gene introduced | Gene origin | Promoter | Principal results | Reference |
|---|---|---|---|---|---|---|
| **Fungal resistance** | | | | | | |
| Gray mold | *F. ananassa* cv Firework | *Thaumatin II* | Nd | CaMV35S | Transgenic lines tested for their resistance to gray mold showed a significant higher level of resistance compared to the control. | Schestibratov and Dolgov 2005 |
| *Botrytis cinerea* | *F. ananassa* cv Pajaro | *Chitinase ch5B* | *Phaseolus vulgaris* | CaMV35S | Two transgenic lines showed a high level of resistance to gray mold disease. | Vellicce *et al*. 2006 |
| | *F. vesca* | *Pectin methyl esterase PaPE1* | *F. ananassa* | CaMV35S | In the ripe transgenic fruit the degree and pattern of methyl esterification as well as the average size of pectin polymer differs. This reduced esterification induced constitutively pathogen defence response in the transgenic lines, resulting in higher resistance to *B. cinerea.* | Osorio *et al*. 2008 |
| *Verticillium dahliae* | *F. ananassa* cv Joliette | *Chitinase pcht28* | *Tomato* | CaMV35S | Significantly increased resistance to *V. dahlia*. | Chalavi and Tabaeizadeh 2003 |
| **Insect resistance** | | | | | | |
| *Otiorhynchus sulcatus* F. (vine weevil) | *F. ananassa* cv *Melody, Symphony* | *Cowpea trypsin inhibitor (CpTi)* | | | Field results for two years confirmed glasshouse finding and demonstrate protection in terms of attack by vine weevil larvae on all the transgenic lines. The *CpTi* has no significant effect on other non-target arthropods. | Graham *et al*. 1997, 2002 |
| **Herbicide resistance** | | | | | | |
| | *F. ananassa* cv *Camarosa* | *CP4.EPSP* | *Agrobacterium* | CaMV35S FMV.34S | 50% of the transgenic lines showed a significant increase of resistance to glyphosate in the field. | Morgan *et al*. 2002 |
| **Ripening, fruit production** | | | | | | |
| Ripening | F. ananassa cv Elsanta | *CHS* (RNai) | *Fragaria* | CaMV35S | Level of anthocyanins was down regulated and precursors of the flavonoid pathway were shunted to the phenylpropanoid pathway. | Hoffmann *et al* 2006 |
| | *F. ananassa* cv Calypso | *CHS* (antisense) | *Fragaria* | CaMV35S | 40% of the transgenic lines showed a reduced activity of CHS. As a result, the level of anthocyanins, flavonols, and proanthocyanidins were down regulated and precursors of the flavonoid pathway were shunted to the phenylpropanoid pathways. | Lunkenbein *et al*. 2006a |
| | | *UDP-glucose: cinnamate glucosyl-transferase (FaGT2)* | *Fragaria* | CaMV35S | Transgenic lines showed that the *FaGT2* gene is only involved in the formation of cinnamoyl glucose and p-coumaroyl glucose during ripening. | Lunkenbein *et al*. 2006b |
| | *F. ananassa* cv Elsanta | *Glycosyltransfera se hairpin (FaGt1)* | *Fragaria* | CaMV35S | Silencing of the *FaGt1* gene induced a reduction of the concentration of anthocyanin pigments in ripe strawberry fruits. In contrast, an increase in epiafzelechin can be observed in these lines. | Griesser *et al*. 2008 |
| Aroma | *F. ananassa* cv Calypso | *O-methyl-transferase FaOMT* (sense and antisense) | *Fragaria* | CaMV35S | FaOMT sequence in sense and antisense orientation induced a near total loss of the volatile compound 2,5-dimethyl-4-methoxy-3(2H)-furanone, whereas the levels of the other volatiles remained unchanged. Repression of FaOMT also affected the ratio of feruloyl 1-*O*-β-D-glucose and caffeoyl-*O*--β-D-glucose. | Lunkenbein *et al*. 2006c |
| Fruit production and fecundity | *F. ananassa* selection AN93.231.53 *F vesca* cv Alpina W. Original | *DefH9-iaam* | Snapdragon and *Pseudomonas syringae* pv *savastanoi* | nd | Transgenic plants showed an increased number of flowers per inflorescence and an increased number of inflorescences per plant. Resulting in an increased number of fruits per plant and of their weight and size. Yield increased by 180% for *F.ananassa* and 140% for *F. vesca*. | Mezzetti *et al*. 2004 |
| Fruit softening | F. ananassa cv Chandler | *Pectate lyase* (antisense) | *Fragaria* | CaMV35S | Most of the transgenic lines at the stage of full ripening display significantly firmer tissue than the control. However, no differences in color, size, shape and weight were observed. | Jimenez-Bermudez *et al*. 2002 |
| | | | | | Jam medium prepared with transgenic strawberries showed a significant reduction in pectate lyase expression, and are similar in firmness but slightly less viscous than control. The texture and content of whole berries increased in the transgenic jam. | Sesmero *et al*. 2007 |
| | | | | | Wall extracts of the transgenic lines showed a reduction in pectin solubility and decreased depolymerization of more tightly bound polyuronides. | Santiago-Domenech *et al*. 2008 |
| | *F. ananassa* cv Calypso | Endo-β-(1-4)glucanases *Cel1* (antisense) *Cel2* (antisense) | *Fragaria* | CaMV35S | Although the *cel1* was down regulated in the transgenic plants, no appreciable effect of this gene activity on fruit firmness was observed. | Woolley *et al*. 2001 |
| | | Endo-β-(1-4)glucanases *Cel1* (antisense) *Cel2* (antisense) *Cel1/cel2* (antisense) | *Fragaria* | FBP7 | *Cel1* gene is not the major determinant of fruit softening. *Cel2* might play a pivotal role on fruit development prior to ripening. | Palomer *et al*. 2006 |
| Cell elongation during ripening | *F. ananassa* cv Chandler | *FaGAST* | *Fragaria* | CaMV35S | Expression of FaGAST in transgenic strawberry, grown under field conditions, caused delayed growth of the plant and fruits with reduced size. Transgenic plants also exhibited late flowering and low sensitivity to exogenous gibberellin. | de la Fuente *et al*. 2006 |

**Table 2** (Cont.)

| Trait | Cultivars | Gene introduced | Gene origin | Promoter | Principal results | Reference |
|---|---|---|---|---|---|---|
| **Environmental stress resistance** | | | | | | |
| Freezing tolerance | *F. ananassa* cv Honeoye | *CFB1* | Cherry | CaMV35S | Leaves of transgenic lines were more tolerant to freezing temperatures (-8/ -10°C) compared to the control (-6°C). | Owens *et al*. 2002 |
| | *F. ananassa* cv Chambly | *Acedic dehydrin WCOR410* | Wheat | | Overexpression of this gene had no apparent deleterious effect on the growth and development of the transgenic plants under both non-acclimated and cold-acclimated conditions. Freezing tests showed an improvement of 5°C in the cold-acclimated transgenic strawberry leaves. | Houde *et al*. 2004 |
| Salt tolerance | *F. ananassa* cv Toyonaka | *Late embryogenesis abundant (Lea3)* | Barley | ActI | The transgenic lines showed an increased tolerance to salt (50 and 100 mM) and the development of damaged symptoms (wilting) caused by salts stress was delayed. | Wang *et al*. 2004 |
| | *F. ananassa* cv Chandler | *osmotin* | | CaMV35S | The transgenic lines shaowed an increased of tolerance to salt stress. These transgenic plants revealed enhanced levels of proline, total soluble protein and chlorophyll content compared to the wild plants. | Husaini and Abdin 2008 |
| **Rooting ability / Dwarfism to rootstock** | | | | | | |
| | *F. ananassa* cv Kaster | *iaglu* | Maize | CaMV35S | Transgenic lines were dwarfish, their leaf laminas smaller, leaf petioles shorter and the crown diameter smaller compared to control. Shoots of all transgenic lines formed more roots. | Wawrzynczak *et al*. 2003, 2005 |
| **Modified metabolism** | | | | | | |
| Sugar composition | *F. ananassa* cv Anther | *ADP-glucose pyrophosphorylase* (antisense) | *Fragaria* | Ascorbate peroxydase | Transgenic fruit showed a decrease in starch and soluble sugar content by 27-47% and 16-37% respectively as compared to the control. | Park *et al*. 2006 |
| **Promoter** | | | | | | |
| | *F. ananassa* cv Chandler | | *Cauliflower mosaic virus* | CaMV35S | CaMV35s promoter is highly expressed in transgenic strawberry pollen. | Cordero *et al*. 2004 |
| Gene expressed in fruit specific tissues | *F. ananassa* cv Chandler | *Luciferase* | *Fragaria* | GalUR | GalUR promoter has an activity restricted to the fruit and is strictly dependent on light. | Agius *et al*. 2005 |
| | *F. ananassa* cv Gariguette and Polka | | *Cauliflower mosaic virus Petunia* | CaMV35S Floral binding protein 7 (FBP7) | FBP7 promoter leads Gus activity in floral and fruit tissues of all developmental stages tested but not in leaf, petiole and root tissue. | Schaart *et al*. 2004 |
| Gene expressed in phloem specific tissues | *F. ananassa* Hecker and La Sans Rivale *F. vesca* cv Alpine | | *Arabidopsis thaliana* | Sucrose H+ symporter (AtSUC2) | AtSUC2 promoter has an activity restricted to the phloem of leaves, petioles and roots in the three strawberry cultivars transformed. | Zhao *et al*. 2004 |
| Gene expressed in root specific tissues | *F. ananassa* cv Calypso | | *Fragaria* | FaRB7 | FaRB7 promoter has a strong constitutive near root-specific activity. | Vaughan *et al*. 2006 |
| | *F. ananassa* cv Chandler | | *Fragaria* | FaEG1 FaEG3 | Transient expression of these two promoters in fruit showed different behaviors in the two endo-β-(1-4)glucanases promoters. | Spolaore *et al*. 2003 |
| | *F. ananassa* cv Calypso | | *Fragaria* | STAG1 | STAG1 promoter shown to be active during the floral development (stamens, base of the receptacles, petals, central pith and vascular tissue) and ripening stage (achenes, pith cells and cortical cells). | Rosin *et al*. 2003 |
| **Selectable marker** | | | | | | |
| Excision by recombination | *F. ananassa* cv Calypso | *Cre-lox* system | | | Antibiotic resistance marker gene free transgenic calypso strawberry lines have been produced with an efficiency of 22%. | Schaart *et al*. 2004 |
| Intracellular symbiosis | *F. ananassa* cv Fertodi | Bombardment of the living nitrogen-fixing bacteria *Azotobacter vinelandii*. | | | Presence of the symbiotic nitrogen-fixing bacteria in the transformed strawberry lines after regeneration. | Preiringer *et al*. 2003 |

from ripe strawberry has been proposed as a new candidate for pectin degradation, contributing to the loss of fruit firmness. The reduction of the steady-state levels of pectate lyase mRNA, by antisense technology, resulted in a great increase in firmness of fully ripe fruit and reduced postharvest softening, without affecting other fruit characteristics such as weight, color, or soluble solids (Jimenez-Bermudez *et al.* 2002). The evaluation of the effect of these transformed lines on the texture of frozen and thawed fruits and the jam obtained from them was recently done by Sesmoro *et al.* (2007). Two independent transgenic lines showing a reduction in pectate lyase mRNA transcript level of 90 and 99% have been grown in the greenhouse. At harvest time, ripened fruits from these two lines were significantly firmer than the control (Sesmoro *et al.* 2007). Control and transformed fruits were processed into jam. It appeared that transformed fruit resisted the cooking process better than the control, as reflected by the higher weight of berries in these jams (Sesmoro *et al.* 2007). The silencing of pectate lyase gene in strawberry fruit improves several quality parameters in jam. However, the effect of pectin modification in these transformed fruits on nutritional and sensory attributes need to be analyzed. These studies can be viewed from commercial and consumer points of view but they also contribute highly to our understanding of the ripening processes in strawberry. Some work is still needed before commercial applications can be realized. Others do not have such an evident potential for application, but merit mention as they contribute to basic knowledge. Salentijn *et al* (2003) studied differential expression of genes involved in fruit firmness. Lunkenbein *et al.* (2006a, 2006b, 2006c) were able to demonstrate the role of several genes (*CHS*, *O-methyltransferase*, and *UDP-glucose:cinnamate glucosyltransferase*) in the ripening of strawberry (**Table 2**).

The downregulation of an individual gene by *Agrobacterium*-mediated genetic transformation has provided some clues to its function in specific ripening pathways (Jimenez-Bermudez *et al.* 2002; Lunkenbein *et al.* 2006b; Palomer *et al*. 2006). However, these traditional ways of studying the functions of various genes, especially ripening related genes

in strawberry, are time consuming, and can take at least 15 months from the transformation experiment until the first ripe fruits are available for analyses (Hoffmann *et al.* 2006). Spolaore *et al.* (2001) have shown strawberry, apple, and peach fruit to be amenable to transient gene expression mediated by *Agrobacterium* using the β-glucuronidase (GUS) reporter gene interrupted by an intron. This system, combined with a biolistic transient transformation protocol, has also been used for the functional analysis of homologous and heterologous promoters in strawberry fruits (Agius *et al.* 2005). Using a similar technique based on injection with a syringe into the receptacles of growing fruit, an inoculum of *Agrobactrerium* carrying a T-DNA expressing an RNAi CHS construct, Hoffmann *et al.* (2006) were able to show a reduction in mRNA levels and enzymatic activity of the *CHS* gene. The use of this technique in combination with metabolite profiling analysis will be useful in future for studying the function of unknown genes during the development and ripening of strawberry fruit.

## *Malus*

### *1. Regeneration and transformation*

Development of an effective system for gene transfer in apple depends largely on the availability of tissue culture techniques that permit efficient regeneration of shoots, selection of transformants, and propagation of transgenic plants. Increasing leaf regeneration efficiency is critical for the development of a transformation system in apple using an *A. tumefaciens* vector (Klee and Rogers 1989) or by a biolistic process.

The first report of *in vitro* regeneration of adventitious shoots from apple was made by Liu *et al.* (1983a, 1983b) using apple seedlings. Several later reports (Barbeieri and Morini 1987; Welander 1988; Fasolo *et al.* 1989; Sriskandarajah *et al.* 1990; Swart *et al.* 1990; Korban *et al.* 1992; James *et al.* 1994) revealed critical factors affecting the frequency of leaf regeneration in apple scion cultivars, rootstocks, and seed explants. They include nitrogen source and concentration, PGRs, incubation conditions, leaf origin, leaf maturity and position on the stem, mode of excision and explants orientation.

**Genetic lines:** *Malus X domestica* cv 'Greensleeves' (James *et al.* 1989) was the first apple cultivar to be transformed, initially with an efficiency of 0.1-0.5% on a per explant basis. The genus *Malus* is comprised of over 40 species. Studies have been centered on development of transgenic procedures for different *M. X domestica* cultivars (30) and rootstocks (7), *M. prunifolia* (2) and one *M. robusta* cultivar (**Table 3**). However, the efficiency of transformation and regeneration capacity is highly dependent on the genetic background (De Bondt *et al.* 1994; Norelli *et al.* 1996).

**Nature of explants:** In a large majority of the procedures published, young expanded apple leaves were used as explants (**Table 3**). The regeneration of transgenic lines from leaves has been enhanced by placing the leaf segment abaxial side up on the medium, possibly due to increased oxygen exchange since stomata are located abaxially (Yepes and Aldwinckle 1994). Sriskandarajah and Goodwin (1998) reported that conditioning of apple shoots for several days in an appropriate liquid medium enhances the regenerative capacity of leaf explants by reducing the need for an extended callus phase. Hovewer, this conditioning process for explants has not been subsequently reported in transformation protocols. Other procedures reported use of internodal (Liu *et al.* 1998, 2001), shoot (Pawlicki-Jullian *et al.* 2002) and stem explants (Yamashita *et al.* 2004), and protoplasts (Hyung *et al.* 1995; Ratnasiri *et al.* 2002). The regeneration of plants from protoplasts is a lengthy process compared with standard tissue transformation. Therefore, the transformation of protoplasts is unlikely to be a source of new transgenic plant material, but the system, if perfected, would be useful as a high-throughput approach for transient assay of gene function or protein targeting.

**Plant growth regulators:** The regeneration medium that has generally produced the greatest shoot regeneration is the MS medium (1962) supplemented with 2-3% sucrose or sorbitol, and standard vitamin complements. The major difference from procedure to procedure is in the combination and concentration of phytohormones (auxin and cytokinin) used to induce callus and adventitious shoots.

The predominant form of cytokinin used in most of the *Malus* regeneration and transformation procedures is TDZ at different concentrations (**Table 3**). In a few reports, the use of BA was also reported (**Table 3**). The type and concentration of auxin is quite variable between procedures (**Table 3**). For the regeneration of *Malus* NAA was used in most of the regeneration protocols, although IBA, and IAA have been used in the regeneration of a few cultivars (**Table 3**).

**Antibiotics:** During the process of transformation, *A. tumefaciens* tends to colonize the explants. To limit the development of this bacterium, explant tissues are commonly regenerated on media containing an antibiotic. Three antibiotics have been used at different concentrations to control the proliferation of *A. tumefaciens* in apple: Carb, Cef and Tic Cef has been the antibiotic most commonly used in apple with a range of concentrations from 200 to 500 mg/L, followed by Tic and Carb (**Table 3**). Other antibiotics used include Claforan (Kotoda *et al.* 2006) and Cefoxitin (Liu *et al.* 1998).

**Inoculation procedure and *Agrobacterium* strain:** While many apple cultivars were transformed with different genes, most of the procedures (95%) relied on *Agrobacterium* mediated transformation gene transfer, and are quite similar (**Table 3, Fig. 2**), the remaining 5% of the transformation procedures used *A. rhizogenes* for the T-DNA integration (Pawlicki-Jullian *et al.* 2002; Yamashita *et al.* 2004). Due to the fact that all genotypes do not respond similarly to the same procedure of transformation, some modifications have been reported. The most common strain of *Agrobacterium,* used in half of the transformation protocols, was EHA105 or 101. Other strains used included LBA4404, AGLO, C58C1, CBE21, and GV3101.

Two inoculation procedures have been used. The first consists of wounding the leaves with a scalpel soaked in the inoculum (Wilson and James 2003). The second is to bathe pre-wounded leaves in the inoculum for a few minutes (De Bondt *et al.* 1994; Norelli *et al.* 1994) to several hours (Mooney and Goodwin 1998). Other modes of inoculation have been tested but did not significantly increase the rate of transformation. For example, sonication of the leaf explants in the presence of inoculum has been reported to increase transformation of the apple cultivar 'Holsteiner' but not of 'Elstar' (Szankowski *et al.* 2001). Vacuum infiltration of 'Royal Gala' leaves was reported to have no effect on transformation (Norelli *et al.* 1996).

**Selectable marker:** Regeneration of transgenic plants depends upon efficient incorporation of the transgenes and the ability to grow in the presence of the selection agent. In most cases (90%) Kan resistance was used as selectable marker. Concentration of Kan used for the regeneration of transgenic apple depended on the cultivar. Two other antibiotics have been used as selection agents: Hyg (Dolgov *et al.* 2000, 2004) and Ppt (Szankowski *et al.* 2003). Explants from different apple cultivars seem to have different sensitivities to Kan. Indeed, depending upon the cultivar, the optimal concentration of Kan to use for selection varied from 25 to 100 mg/L.

Based on the sensitivity of apple cultivars, selection has been performed for some apple cultivars with a low concentration of Kan after co-cultivation, followed by an increase in concentration in subsequent subcultures (Welander *et al.*

**Table 3** Apple varieties transformed genetically.

| Cultivars | Explants used | Hormones used for regeneration, nutrients and sugar | Antibiotic | Inoculation procedure | *Agrobacterium* strains | Selectable marker | Efficiency of transformation | References |
|---|---|---|---|---|---|---|---|---|
| ***Malus X domestica*** | | | | | | | | |
| 1) Ariane | Leaf | TDZ, IBA<br>MS with sucrose | Cefotaxime<br>350 mg/L | Leaf wounded by crushing with non-traumatic forceps and immersed 5 min in inoculums. | EHA105 | Kan 100 mg/L | 2% | Faize *et al*. 2003, 2004 |
| 2) Braeburn | Leaf | IBA, TDZ, 2iP, $GA_3$<br>MS with sucrose | Ticarcillin<br>400 mg/L | Leaf cut 3 times perpendicularly to the midrib and shaken 1 min in inoculum. | EH101 | Kan 50 mg/L | 1.2% | DeBondt *et al*. 1994, 1996 |
| | Protoplast | | | Protoplast transformation was via a PEG mediated protocol. | none | none | 0-7.4% GFP protoplast transformant | Ratnasiri *et al*. 2002 |
| 3) Delicious Red | Leaf | BAP<br>MS with sucrose | Cefotaxime<br>500 mg/L | Leaf cut transversaly into strips (2-3 mm) and transferred to inoculum for 36 h. | C58C1/pGV3850 | Kan 100 mg/L | 1-2% | Mooney and Goodwin 1988 |
| Delicious | | NAA, IAA TDZ<br>MS with sucrose | Cefotaxime<br>200 mg/L | Leaf explants shaken for 20 min 75 rpm in the inoculum at 35°C. | nd | Kan 100 mg/L | 1-2% | Sriskandarajah *et al*. 1994, 1998 |
| | | TDZ, BA, NAA, IBA<br>MS with sucrose for 1st regeneration media and with sorbitol for the 2nd regeneration medium | Cefotaxime<br>400 mg/L | Leaf cut 3 times perpendicularly to the midrib and shaken in inoculum. | EHA101 | Kan 50 mg/L | Cell transformed<br>No plants regenerated | Maximova *et al*. 1998 |
| 4) Elstar | Leaf | IBA, TDZ, 2iP, $GA_3$<br>MS with sucrose | Ticarcillin<br>400 mg/L | Leaf cut 3 times perpendicularly to the midrib and shaken 1 min in inoculum. | EH101 | Kan 50 mg/L | 1.2% | DeBondt *et al*. 1994, 1996 |
| | | NAA, TDZ<br>MS with sorbitol | Cefotaxime<br>250 mg/L | Leaf immersed in inoculum for 4-6 min. | AGLO | Kan 50 mg/L | 0.4-0.8% | Schaart *et al*. 1995; Puite and Schaart 1996 |
| | | TDZ, NAA<br>MS with sorbitol | Ticarcillin<br>150 mg/L | Leaf gently shaken in inoculum for 10 min. | EHA105 | Phosphonothricin 1 then 10 mg/L | 0.17% | Szankowski *et al*. 2003 |
| 5) Falstaff | Leaf | IBA, TDZ, BAP 2iP, $GA_3$<br>MS with sorbitol | Cefotaxime<br>200 mg/L | Leaf discs immersed 20-30 min in inoculum. | LBA4044 | Kan | 7.5%<br>3.5% | Wilson and James 1998 |
| 6) Florina | Leaf | BA, NAA<br>MS with sucrose | Cefotaxime<br>300 mg/L | Leaf explants dipped in inoculum for 5 min. | C58C1 | Kan 100 mg/L | 7.9% | Radchuck and Korkhovoy 2005 |
| 7) Fuji | Leaf | IBA, TDZ, 2iP, $GA_3$<br>MS with sucrose | Ticarcillin<br>400 mg/L | Leaf cut 3 times perpendicularly to the midrib and shaken 1 min in inoculum. | EH101 | Kan 50 mg/L | 1.2% | DeBondt *et al*. 1994, 1996 |
| | | TDZ, NAA<br>MS with sucrose | Carbe<br>250 mg/L | Leaf wounded. | LBA4404 | Kan 25 mg/L then 50 mg/L | 1.1% | Murata *et al*. 2001 |
| | | TDZ, IBA<br>MS with sorbitol | Cefotaxime<br>250 mg/L | Nd | LBA4404 | Kan 100 mg/L | 1.4-6.5% | Seong *et al*. 2005 |
| | Protoplast | | | Protoplast electroporation. | none | none | nd | Hyung *et al*. 1995 |
| 8) Gala<br>Galaxy<br>Royal Gala | Leaf | IBA, TDZ, BAP 2iP, $Ga_3$<br>MS with sorbitol | Ticarcillin<br>400 mg/L | Leaf cut 3 times perpendicularly to the midrib and shaken 1 min in inoculum. | EH101 | Kan 50 mg/L | 1.2% | DeBondt *et al*. 1994, 1996 |
| | | TDZ, IBA<br>N6 + MS with sucrose | Cefotaxime<br>350 mg/L | Leaf wounded by crushing with non traumatic forceps and immersed 5 min in inoculum. | EHA105 | Kan 50 mg/L | 5-20% | Norelli *et al*. 1999; Ko *et al*. 2000 |
| | | NAA, TDZ<br>MS with sorbitol | Cefotaxime<br>250 mg/L | Leaf immersed in inoculum for 4-6 min<br>Leaf cut and floating in inoculum for 15-30 min. | AGLO | Kan 50 mg/L | 0.7-0.9%<br>0.7-8.7% | Schaart *et al*. 1995; Puite and Schaart 1996 |
| | | BAP, NAA, IBA, TDZ<br>MS with sucrose | Cefotaxime | Leaf cut transversally into strips (2-3 mm) and transferred to inoculum for 5 min. | LBA4404 | Kan 50 mg/L | 0.4-4.6% | Yao *et al*. 1995 |
| | | TDZ, IBA<br>MS with sucrose | Cefotaxime<br>350 mg/ | Leaf wounded by crushing with non-traumatic forceps and immersed 5 min in inoculum. | EHA105 | Kan 100 mg/L | 1-1.4% | Faize *et al*. 2003, 2004 |
| | Internodal explants | TDZ, NAA<br>N6 with sucrose | Carbe<br>100 mg/L<br>Cefoxitin<br>100 mg/L | Internodal explant from etiolated *in vitro* shoot was dipped in inoculum. | EHA105 | Kan 100 mg/L | 1.7% | Liu *et al*. 1998, 2001 |
| 9) Golden Delicious | Leaf | NAA, TDZ<br>MS with sorbitol | Cefotaxime<br>250 mg/L | Leaf immersed in inoculum for 4-6 min<br>Leaf cut and floating in inoculum for 15-30 min. | AGLO | Kan 50 mg/L | 0.2-6%<br>1.6% | Schaart *et al*. 1995<br>Puite and Schaart 1996 |
| | | | Cefotaxime<br>400 g/L | Leaf cut 3 times perpendicularly to the midrib and shaken in inoculum. | EHA101 | Kan 50 mg/L | Cell transformed<br>No plants regenerated | Maximova *et al*. 1998 |

**Table 3** (Cont.)

| Cultivars | Explants used | Hormones used for regeneration, nutrients and sugar | Antibiotic | Inoculation procedure | *Agrobacterium* strains | Selectable marker | Efficiency of transformation | References |
|---|---|---|---|---|---|---|---|---|
| ***Malus X domestica*** | | | | | | | | |
| 10) Greensleeves | Leaf | BA, NAA MS with sorbitol and sucrose | Cefotaxime 200 mg/L | Leaf discs immersed 20-30 min in inoculum. | LBA4404 C58C1/ pGV385 | Kan 25 mg/L | 0.1-0.5% | James *et al*. 1989, 1993 |
| | | | Cefotaxime 400 g/L | Leaf cut 3 times perpendicularly to the midrib and shaken in inoculum. | EHA101 | Kan 50 mg/L | Cell transformed No plants regenerated | Maximova *et al*. 1998 |
| | | BAP, NAA, TDZ MS with sorbitol and sucrose | Cefotaxime 200 mg/L | Leaf disc immerged 20-30 min in inoculum. | EH101 | Kan 25 mg/L | nd | Gittins *et al*. 2000, 2001, 2003 |
| 11) Holsteiner cox | Leaf | TDZ, IBA MS with sorbitol | Ticarcillin 150 mg/L | Leaf gently shaken in inoculum for 10 min. | EHA105 | Phosphonothric in 1 then 10 mg/L | 0.5–2.68% | Szankowski *et al*. 2003 |
| 12) Jonagold | Leaf | IBA, TDZ, 2iP, $Ga_3$ MS with sucrose | Ticarcillin 400 mg/L | Leaf cut 3 times perpendicularly to the midrib and shaken 1 min in inoculum. | EH101 | Kan 50 mg/L | nd | DeBondt *et al*. 1994 |
| | | BAP, NAA, zeatin MS with sorbitol | Cefotaxime 300 g/L | Leaf strips of 3-4 mm were immersed for 10-15 min in inoculum. | EH101 | Kan 50 mg/L | nd | Viss *et al*. 2003 |
| 13) Jonagold king | Leaf | IBA, TDZ, 2iP, $Ga_3$ MS with sucrose | Ticarcillin 400 mg/L | Leaf cut 3 times perpendicularly to the midrib and shaken 1 min in inoculum. | EH101 | Kan 50 mg/L | nd | DeBondt *et al*. 1994 |
| 14) Jonagored | | | | | | | | |
| 15) Liberty | Leaf | TDZ, NAA N6 + MS with sucrose | Cefotaxime 350 mg/L | Leaf wounded by crushing with non-traumatic forceps and immersed 5 min in inoculum. | EHA105 | Kan 100 mg/L | 0.03-0.4% | Hanke *et al*. 2000 |
| 16) McIntosh Wijcik | Leaf | TDZ, NAA N6 + MS with sucrose | Cefotaxime 350 mg/L | Leaf wounded by crushing with non-traumatic forceps and immersed 5 min in inoculum. | EHA105 | Kan 50 mg/L | 5-20% | Norelli *et al*. 1996 |
| | | IBA, TDZ, BAP 2iP, $Ga_3$ MS with sorbitol | Cefotaxime 200 mg/L | Leaf discs immersed 20-30 min in inoculum. | LBA4044 | Kan | 3.5% | Wilson and James 1998 |
| | | NAA, TDZ N6 with sucrose | Cefotaxime 350 mg/L | Leaf wounded by crushing with non-traumatic forceps and immersed 5 min in inoculum. | EHA105 | Kan 25 mg/L | 2.6-3.1% | Bolar *et al*. 1999 |
| 17) Melba | | NAA, TIBA MS sucrose | Cefotaxime 500 mg/L | Leaf pieces 1cm long immersed in inoculum. | CBE21 | Kan 35 then 50 mg/L | 13.3% | Dolgov *et al*. 2000 |
| 18) Merlijn | Leaf | IBA, TDZ, 2iP, $Ga_3$ MS with sucrose | Ticarcillin 400 mg/L | Leaf cut 3 times perpendicularly to the midrib and shaken 1 min in inoculum. | EH101 | Kan 50 mg/L | nd | DeBondt *et al*. 1994 |
| 19) Orin | Leaf | TDZ, NAA MS with sucrose | Carbe 250 mg/L | Leaf wounded. | LBA4044 | Kan 25 then 50 mg/L | 0.1% | Murata *et al*. 2000 |
| | | TDZ, NAA MS sucrose | Cefotaxime 500 mg/L | Leaf wounded. | LBA4004 | Kan 50 mg/ | 0.5% | Kanamaru *et al*. 2004 |
| | | TDZ, NAA MS with sucrose | Clofaran 500 mg/L | Leaf immersed in inoculun for 30min. | EHA101 | Kan 25 mg/L | 0.25% | Kotoda *et al*. 2006 |
| 20) Pink Lady | Leaf | NAA, TDZ MS with sucrose and glucose | Cefotaxime 200 mg/L | Leaf explants shaken for 20 min 75 rpm in inoculum at 35C. | nd | Kan 100 mg/L | 1-2% | Sriskandarajah and Goodwin 1998 |
| 21) Pinova | Leaf | BAP, NAA N6 + MS with sucrose | Cefotaxime 350 mg/L | Leaf wounded by crushing with non-traumatic forceps and immersed 5 min in inoculum. | EHA105 | Kan 100 mg/L | 0.03-0.4% | Hanke *et al*. 2000 |
| 22) Pilot | | | | | | | | |
| 23) Pirol | | | | | | | | |
| 24) Pingo | | | | | | | | |
| 25) Pi-AU 56-83 | | | | | | | | |
| 26) Queen Cox | Leaf | NAA, TDZ DKW with sorbitol | Cefotaxime 200 mg/L | Leaf scored transversely 3-8 times with scapel dipped in inoculum. | EHA101 | Kan 50 then 75 mg/L | 0.5-2.2% | Wilson and James 2003 |
| 27) Remo | Leaf | BAP, NAA N6 + MS with sucrose | Cefotaxime 350 mg/L | Leaf wounded by crushing with non-traumatic forceps and immersed 5 min in inoculum. | EHA105 | Kan 100 mg/L | 0.03-0.4% | Hanke *et al*. 2000 |
| 30) Reka | Leaf | BAP, NAA N6 + MS with sucrose | Cefotaxime 350 mg/L | Leaf wounded by crushing with non-traumatic forceps and immersed 5 min in inoculum. | EHA105 | Kan 100 mg/L | 0.03-0.4% | Hanke *et al*. 2000 |
| **Rootstocks** | | | | | | | | |
| 1) A2 | Leaf | NAA, TDZ MS with sucrose | Cefotaxime 500 mg/L | Leaf gently crushed by forceps and incubated in the inoculum for 20 min. | GV31101 | Kan 50 mg/L | 0.33% | Zhu *et al*. 2001a |
| 2) JET-H | Leaf | TDZ, IAA N6 with sucrose | | Leaf immersed in inoculum for 15 min. | LBA4404 | Kan 50 mg/L | nd | Sule *et al*. 2002 |
| 3) Jork 9 | Leaf | NAA, TDZ MS with sorbitol | Cefotaxime 500 mg/L | Leaf wounded with a scalpel and gently shaken in inoculum for 20 min. | EHA101 C58C1 | Kan 50 mg/L | 6.5% 3.3% | Sedira *et al*. 2001, 2005 |
| | Shoot | TDZ, NAA MS with sorbitol | Cefotaxime 500 mg/L | Base of 5 week old *in vitro* shoots were dipped in inoculum. | A. *rhizogenes* C58C1 | Kan 50 mg/L | nd | Pawlicki-Jullian *et al*. 2002 |

**Table 3** (Cont.)

| Cultivars | Explants used | Hormones used for regeneration, nutrients and sugar | Antibiotic | Inoculation procedure | *Agrobacterium* strains | Selectable marker | Efficiency of transformation | References |
|---|---|---|---|---|---|---|---|---|
| **Rootstocks** | | | | | | | | |
| 4) M.7 | Leaf | BAP, NAA N6 + MS with sucrose | Cefotaxime 350 mg/L | Leaf wounded by crushing with non-traumatic forceps and immersed 5 min in inoculum. | EHA105 | Kan 50 mg/L | 5-20% | Norelli *et al.* 1996 |
| 5) M.26 | Leaf | BAP, NAA N6 + MS with sucrose | Cefotaxime 350 mg/L | Leaf wounded by crushing with non traumatic forceps and immersed 5 min in inoculum.. | LBA4404 | Kan 50 mg/L | 15-80% | Norelli *et al.* 1994 Aldwinckle pers. comm. |
| | Leaf | BA, NAA MS with sucrose | Cefotaxime 500 mg/L | Leaf wounded with a scalpel and gently shaken in inoculum for 5 min. | C58C1 | Kan 25 then 50 mg/L | 2.5-7.1% | Welander *et al.* 1998 |
| | Leaf | TDZ, NAA MS with sucrose | Cefotaxime 250 mg/L | Leaf wounded and infected with inoculum. | GV3101 | Kan 50 mg/L | 0.9-3.9% | Holefors *et al.* 1998, 2000 |
| 6) M9/29 | Leaf | NAA, TDZ MS with sorbitol | Cefotaxime 250 mg/L | Leaf wounded and infected with inoculum. | C58C1 EHA 101 | Kan 50 mg/L | 0.02% 0.06% | Zhu *et al.* 2001b |
| 7) N545 | Leaf | TDZ, NAA, IBA MS with sucrose | Cefotaxime 500 mg/L | Leaf pieces of 1cm immersed in inoculum. | CBE21 | Kan 35 then 50 mg/L | 1.5-7.2% | Dolgov *et al.* 2000, 2004 |
| | | | | | EHA105 | Hygromycin 5 mg/L | 8.3% | |
| ***Malus prunifolia*** | | | | | | | | |
| Maruba-kaidou | Leaf | TDZ, ABA, IAA MS with sucrose | Carbe 300 mg/L | Leaf soaked in inoculum | EHA101 | Hygromycin 10 mg/L/ | nd | Igarashi *et al.* 2002 |
| Ringo Asami | Stem | BAP, IBA, $GA_3$ MS | | Stem infected with a needle dipped in inoculum. | A. *rhizogenes* MAFF-02-10265 | | 2% | Yamashita *et al.* 2004 |
| ***Malus robusta*** **Rehd.** | | | | | | | | |
| Baleng-haitang | Leaf | BA, NAA MS with sucrose | Carbe 300 mg/L | Leaf cut in 3 pieces and soaked in inoculum for 5 min. | LBA4404 | Kan 50 mg/L | 1.2% | Qu *et al.* 2005 |
| ***Malus micromalus*** **Makino** | | | | | | | | |
| | Leaf | BA, NAA MS with sucrose | Carbe 300 mg/L | Leaf segments soaked in inoculum for 5 min. | LBA4404 | Kan 50 mg/L | nd | Zhang *et al.* 2006 |
| ***Malus siervesii TNR 31-35 neidzwetzkyana*** | | | | | | | | |
| | Leaf | $GA_3$, IBA MS with sucrosel | Ticarcillin 150 mg/L | Leaf soaked in inoculum | EHA105 | Glufosinate ammonium increasing concentration | 0.3% | Szankowski *et al.* 2009 |

1998; Murata *et al.* 2000, 2001; Wilson and James 2003; Dolgov *et al.* 2004). The lower concentration of antibiotic permits a rapid growth of callus and early shoot initiation, while the eventual higher concentration eliminates non-transgenic shoots. The same procedure has been used with the Ppt herbicide by Szankowski *et al.* (2003).

Alternatively, transgenic apple can be produced by the use of marker genes that do not rely on antibiotic or herbicide resistance but instead promote regeneration after transformation. Examples are phosphomannose isomerase (*pmi*; Flachowsky *et al.* 2004; Zhu *et al.* 2004; Degenhardt *et al.* 2006; Szankowski and Degenhardt 2007), and Vr-ERE (Chevreau *et al.* 2007) (**Table 4**). Only Degenhardt *et al.* (2006) reported the regeneration of transgenic apple lines using the *pmi* gene as selectable marker with a rate of transformation from 1 to 24%. The other reports on *pmi* showed expression of the reporter gene in the transformed leaves, but no regeneration into plants was reported. Effective production of marker-free transgenic apple plants was also reported using a plant-adapted inducible R recombinase gene and a bifunctional, positive/negative selectable marker to reduce the appearance of chimeras due to incomplete DNA excision (Schaart *et al.* 2004). The positive selection was provided by *nptII* whereas the negative selectable marker was the *codA*, a conditionally lethal dominant gene encoding an enzyme that converts the non-toxic 5-FC to cytotoxic 5-FU. However, no efficiency of transformation was reported for the apple cultivar 'Elstar', and a downside to these procedures is that in some lines the selectable marker is not excised and is still present in the plant genome (Schaart *et al.* 2004; Kondrat *et al.* 2006).

Other methods based on the transformation of apple without any selectable marker have been reported (Malnoy *et al.* 2007b). In this case GE apples are produced by *Agrobacterium* inculation followed by regeneration of shoots without the use of a selection agent. This produces many plants, some of which are non-transgenic (*ca.* 70% non-transgenic depending on the cultivar). However, depending on the regeneration and gene transfer frequencies, some plantlets are transgenic and can be identified by PCR screening. A prerequisite is a regeneration/transformation protocol of high efficiency. So far, this method has been limited to model plants and few specific crop cultivars, e.g., in potato. Due to the very high efficiency of transformation of the apple genotypes 'Galaxy' and 'M.26', Malnoy *et al.* (2007b) have been developing this technique in apple. They reported regeneration via this procedure for both genotypes with an efficiency of transformation (% of regenerants that are transgenic) close to 12% for 'Galaxy' and 25% for 'M.26'. Recently, the technique has been extended successfully to other apple cultivars, including Fuji (H. S. Aldwinckle, pers. comm.).

**Transformation efficiency:** Transformation efficiency is typically described as the percentage of explants that produce a transgenic shoot. Early procedures of transformation reported an efficiency of transformation of 0.2 to 15% (**Table 4**). After improvement in this procedure, the rate of transformation can be as great as 80% (H.S. Aldwinckle, E. Borejsza-Wysocka, and M. Malnoy, pers. comm.).

**Other modifications:** Other modifications have been made to these procedures to increase their transformation efficiency. These included the use of i) plant phenolic compounds (acetosyringone, betaine phosphate) to increase the expression of several virulence genes in *A. tumefaciens*, ii)

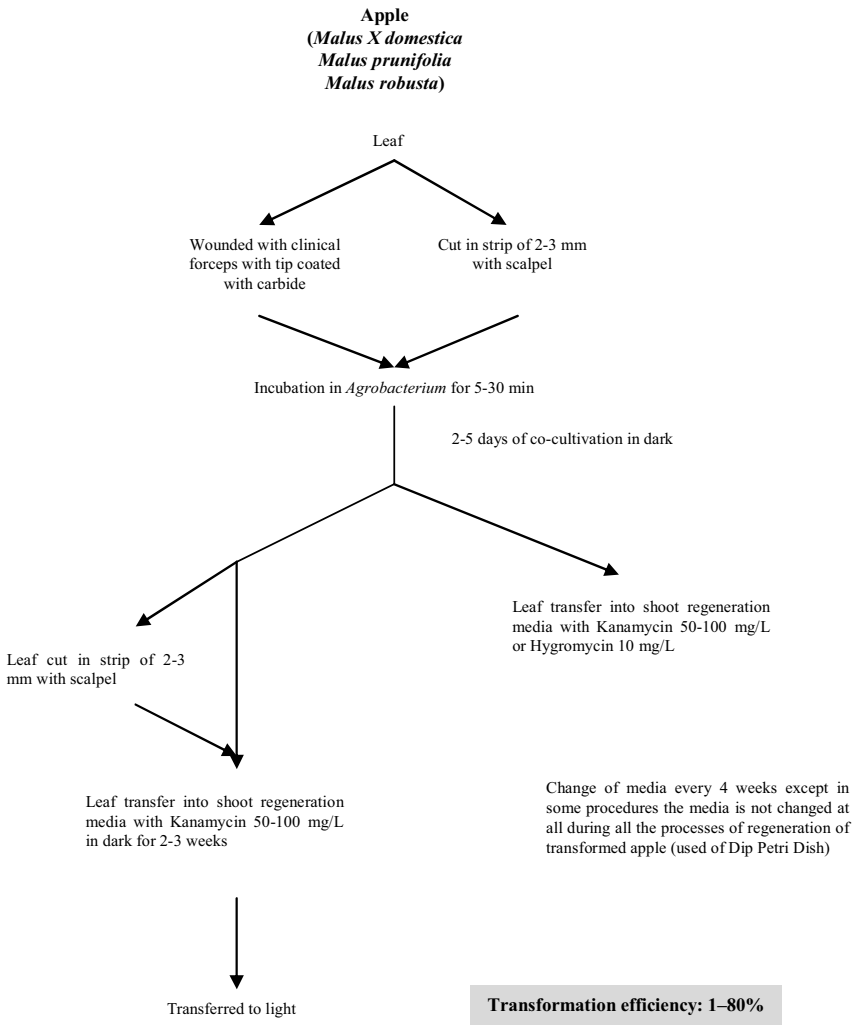


**Fig. 2 Representation of the apple (*Malus X domestica*, *M. prunifolia*, *M. robusta*) transformation procedure.**

gelling agent, iii) nitrogen source, iv) concentration of $AgNO_3$ (Seong *et al.* 2005), and v) binary vector. Hammerschlag *et al.* (2000) reported the generation of *Agrobacterium*-free transgenic apple by vacuum infiltration of explants inoculated with *Agrobacterium* with an acidified medium with a high concentration of antibiotics (500 mg/l Carb, Cef and Cefoxitin). Another way to avoid contamination with *Agrobacterium* is to bathe pre-wounded leaves in the inoculum for a short time.

Zhu *et al.* (2005) has used TIB as a part of propagation of the apple rootstock 'M.26'. This system can also be used for transforming apple.

## *2. Application of these procedures for Malus improvement*

The first report of transformed apple plants in 1989 showed promise for new apple cultivars that would be superior in taste, healthier and easier to grow. Although, many different traits have now been introduced successfully into apple (**Table 4**), no transformed apple cultivars have yet entered commercial production. Most early reports on transformed apple described 'proof of concept' experiments involving the development of regeneration protocols, and the choice of appropriate promoters and selectable markers. For gene activation, in many cases researchers relied on the well characterized constitutively expressing CaMV 35S promoter (**Table 4**). Although not mentioned specifically in conjunction with a target gene, promoters linked with targeted expression patterns in apple have been identified, for example the 940 *extA* promoter, which is active in young tissue (Gittins *et al.* 2001), two promoters, RBCS3C and SRS1, suitable for the expression of transgenes in green photosynthetic tissues of apple (Gittins *et al.* 2000), a pathogen inducible promoter (Malnoy *et al.* 2006) and others with particular expression patterns that are under development and characterization (Gittins *et al.* 2003; Norelli *et al.* 2007). A major problem for the perception of GE apple is the use of the *npt*II selectable marker gene (which might conceivably be horizontally transferred to humans after ingestion of transgenic food) is still used extensively. Recently, some groups have started to develop alternatives (**Table 4**).

More recently the focus has moved onto functional testing of traits of potential commercial interest. These traits can be grouped into two categories: production and consumer traits. Production traits include bacterial, fungal and pest resistance, dwarfing, propagation, stress resistance, precocity, storage life and self fertility (**Table 4**). Examples of consumer traits include novel health properties, improved flavor, reduced browning after slicing, and reduced allergenicity (**Table 4**). In this section, we will describe a few examples of the use of GE to improve some characteristics of apple.

**Disease resistance:** The first and probably most important target trait for GE of apple was fire blight, which was pioneered by the Cornell University group led by H.S. Aldwinckle (**Table 4**). A large number of genes have been expressed in apple to improve their resistance to the bacterium *Erwinia amylovora*, with varying degrees of success (**Table 4**). Three different strategies have been used: producing antimicrobial proteins, inhibiting bacterial pathogenicity factors, and silencing or overexpression of defense related genes of apple.

Attacin E, cecropin and lysozyme are three distinct anti-

**Table 4** Traits expressed in transformed apple.

| Trait | Apple cultivars | Gene introduced | Gene origin | Promoter | Principal results | Reference |
|---|---|---|---|---|---|---|
| **Bacterial resistance** | | | | | | |
| *Agrobacterium tumefaciens* (Crown gall) | Jonagold | *iaaM and ipt* (silencing) | *A. tumefaciens* | CaMV35S FMV | Silencing of iaaM gene expression was observed. Reduction and abolition of Crown Gall formation was observed. | Viss *et al*. 2003 |
| *Erwinia amylovora* (Fire blight) | Gala | SB37 (+/-sp Shiva 1 (-sp) | Synthetic peptide | Pin2 CaMV35S | Some SB-37 transgenic lines with partial resistance in the orchard. | Norelli *et al*. 1999, Aldwinckle *et al*. 2003 |
| | Royal Gala | MB39 (+sp) | Modified cecropin | Osmotin (wound inducible) | 3 of 7 transgenic lines showed an increase in resistance in greenhouse (2.5 to 3.3 fold more resistance than the control). | Liu *et al*. 1999, 2001 |
| | Royal Gala | MB39 (+sp) | Modified cecropin | Osmotin (wound inducible) | 3 of 7 transgenic lines showed an increase in resistance in greenhouse (2.5 to 3.3 fold more resistance than the control). | Liu *et al*. 1999, 2001 |
| | M.26 | *Attacin E* | *Hyalophora cecropia* (giant silk moth) | Pin2 CaMV35S | Some transgenic lines showed partial resistance in greenhouse and in the orchard. Increased resistance when signal peptide and translation enhancer (AMV) were used. | Norelli *et al*. 1994, Ko *et al*. 2000; Aldwinckle *et al*. 1998, 2003 |
| | Pinova, Pilot, Pirol, Pingo, Elstar, Remo, Liberty, Reka, Pi-AU 56-83 | Attacin E (-sp) T4 Lysozyme | *Hyalophora cecropia* (giant silk moth) *T4 Bacteriophage* | Pin2 CaMV35S | Some transgenic lines showed partial resistance in greenhouse. | Hanke *et al*. 2000 |
| | Gala | T4 Lysozyme alone or associated with attacin E | *T4 Bacteriophage Hyalophora cecropia* | Pin2 (attacin E) CaMV35S (T4ly) | Some transgenic lines showed partial resistance in greenhouse. No increase in resistance when this gene was combined with attacin gene. | Ko *et al*. 1998; 2002; Aldwinckle *et al*. 1998, 2003 |
| | Pinova | *Dpo* | phage ϕEa1h | CAMV35S | 7 of 33 lines show less disease than the non transformed genotype in greenhouse, but this difference was not statistically different, except for one line. | Flachowsky *et al*. 2008 |
| | M.26 | | | CAMV35S Gst1 | Increased resistance to fire blight. Higher resistance observed when the *Dpo* gene was driven by CaMV35S promoter. | Boresjza-Wysocka *et al*. 2007 |
| | M.26 | *HrpN* | *Erwinia amylovora* | Gst1(wound inducible) | Most of the transgenic lines had partial resistance to *E. amylovora* in greenhouse and in the field. Two of these lines showed a level of resistance similar to the resistant rootstock M.7. | Abdul-Kader *et al*. 1999; Aldwinckle *et al*. 2003 |
| | Galaxy M.26 | *MpNPR1* | Apple | Pin2 CaMV35S | Significant reduction in susceptibility to *E. amylovora* of 33-86% for Galaxy; M.26 showed a less substantial reduction in susceptibility compared to Galaxy (0 to 70%). | Malnoy *et al*. 2007 |
| | Galaxy | *DIPM (*Silencing) 4 different genes | Apple | CaMV35S | Some transgenic lines showed silencing of the DIPM genes and an increase in resistance to *E. amylovora*. | Boresjza-Wysocka *et al*. 2006 |
| | Greensleeves | *Aldose 6 phosphate (A6PR) antisense* also called *S6PDH* | Apple | CaMV35S | Sorbitol content in transgenic lines repressing or overexpressing sorbitol have no influence on disease severity or progression regardless of E. amylovora genotype or inoculums concentration. | Duffy and Dandekar 2008 |
| **Insect resistance** | | | | | | |
| | Royal Gala | *Avidin Streptavidin* | nd | CaMV35S | High level of resistance to larvae of lightbrown apple moth (*Epiphyas postvittana)*. Mortality of the larvae was between 80% - 90% in the transgenic lines compare to 14% in the untransformed apple. | Markwick *et al*. 2003 |
| | | *Proteinase inhibitor* | *Nicotiana alata* | CaMV35S Ribuloase-1,5-biphosphate carboxylase | Lightbrown apple moth (*E. postvittana)* feeding on the transgenic apple had significantly reduced body weight after 7 days and female pupae were 19-28% smaller than control. In addition, morphological changes such as pupal cases attached to the wings, deformed wings, deformed body shape, and pupal cases and curled wings attached to body were observed in adults that developed from larvae fed with transgenic apple leaves expressing PI when compared to larvae fed with non-transformed apple leaves. | Maheswaran *et al*. 2007 |
| **Fungal resistance** | | | | | | |
| Fungal resistance *Alternaria mali* | Melba McIntosh | *thaumatin Rab* | *Thaunatococcus danielli* (Plant) Plant nd | CaMV35S Wound inducible promoter | nd Smaller disease lesion and spot size in transgenic lines compared to untransformed. | Dolgov *et al*. 2004 Kim *et al*. 2006 |
| *Venturia inaequalis* (apple scab) | McIntosh | *Endochitinase (ech42) Exochitinase (nag70)* | Trichoderma atroviride (fungus) | 2CaMV35S | Negative correlation between growth of transgenic lines and endochitinase activity was observed. 6 of 8 transgenic lines expressing endochitinase were more resistant than control. Disease severity was reduced by 0 to 99.7% (number of lesions) and 0 to 90% (% of leaf area infected). Exochitinase was less effective than endochitinase. Some plants expressing both genes were more resistant than plants expressing either single gene. | Bolar *et al*. 2000, 2001 |

**Table 4** (Cont.)

| Trait | Apple cultivars | Gene introduced | Gene origin | Promoter | Principal results | Reference |
|---|---|---|---|---|---|---|
| **Rooting ability / Dwarfism to rootstock** | | | | | | |
| | A2 | *rolA* | *A. rhizogenes* | rolA promoter | The 2 transformed lines showed a reduction in height and shortened internode length. | Zhu *et al*. 2001a |
| | Florina | *rolB* | *A. rhizogenes* | CaMV35S | All transgenic lines produced roots *in vitro* in hormone free medium. Transgenic plant does not show any phenotypical difference with the exception of root parts after two months of growth *in vitro* or in greenhouse compared to the control. | Radchuck and Korkhovoy 2005 |
| | Jork 9 | *rolB* | *A. rhizogenes* | nd | Increase in adventitious root production. Some clones showed reduced apical dominance, slow initial growth and shorter internodes. | Pawlicki-Jullian *et al*. 2002 |
| | Jork 9 | *rolB* | *A. rhizogenes* | rolB promoter | Increase in root production. | Sedira *et al*. 2001, 2005 |
| | M.26 | *rol A* | *A. rhizogenes* | rolA promoter | All transformants had reduced stem growth. Some showed reduced length, leaf area and dry weight (shoot, root and plant). No decrease in rooting ability of the transformed lines. | Holefors *et al*. 1998 |
| | | | | | 2 forms of *rolA* mRNA was found in the transgenic lines: one form unspliced and the other spliced in which an 85 bp intron sequence was spliced out. This can affect the expression of *rolA*. | Xue *et al*. 2008 |
| | M.26<br>M.9/29 | *Phytochrome B* | *Arabidopsis thaliana* | CaMV35S | Shoot, root and plant dry weight were reduced in all transformed lines. 9 of 13 transgenic lines showed a stem length reduction | Holefors *et al*. 2000 |
| | | *rolB* | *A. rhizogenes* | rolB promoter | Transgenic lines had greater rooting ability and were higher than the control. | Welander *et al*. 1998<br>Zhu and Welander 2000a |
| | | *rolB* | *A. rhizogenes* | rolB promoter | All transgenic lines produced roots *in vitro* in hormone-free medium. 15% of the transgenic lines showed reduction in node number and stem length. | Zhu *et al*. 2001b |
| | Marubakaidou | *rolC* | *A. rhizogenes* | rolC promoter | Introduction of *rolC* results in short plants with shortened internodes, smaller leaves and reduced apical dominance compared to the non transformed plants. | Igarashi *et al*. 2002 |
| | Micromalus Makino | *rolC* | *A. rhizogenes* | rolC promoter | Increased rooting ability without auxin treatment, and reduced height, internode length and leaf areas. | Zhang *et al*. 2006 |
| | Greensleves | *GA 20-oxidase* (sense or antisense) | Apple | CaMV35S | Reducing the expression of *MpGa20ox1* using either sense or antisense suppression resulted in a dwarf phenotype. | Bulley *et al*. 2005 |
| | Gravenstein<br>McIntosh<br>A2 | *Gai* gibberellic acid insensitive | *A. thaliana* | CaMV35S | Most of the transgenic plants showed reduced growth *in vitro*. Growth analyses in the greenhouse showed a clear reduction in stem length, internode length and node number for the dwarf clones. The normal phenotype of some transgenic clones appears to be associated with silencing of the introduced *gai* gene. In general, transgenic clones showed reduced rooting ability, especially for the extremely compact ones | Zhu *et al*. 2008 |
| **Herbicide resistance** | | | | | | |
| | N545 | *bar* | *Streptomyces* | CaMV35S | Greenhouse plants treated with 1% solution of BASTA showed no damage. | Dolgov and Skryabin 2004 |
| **Environmental stress resistance** | | | | | | |
| | Ariane<br>Galaxy | *Endohitinase (ech42)*<br>*Exochitinase (nag70)* | Trichoderma atroviride | 2CaMV35S | Negative correlation between growth of transgenic lines and endochitinase activity was observed. Reduced growth appeared to be associated with high lignin content, peroxidase and glucanase activity.<br>All the lines with high endochitinase activity exhibited significant reduction of scab symptoms. | Faize *et al*. 2003 |
| | Jonagold | *Ace-AMP1*<br>*Rs-AFP2* | Onion<br>Radish | CaMV35S | *Rs-AFP2* expressing shoot showed 8 to 32 fold antifungal activity compared to the control. *Ace-AMP1* expressing shoot showed 4 fold increased antifungal activity relative to control plants. | De Bondt *et al*. 2000 |
| | Jonagold | *Ai-AMP* | nd | CaMV35S | nd | Broothaerts *et al*. 2000 |
| | Golden Delicious<br>Gala<br>Elstar | *hordothionin* | Barley | CaMV35S | Decrease in scab symptom development. | Jense *et al* 2002 |
| | Ariane<br>Galaxy | *Puroindoline-b* | Wheat | CaMV35S | Reduction of symptoms in transgenic Galaxy (55% for best lines) and in Ariane (64%) after inoculation with the apple scab race 6. No increase in resistance was observed in the transgenic Galaxy lines after inoculation with the apple scab race 1. | Faize *et al*. 2004 |
| | Gala | *HcrVf2* | **Apple** | CaMV35S | *Hcrvf2* confers scab resistance to the susceptible apple cultivar Gala. Acquired resistance is race-specific. | Barbieri *et al*.2003; Belfanti *et al*. 2004; Silfverberg-Dilworth *et al*. 2005 |
| | Galaxy<br>McIntosh | *Vfa1, Vfa2* and *Vfa4* | **Apple** | Their own native promoter | Transgenic lines expressing either *Vfa1* or *Vfa2* showed a significant increase in resistance to *Venturia inaequalis*. Transgenic lines expressing *Vfa4* gene were as, or more susceptible than control. | Malnoy *et al*. 2008 |

**Table 4** (Cont.)

| Trait | Apple cultivars | Gene introduced | Gene origin | Promoter | Principal results | Reference |
|---|---|---|---|---|---|---|
| **Environmental stress resistance** | | | | | | |
| High and low temperature stress | Royal Gala | *Ascorbate peroxidase* | pea | nd | Transgenic lines showed different degrees of increased resistance to high and low temperature stress. Resistance to freezing injury was 1-3 degrees greater and up to seven degrees increase to acute heat stress. | Artlip *et al*. 2007 |
| Drought and cold stress | Greensleeves | *Osmyb4* | rice | CaMV35S | Expression of the MYB4 transcription factor improved physiological and biochemical adaptation to cold and drought stress and modified metabolite accumulation (glucose, fructose, and proline) in apple. | Pasquali *et al*. 2008 |
| Iron deficiency tolerance | Balenghaitang Malus robusta | *LeIRT2* | tomato | CaMV35S | 2 of 9 transgenic lines were tested for their iron deficiency tolerance. One of these 2 lines showed a higher resistance to iron deficiency (21-34% greater than control). | Qu *et al*. 2005 |
| Zinc stress | M.26 | *ZNT1* *ZIP4* | **Apple** | CaMV35S | Transgenic plant, overexpressing the *ZIP4* or *ZTN1* gene, grown under low level of Zn had very limited and no effect on the plant, respectively, compared to the control. The overexpression of *ZIP4*, but not of *ZTN1* transproter gene, increased the concentration of Ca and Cu in the root and underground tissues. | Swietlit *et al*. 2007 |
| **Flowering time** | | | | | | |
| | Orin | *MdTFL1* | **Apple** | CaMV35S | Transgenic apple expressing MdTFL1 antisense RNA first flowered 8-22 months after transfer to the greenhouse, whereas non-transgenic plants flowered 69 months after transfer to the greenhouse. | Kotoda *et al*. 2006 |
| **Self-fertility** | | | | | | |
| | Pinova | *BpMADS4* | Silver birch | CaMV35S | 13 weeks after transformation first flower was observed *in vitro*; Flower appeared in greenhouse after a few weeks but abnormal growth was observed. | Flachowsky *et al*. 2007 |
| | Elstar | *S3* gene silencing | **Apple** | CaMV35S | Production of transgenic apple tree with true self-fertility. This self-fertility was stable for several years without any obvious adverse effects on tree growth or fruit appearance. | Broothaerts *et al*. 2004 |
| **Colors** | | | | | | |
| | Royal Gala | MdMYB10 | Apple | CaMV35S | Regenerated callus and transformant were highly pigmented | Espley *et al*. 2007 |
| **Modified metabolism** | | | | | | |
| Production of the phytoalexin Resveratrol | Elstar Holsteiner cox | *Stilbene synthase* | *Vitis vinifera* | Stilbene promoter (UV, wound and pathogen inducible) | Transgenic lines produced a resveratrol glucoside (piceid) which is accumulated in the plant, skin and flesh of the fruit. Accumulation of piceid after induction by UV treatment does not affect the accumulation of other compounds of the flavonoid and phenylpropanoid pathway. Expression of the stilbene synthase gene does not affect the leaf shape, flower morphology or color, or fruit shape and size compared to control plants and fruit. | Szankowski *et al*. 2003 Ruhmann *et al*. 2006 |
| Flavonoids production | Holsteiner cox | *Lc* (leaf color) | *Zea mays* L. | CaMV35S | Transgenic showed increased expression for some keys enzyme of the phenylpropanoids pathways (PAL, CHS, FHT, DFR, LAR and ANS). *Lc* overexpression in *M. domestica* resulted in enhanced biosynthesis of specific flavonoid classes, which play important roles in both phytopathology and human health. | Li *et al*. 2007 |
| | TNR31-35 Malus sierversii niedzwetzkyana | *Ans RNAi* *Anthocyanidin synthase* | Apple | CaMV35S | Strong reduction of the anthocyanin content in the transgenic lines. The silencing of the NAS gene results in increase of the content of hydroxycinnamic acids, flavonols and cathechin. The *in vitro* plant showed some severe symptoms of browning and necrotic lesions accompanied by strong reduction in viability due to the use of a constitutive promoter. | Szankowski *et al*. 2009 |
| Decreased flesh browning | Orin Fuji | *Polyphenol oxidase* antisense | Apple | CaMV35S | Transgenic lines, in which PPO expression is reduced, had less flesh browning. | Murata *et al* 2000, 2001 |
| Sugar accumulation | Orin | *Sorbitol 6 phosphate dehydrogenase (S6PDH)* | Apple | CaMV35S | Transgenic lines showed different expression level of S6PDH. Lines with less activity contained only a low level of sorbitol but showed 6 to 7 fold increase in sucrose. The growth of this plant stopped early during the summer due to a deficiency of sugar. Lines with increased amount of S6PDH activity had increased sorbitol and sucrose content. | Kanamaru *et al*. 2004 |
| | Greensleeves | *Aldose 6 phosphate (A6PR)* antisense also called *S6PDH* | Apple | CaMV35S | Antisense inhibition of A6PR expression significantly decreased A6PR activity and sorbitol synthesis, but increased concentration of sucrose and starch at both dusk and predawn. Silencing leaf sorbitol synthesis alters long-distance partitioning and apple fruit quality. | Cheng *et al*. 2005 Zhou *et al*. 2006 Teo *et al*. 2006 |
| Down regulation of ethylene production | RRoyal Gala | *Aminocyclopro* AApple *pane 1 carboxylic (ACC) synthase 2* antisense | | CCaMV35S | Production of apple tree with down regulation of ethylene production. Some lines had fruits with delayed softening. | Hrazdina *et al*. 2003 |

**Table 4** (Cont.)

| Trait | Apple cultivars | Gene introduced | Gene origin | Promoter | Principal results | Reference |
|---|---|---|---|---|---|---|
| **Modified metabolism** | | | | | | |
| | GGreensleeves | *ACC synthase* antisense *ACC oxidase* antisense | Apple | CaMV35S | Some transgenics were significantly suppressed in ethylene production. The fruit of these apple trees was firmer and displayed increased shelf-life. No difference was observed in sugar or acid accumulation in these fruits compared to the control. However, a significant and dramatic suppression of the synthesis of volatile esters was observed. | Dandekar *et al*. 2004; Defilippi *et al*. 2004, 2005a, 2005b |
| | Royal Gala | *ACC synthase* antisense | Apple | | Royal Gala' apple that produces no detectable levels of ethylene using antisense ACC oxidase, resulting in apples with no ethylene-induced ripening attributes. In response to external ethylene these antisense fruits undergo a normal climacteric burst and produced increasing concentrations of ester, polypropanoid, and terpene volatile compounds over an 8-d period. Using an microarray approach 17 candidate genes have been identified to be ethylene control point for aroma production in apple. | Schaffer *et al*. 2007 |
| **Cell adhesion** | | | | | | |
| | Royal Gala | *Polygalac-turonase* | Apple | CaMV35S | Phenotypic modification of apple tree (silvery colored leaves and leaf shedding). Mature leaves with malfunctioning and malformed stomata. | Atkinson *et al*. 2002 |
| **Apple allergen** | | | | | | |
| | Elstar | *Mal d1* Rnai | Apple | CaMV35S | Reduction of *Mal d1* expression in the transgenic silenced apple. This translated into significantly reduced *in vivo* allergenicity. | Gilissen *et al*. 2005 |
| **Promoter** | | | | | | |
| Gene expressed in restricted area | Greensleeves | | Tomato Soybean | Rubisco small subunit (SSU) promoter CaMV35S | SSU promoters are active in the photosynthetically active leaf mesophyll and palisade cells. The mean activity of the two SSU promoters in apple leaves appeared to be approximately half that of the constitutive promoter CAMV35S. SSU soybean promoter was strictly light dependent. | Gittins *et al*. 2000 |
| Gene expressed in young stem tissue and load bearing regions | Greensleeves | | *Brassica napus* | -940 extA promoter | (-)940 extA promoter has an activity in apple stem comparable to that obtained with CaMV35S. Its activity was lower in petioles (50%), roots (20%) and leaves compared to the activity of CaMV35S. | Gittins *et al*. 2001 |
| Gene expressed in vegetative tissues | Greensleeves | | *A. rhizogenes* *Commelina* yellow mottle virus | RolC promoter CoYMV promoter | The two promoters showed an activity lower than the CaMV35S. RolC promoter was less active than CoYMV promoter in apple. These promoters are active in the vascular system, particularly the phloem. | Gittins *et al*. 2003 |
| Gene expressed in response to pathogen attack | M.26 Royal Gala | | Potato | Gst1 promoter | In the two apple genotypes, Gst1 exhibited a low level of expression after bacterial and fungal inoculation compared to CaMV35s (15% and 8% respectively). It is activated by salicylic acid but not by wounding. | Malnoy *et al*. 2006a |
| Chemical inducible and light inducible promoter | M.26 | | nd | XVE promoter | Promoter currently being evaluated. | Norelli *et al*. 2007 |
| **Selectable marker** | | | | | | |
| Non-antibiotic based selection | M.26 | Phosphomannose isomerase (*pmi*) | nd | CaMV35S | Mannose (2 g/L) can be used as selection agent but the selection efficiency seems to be lower than that of Kan. | Zhu *et al*. 2004 |
| Excision by recombination | Pinova Pilot Elstar Regine | *pmi* *GFP* | | CaMV35S | Some putative transgenic lines were regenerated with the GFP gene as reporter gene. No transgenic lines were regenerated with the pmi gene as selectable marker. | Flachowsky *et al*. 2004 |
| | | *pmi* *UDP-glucose:galactose-1-phosphate uridyltransferase (galT)* | nd | | First regenerants were obtained, with the pmi gene as selectable marker, and tested gus positive. No transgenic lines yet regenerated with the *galT* gene. | Szankowski and Degenhardt 2007; Degenhardt *et al*. 2006 |
| | Galaxy | *Vr-ERE* | *Vigna radiata* | CaMV35S | Benzaldehyde (BA) is able to inhibit adventitious bud regeneration. BA can be used instead of Kan but the efficiency of the selection pressure is not yet proven. | Chevreau *et al*. 2007 |
| | Elstar/ Gala | *R-Rs* system | | | Antibiotic resistance marker gene free transgenic Elstar apple lines have been produced. | Schaart *et al*. 2004 |
| No selectable marker | M.26 Galaxy | none | | | Transgenic lines of M.26 and Galaxy were regenerated without any selectable marker with an efficiency of 22 and 12%, respectively. | Malnoy *et al*. 2007b |

microbial genes used to increase resistance to fire blight (**Table 4**). Several groups reported an increase of resistance, *in vitro* and in the greenhouse, to fire blight in the different apple cultivars expressing these genes (Norelli *et al.* 1994, 1999; Hanke *et al.* 2000; Ko *et al.* 2000; Liu *et al.* 2001; Ko *et al.* 2002). However, the best results were obtained with the attacin E gene. One particular line displayed only 5% shoot blight infection compared with approximately 60% of the non-transformed 'Royal Gala' control plants in 1998 field trials (Norelli *et al.* 1999). A short overview of the field trials with these various lytic proteins transformed lines was presented by Aldwinckle *et al.* (2003).

The second strategy currently used to improve resistance to fire blight is by inhibiting the bacterial pathogenicity factors. The extracellular polysaccharide (EPS) produced by *E. amylovora* has a role in bacterial pathogenicity, bacterial survival, as well as binding and absorbing nutrients, and avoiding host detection. The polysaccharide depolymerase gene (*Dpo*) from the ΦEa1 *E. amylovora* phage (driven by various promoters, e.g. CaMV35S or Gst1) was inserted into the apple rootstocks 'JTEH' and 'M.26' and into the apple cultivars 'Pinova' (Sule *et al.* 2002; Boresjza-Wysocka *et al.* 2007; Flachowsky *et al.* 2008) (**Table 4**). In the first reports, 4 out of 5 transformed plants were completely resistant to fire blight (Sule *et al.* 2002). These results were confirmed by Hanke *et al.* (2003), who showed that 7 out of 33 transformed Pinova lines showed less susceptibility to fire blight than the parent 'Pinova', but no statistical difference was observed, except for one transgenic line. However, Boresjza-Wysocka *et al.* (2007) observed a greater resistance to fire blight when *Dpo* was fused to a signal peptide and driven by the psthogen inducible promoter *gst1* rather than by *CaMV35S*.

During the infection process pathogens secrete compounds that elicit a range of plant host defense responses that occur at different rates. Researchers have tried to induce plant defense responses by introducing elicitors or by speeding up the defense response with various promoters. The *E. amylovora* effector protein, harpin N, is able to partially protect against subsequent *E. amylovora* infection when sprayed on flowers, probably by inducing the systemic acquired resistance (SAR) response. The gene encoding for harpin N was introduced into the susceptible rootstock 'M.26' under the control of either the weak constituitive *nos* or the pathogen-inducible *gst1* promoter (Abdul-Kader *et al.* 1999; Malnoy *et al.* pers. comm.). Two years of field testing of own rooted plants of the transgenic lines of 'M.26' containing the *harpin* $N_{Ea}$ gene with the *gst1* promoter, showed a significant reduction in susceptibility to fire blight. Two of these lines showed a level of resistance equivalent to that of the apple rootstock 'M.7', which is usefully resistant to fire blight. These transgenic lines can also help promote a better understanding of the role of harpin $N_{Ea}$ in the induction of fire blight resistance in the transgenic clones. It will also be interesting to look at the behavior of these transgenic lines in response to other pathogens of apple.

In all of the above reported approaches to creating a fire blight resistant plant, the resistance induction was entrusted to a non-*Malus* gene. Besides 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 (*DIPMs*). If this interaction does not take place *E. amylovora* will not be able to infect that host. With the aim of silencing the *DIPM* genes and preventing disease-causing interaction with DspE, 400 bp sense sequences from non-conserved regions of each gene, with homology among each other of <50% were introduced into the genome of the apple cv. 'Galaxy' (Borejsza-Wysocka *et al.* 2006). In addition, three constructs containing the four 400 bp sequences in tandem, a full length sense sequence of one gene, and a hairpin sequence of that gene were also introduced into 'Galaxy' apple. RT-PCR assays of the transgenic plants for transcript expression of the target DIPMs have shown evidence of silencing at the mRNA level in some lines. Some of the transgenic lines have also been evaluated for resistance to fire blight by inoculation of shoots of own-rooted potted plants with the virulent strain Ea273 of *E. amylovora*. Preliminary results indicate that some lines have increased resistance (Borejsza-Wysocka *et al.* 2006).

Using the same approach used to identify the DIPM protein, Oh and Beer (2007) were able to identify a protein that interacts with the harpin $N_{Ea}$ elicitor factor of *E. amylovora*. This small protein (60 aa) has a functional signal peptide and is associated with plant plasma membranes. With the aim of silencing the gene encoding for the harpin $N_{Ea}$ Interacting Protein of Malus (*HIPM*), an RNAi construct containing the full length of this gene was introduced into the apple cv. 'Galaxy'. Preliminary results showed partial resistance to fire blight (M. Malnoy, E. Borejsza-Wysocka, and H.S. Aldwinckle, pers. comm.).

Another approach has been to overexpress a master regulator of the plant disease response. The nonexpressor of PR (*NPR1*) gene is a key mediator of SAR (Cao *et al.* 1994, 1997). An additional copy of the apple ortholog, *MpNPR1*, was introduced into 'Galaxy' and 'M.26' (Malnoy *et al.* 2007a). In growth chamber challenges with *E. amylovora*, the transformed 'Galaxy' clones had 17.5 to 35.5% shoot length infected compared to 80% in controls. In addition, there was increased resistance to two other pathogens [*Venturia inaequalis* (scab) and *Gymnosporangium juniperi-virginianae* (cedar apple rust)] (Malnoy *et al.* 2007a). The increased, broad spectrum resistance produced by the introduction of an additional copy of a gene sourced within apple, makes the use of *MpNPR1* (and similar strategies) very attractive, as all the other genes for resistance employed previously have been of viral, bacterial, fungal or animal origin.

Apple scab, caused by the ascomycete *V. inaequalis* (Cke.), is the most important fungal disease of apple in most apple-growing regions with high spring and summer rainfall. The control of this disease in commercial orchards can require up to 15 or more fungicide treatments per year. However, because of increasing fungicide resistance within the pathogen, use of modern specific fungicides must be strictly limited and/or alternated. An alternative approach is growing resistant cultivars that use scab resistance genes from (mostly small fruited) wild species. However, the production of high quality resistant cultivars by classical breeding is difficult because of the long juvenile phase and self-incompatibility of apple. Starting with the wild species *M. floribunda* 821 carrying the *Vf* gene for resistance to apple scab, breeders have developed many scab resistant cultivars, but few have met with any measure of commercial success mainly because of lack of high quality or storeability. The direct transfer of a scab resistance gene offers a new alternative for cultivar improvement.

Several groups have attempted to improve the scab resistance (**Table 4**) of highly susceptible apple cultivars such as 'Gala' (Janse *et al.* 2002; Belfanti *et al.* 2004), 'Galaxy' (Faize *et al.* 2003, 2004; Malnoy *et al.* 2008), 'Jonagold' (De Bondt *et al.* 1999), and 'McIntosh' (Bolar *et al.* 2000; Malnoy *et al.* 2008) or partially resistant cultivars such as 'Ariane' (Faize *et al.* 2003, 2004), by the integration of heterologous antifungal genes (*chitinases*, *Amp1*, *AFP2*, *puroindolin*, or *hordothionin*) or *Vf* gene orthologs. The biocontrol fungus *Trichoderma atroviride* (previously *T. harzianum*) produces many chitinolytic enzymes, including endochitinase, which randomly cleaves chitin, a major component of the fungus cell wall. *Trichoderma* endochitinase encoded by the gene *ech42* inhibits spore germination and hyphal elongation. Bolar *et al.* (2000) obtained several lines of the scab susceptible 'McIntosh' apple with varying levels of *ech42* expression. Some of the transgenic lines exhibited increased resistance to *V. inaequalis*. However, transgenic lines with high endochitinase activity had reduced vigor. Similar results were also observed by Faize *et al.* (2003) when the *ech42* gene was introduced into the apple cultivars 'Galaxy' and 'Ariane'. This reduction of growth appeared to be associated with lignin content, and peroxidise and glu-

canase activity in the transgenic lines (Faize *et al.* 2003). Endochitinase from the *ech42* gene interacts synergistically with other chitinolytic enzymes of *T. atroviride* such as N-acetyl-β-glucosaminidase (exochitinase *nag70*). Bolar *et al.* (2001) and Faize *et al.* (2003) studied transgenic lines of three different apple cultivars expressing *ech42* and *nag 70* alone or in tandem and demonstrated *in planta* synergy between these enzymes. Indeed, the transgenic lines expressing these two genes were more resistant than plants expressing either single enzyme at the same level.

A significant increase of resistance to *V. inaequalis* was also observed with the expression of other antifungal genes. The puroindolin B gene, a member of the plant lipid transfer proteins (LTPs), was expressed in the apple cultivar, 'Ariane,' which is resistant to races 1-5 of *V. inaequalis* and susceptible to races 6 and 7, and 'Galaxy', which is susceptible to all races of *V. inaequalis*). After inoculation with *V. inaequalis* race 6 symptoms were reduced by of 55% and 64% in the most resistant transgenic lines of 'Galaxy' and 'Ariane', respectively (Faize *et al.* 2004). Preliminary results showed that the *hordothionin* genes (Janse *et al.* 2002), *Ace-AMP1*, and *Rs-AFP2* (De Bondt *et al.* 2000) have potential for increasing resistance of apple to *V. inaequalis*.

The scab-resistance locus *Vf* was identified in the wild apple species, *M. floribunda* 821, and has been widely introgressed into susceptible apple cultivars. The *Vf* locus confers resistance to five races of *V. inaequalis*, but not to races 6 and 7, identified thusfar only in Europe. A cluster of four receptor-like sequences have been identified at the *Vf* locus, which resemble the *Cf* resistance genes in tomato (Vinatzer *et al.* 2001; Xu and Korban 2002). Three of them, designated *Vfa1*, *Vfa2*, and *Vfa4*, have intact ORFs, whereas, the fourth, *Vfa3*, is truncated and an obvious pseudogene. Differential expression has been observed among the four *Vf* orthologs during leaf development, whereby *Vfa1*, *Vfa2*, and *Vfa3* are highly expressed in immature leaves, but only slightly detectable in mature leaves; whereas *Vfa4* is expressed in immature leaves, and highly expressed in mature leaves (Xu and Korban 2002). Barbieri *et al.* (2003) and Belfanti *et al.* (2004) have proved that overexpression of the *Hcrvf2* (= *Vfa*2) gene, under the control of the constitutive promoter CaMV35S, was sufficient to confer scab resistance to a susceptible apple cultivar. However, this resistance is specific to *V. inaequalis* races, effective toward races 1-5, but not toward race 6 (Silverberg-Dilworth *et al.* 2005). Malnoy *et al.* (2008) also showed that the gene *Vfa2* is sufficient to confer partial resistance to *V. inaequalis* in 'Galaxy' and 'McIntosh' when it is expressed under the control of its own promoter. They also showed that the *Vfa1* gene is able to confer partial resistance to a mixture of apple scab races (races 1 to 5). These two studies showed that the *Vfa1* and *Vfa2* genes are involved in the resistance of apple to *V. inaequalis*, whereas the *Vfa4* gene is not involved in resistance. Indeed, the transformed 'Galaxy' and 'McIntosh' lines expressing the *Vfa4* gene were as, or more susceptible than the control (Malnoy *et al.* 2008). It will be interesting to transform scab susceptible apple cultivars with the *Vfa1* and *Vfa2* genes in tandem, to determine their synergetic effect. It will also be informative to study the specificity of the different *V. inaequalis* races in 'Galaxy' and 'McIntosh' transgenic lines expressing *Vfa1* or *Vfa2*. The overexpression of *Hcrvf2* in apple induced the expression of many defense related genes (Paris *et al.* 2009).

Resistance in apple to two other diseases (crown gall and *Alternaria* blotch) and to an insect (lightbrown apple moth) have been reported to be increased by GE (**Table 4**).

Lightbrown apple moth (LBAM), *Epiphyas postvittana*, is a serious pest of pome and stone fruits and of many other horticultural crops, including grapevine, citrus, kiwifruit, berry fruits, avocados and some vegetable and flower crops in New Zealand. Markwick *et al.* (2003) produced some 'Royal Gala' transgenic lines expressing the *avidin* or *strepavidin* gene. ELISA assays showed that *avidin* expression ranged from 1.9 to 11.2 μM and *streptavidin* expression ranged from 0.4 to 14.6 μM. Expressed at these levels, both biotin-binding proteins conferred a high level of insect resistance on transformed apple plants to larvae of the LBAM (Markwick *et al.* 2003). Mortality of LBAM larvae was significantly higher ($P < 0.05$) on three avidin-expressing (89.6, 84.9 and 80.1%) and two streptavidin-expressing (90 and 82.5%) apple lines than on non-transformed control plants (14.1%) after 21 days. Weight of LBAM larvae was also significantly reduced by feeding on all apple shoots expressing *avidin* and on apple shoots expressing *streptavidin* at levels of 3.8 μM and above (Markwick *et al.* 2003).

Crown gall is a significant agricultural problem worldwide. *A. tumefaciens*, a ubiquitous soil bacterium, genetically transforms plant cells to grow as tumors; therefore, after a few hours of infection the disease will progress even if the tumor-inducing bacteria are killed with antibiotics. Thus, prevention is the only effective way to control crown gall. Tumors on stems and leaves result from excessive production of the phytohormones auxin and cytokinin in plant cells genetically transformed by *A. tumefaciens*. High phytohormone levels result from expression of three oncogenes transferred stably into the plant genome from *A. tumefaciens*: *iaaM*, *iaaH*, and *ipt*. The *iaaM* and *iaaH* oncogenes direct auxin biosynthesis, and the *ipt* oncogene causes cytokinin production. In contrast to other tissues, roots do not respond to high cytokinin levels, and auxin overproduction is sufficient to cause tumor growth on roots. Inactivation of *iaaM* abolished gall formation on apple tree roots. Transgenes designed to express double-stranded RNA from *iaaM* and *ipt* sequences in 'Jonagold' prevented crown gall disease on roots of transgenic apple trees (Viss *et al.* 2003). Thus, silencing the *A. tumefaciens iaaM* oncogene could provide an effective means to prevent crown gall disease in perennial plants such as apple trees.

**Dwarfing and rooting ability:** Dwarfing is the second major trait to have been GE in apple.

Vegetatively propagated plants depend on a high rooting capacity or have to be grafted on rootstocks. Clearly such a rootstock cultivar has to have, besides its specific growth characteristics, including induction of the desired size of tree, a good rooting ability. Apple rootstocks are propagated by stool layering, seldom by rooting of cuttings, which is weak in most rootstocks, even with the use of auxin. Root-inducing genes have been characterized in *Agrobacterium rhizgenes* (*rol*A, *rol*B, and *rol*C), and cause the "hairy root" disease in the host. The principal research on GE of apple rootstocks with the *rol* genes have been carried out by Welander's group at the Department of Horticulture, Swedish University of Agricultural Science, Alnarp (**Table 4**). The *rolB* gene has been successfully expressed in several apple rootstocks: 'M.26' (Welander *et al.* 1998), 'M.9' (Zhu *et al.* 2001b), and 'Jork9' (Sedira *et al.* 2001; Pawlicki-Julian *et al.* 2002; Sedira *et al.* 2005) and in the apple cultivar 'Florina' (Radchuck and Korkhovoy 2005). Resulting transgenic apple rootstocks and cultivars showed enhanced rooting in general, and an increased number of roots per explant (Welander *et al.* 1998; Sedira *et al.* 2001; Zhu *et al.* 2001b; Pawlicki-Julian *et al.* 2002; Radchuck and Korkhovoy 2005). However, growth was reduced in some rootstocks compared to the controls (Zhu *et al.* 2001b; Pawlicki-Julian *et al.* 2002). In contrast, the transgenic apple 'Florina' does not show any phenotypical difference after several months of growth *in vitro* or in the greenhouse compared to the control (Radchuck and Korkhovoy 2005). The possible explanation for the differential effect of *rolB* on growth may be that Radchuck and Korkhovoy (2005) grew plants for two years, whereas Zhu *et al.* (2001b) grew them for four months and under a limited supply of nutrients compared with steady-state nutrient supply conditions (Zhu and Welander 2000). The apple rootstocks expressing the *rol*B gene are more sensitive to auxin (IBA) compared to the controls (Zhu *et al.* 2001b; Sedira *et al.* 2005). Zhu *et al.* (2001a) also reported that the expression of the *rolA* gene increased sensitivity to auxins of the transgenic 'M.26' lines compared to the controls. Expression of *rolA* altered the growth

capacity of transgenic 'M.26' (Holsfors *et al.* 1998) and A2 (Zhu *et al.* 2001a). Some of these transgenic rootstocks showed a reduction in length, leaf area and dry weight. Similar results were observed with the expression of either the *rol*C or *phytochrome B* gene in the apple rootstocks 'Marubakaidou' (Igarashi *et al.* 2002) and 'M.26' (Holefors *et al.* 2000), respectively.

A delay in flowering and strongly reduced fertility had been previously reported in some *rolA* transgenic plants. However, this was not observed in the apple cv. 'Gravenstein', grafted on the *rolA*-transformed 'M.26' rootstock (Zhu *et al.* 2001a). 'Gravenstein' flowered on both the *rolA* transformed rootstock and on the non-transformed rootstock in the second year after grafting, and flowers were normal in greenhouse (Zhu *et al.* 2001a). In apple, the side effects of the *rolA* gene might not be transmissible to a scion cultivar, as shown by the preliminary results of Zhu *et al.* (2001a). Currently, Zhu *et al.* (2007) have reported that the transgenic rootstocks 'A2'-*rolA,* 'M.26'-*rolB*, and 'M9'/29-*rolB*, grafted with different apple cultivars are already in field trials to evaluate effects of rootstocks on growth and development of scion cultivars, and the possible transport of the *rolB* protein from rootstock to scion cultivars. Their preliminary results showed that, for the same cultivar, no differences in bud break, flowering and flower numbers were found between the transgenic and non-transgenic rootstocks. The plant height and stem diameter were reduced for the vigorous cultivars grafted on the *rolB* rootstocks compared to the non-transformed rootstocks (Zhu *et al.* 2007). In addition, no transgene was found in the non-transformed scion cultivars grafted on the *rolB* rootstocks (Zhu *et al.* 2007).

With the same goal in mind, dwarf (reduced internode length) 'Greensleeves' scions were produced by silencing an endogenous GA 20-oxidase (Bulley *et al.* 2005). The size of the transformed trees ranged from 50 to 80% of non-transformed controls and interestingly the dwarfing effect was retained after grafting onto normally vigor-inducing rootstocks ('M.25' and 'MM.106').

**Fruit ripening:** Good storage potential is an important quality of any modern dessert apple cultivar. While controlled atmosphere storage technology and chemicals such as 1-methylcyclopropene (MCP) can mitigate poor storage in some cultivars, these technologies have drawbacks in terms of energy use, refrigeration and atmosphere control, and expense, and chemical application is subject to regulatory control. A number of genes have been identified which play a determinate role in the ripening and softening of fruit; but 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.

Transformed 'Gala', 'McIntosh' and 'Greensleeves' apple cultivars down-regulated for either ACS or ACO have been produced (Hradzina *et al.* 2003; Dandekar *et al.* 2004; Schaffer *et al.* 2007). Both Dandekar *et al.* (2004) and Defilippi *et al.* (2004) analyzed fruit from the same or similar transformed apple lines grown with greatly reduced ethylene biosynthesis. They both observed greatly increased shelf life and increased firmness compared to controls. Dandekar *et al.* (2004) reported no significant difference in soluble solids (sugar) and acidity at harvest and after storage, whereas sugar and acid composition was different from controls in fruit stored without ethylene treatment but was no different from controls for ethylene treated fruit (Defilippi *et al.* 2004). Volatile ester production was suppressed. These volatile esters are important components of the fruit flavor complex, but a similar level of suppression of volatile ester production was observed when control fruit was treated with MCP (Defilippi *et al.* 2005a, 2005b). Ethylene treatment allowed ester and alcohol synthesis to recover to 70% of that of control fruit values, whereas ester and alcohol synthesis only slightly recovered in MCP treated fruit (Defilippi *et al.* 2005a, 2005b). In a separate study, Schaffer *et al.* (2007) found that in ACC oxidase down-regulated 'Royal Gala', ester and alcohol synthesis completely recovered after ethylene treatment. Their microarray data revealed that ethylene controlled only the last biosynthetic steps of aroma biosynthesis. Genotypic factors such as ethylene sensitivity might explain the difference in recovery of ester and alcohol synthesis. Being able to apply ethylene to control ripening offers a situation similar to that already being used for banana, and increased storage life without refrigeration could allow large energy savings. These studies have provided valuable insights into the complexities of fruit ripening and flavor development. However, as ethylene is an important phytohormone that is also involved in mediating plant response to stress (flooding, drought, chilling, wounding, and pathogen attack), flower opening and abscission, more work is needed for commercial applications to be realized. These studies can be viewed from the point of view of commercial use but they also contribute highly to our understanding of the ripening processes in apple. Others do not have such an evident potential for commercial application, but merit mention as they contribute to knowledge of physiological processes in apple. Cheng *et al.* (2005) demonstrated the plasticity of the apple photosynthesis system by using antisense inhibition of sorbitol synthesis in GE apple; Kanamuaru *et al.* (2004) were able to determine that sorbitol 6 phospahte dehydrogenase genes (S6PDH) is a key enzyme regulating partitioning between sorbitol and sucrose in apple leaves. Atkinson *et al.* (2002) overexpressed polygalacturonase and obtained a range of new phenotypes, altering leaf morphology, plant water relation, stomatal structure and function, as well as leaf attachment.

Underexpression of polyphenoloxidase (PPO) (catechol oxidase), the enzyme responsible for enzymatic browning of apples, by use of an antisense PPO gene clearly led to reduced calli browning (Murata *et al.* 2000) and shoots had a similarly lower tendency for browning through the PPO activity (Murata *et al.* 2001). Such fruit would be attractive to both the consumer and the food processing industry. Various commercial apple cultivars were transformed with a chimeric gene with the aim of producing a non-browning phenotype.The gene was comprised of sequence elements from four divergent apple PPO genes under control of a constitutive promoter. Coordinated down regulation of the entire apple PPO gene family, reduced levels of total PPO enzyme activity in the leaf and fruit of these transformed lines (>90%) and non-browning phenotypes were achieved in multiple lines of several apple cultivars [Okanagan Specialty Fruits ("OSF"), unpublished results]. Multiple years of field testing of this material confirmed the stability of the non-browning phenotype and have identified no negative impacts on horticultural traits, or on resistance to diseases and insects when grown under field conditions. The non-browning technology developed at OSF has been incorporated into a new enabling platform that: (i) eliminates the selectable marker, (ii) removes all interfering IP, (ii) uses only plant derived gene sequences and control elements, and (iv) improves the efficiency of gene silencing. Plants arising from this series of transformations are now entering field trials (Armstrong and Carter 2008).

**Novel healthful properties:** Polyphenol constituents derived from fruits like apple are more effective antioxidants *in vitro* than vitamins C and E, and thus may be more valuable for protection *in vivo*. Therefore, the active ingredient in "an apple a day keeps the doctor away" may well be among the phytochemical components of the apple fruit. The phytochemical components include flavonoids, phenylpropanoids, and phenolic acids and have been highlighted as important contributing factors in the antioxidant activity in our diet (Rice-Evans *et al.* 1997). In apple, flavonols like quercetin and flavones like rutin may be important (Rice-

Evans *et al.* 1997). Indeed, quercetin derived from apple has potential anticarcinogenic and neuroprotective effects (Lee *et al.* 2005). Stilbene synthase is an enzyme responseble for the synthesis of phytoalexin resveratrol. The production of resveratrol is related to fungal infection and abiotic stress (Chung *et al.* 2003; Rudolf and Resurreccion 2005) such as UV light and ozone. Stilbenes, in general, and resveratrol, in particular, are biologically active compounds, which have antifungal activities against various pathogens, among them *V. inaequalis* (Schulze *et al.* 2005). In addition to these implications for disease resistance, resveratrol and its glycosides have attracted interest as health promoting agents because of their anti-inflammatory, estrogenic, antiplatelet, and anticarcinogenic activities. The biological and pharmacological activities of resveratrol are thought to be due to strong antioxidant properties. Due to the fact that apple has long been recognized as an excellent source of antioxidants, the synthesis of resveratrol in transgenic apple would expand the antioxidant capacity and could therefore be regarded as an additional factor for improving the intrinsic quality of the fruit. Szankowski *et al.* (2003) have produced transgenic apple plants expressing the stilbene synthase gene from grapevine under the control of its own wound-, pathogen-, and UV-inducible promoter. Under greenhouse conditions, these transgenic apple lines are phenotypically normal, and flowered within the first and second years after grafting (Ruhmann *et al.* 2006). Transgenic apple fruit was phenotypically indistinguishable from non-transgenic fruit of the same cultivars (Ruhmann *et al.* 2006). The stilbene synthase gene is expressed in the fruit skin and in the flesh after promoter induction. The introduction of this novel pathway did not dramatically influence the accumulation of other phenolic compounds naturally present in apple fruits (Ruhmann *et al.* 2006). Because of the high antioxidant activity of resveratrol, its synthesis in apple will contribute to fruit quality and might also have positive effects on fruit stability during storage (Ruhmann *et al.* 2006).

Allergy to apple fruits is a common phenomenon in patients with birch pollen allergy. Approximately 90% of patients allergic to birch pollen have IgE antibodies against the birch pollen allergen Bet v 1. This allergen belongs to a group of pathogenesis related proteins, more specifically the PR10 proteins (Puhringer *et al.* 2000). Many plant foods, in particular fruits and tree nuts, contain homologous proteins that are recognized by the same Bet V 1- specific IgE antibodies. In apple, this allergen was designated Mal d 1 (Vanek-Krebitz *et al.* 1995). Approximately 70% of patients allergic to birch pollen have been reported to have adverse reactions to apple as a consequence of the cross-reactive IgE antibodies (Ebner *et al.* 1991). Although birch pollen-related apple allergy is almost exclusively mild and restricted to the oral cavity, most patients allergic to apples avoid the fruit in their diet (Gilissen *et al.* 2005). Related fruits of the Rosaceae family, such as pear, cherry and peach can also induce adverse reactions on the basis of the same cross-reactive IgE antibodies (Ortolani *et al.* 1988). Therefore, avoidance often results in deprivation in the diet of a wide range of common plant foods that have important nutritional value. Production of an apple with a significant reduction of the overall expression of *Mal d 1* from existing economically successful cultivars seems to be an attractive approach. Gilissen *et al.* (2005) chose the approach of RNA interference (RNAi) for post-transcriptional silencing of the gene *Mal d1*. They have isolated one *Mal d 1* gene from 'Gala' and transformed the 'Elstar' apple cultivar with a *Mal d 1* RNAi vector. Normally it takes 3-5 years to grow an apple fruit-producing tree from seed or *in vitro* culture. Because *Mal d 1* genes are expressed in leaves as well as in the apple fruit, Gilissen *et al.* (2005) were able to evaluate *Mal d 1* gene silencing in the leaves of young apple shoots growing *in vitro*. Their results showed a reduction of expression of the *Mal d 1* by immunoblotting. This translated into significantly reduced *in vivo* allergenicity (Gilissen *et al.* 2005). These observations support the feasibility of production, by gene silencing, of hypoallergenic apple cultivars deficient in Mal d 1 (Gilissen *et al.* 2005). These data will need to be confirmed further by analyzing the expression of *Mal d 1* in transgenic fruit and by testing their allergenicity. Since Mal d 1 is a PR protein, *Mal d 1* silenced plants must also be evaluated for undersirable reduction in disease resistance.

Other traits that have been improved with varying success include environmental stress resistance, herbicide resistance, flowering time, self-fertility, colour, and cell adhesion. All these studies are listed and described in **Table 4**.

Almost all work cited in **Table 4**, excluding the few specially mentioned exceptions, relied on the selection of the *npt*II gene, on non-apple gene promoters (CaMV 35S amongst others), and on *A. tumefaciens*-mediated transformation. Often, for experimental purposes, genes not influencing the target trait were used, such as the gene producing GUS. 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. At this time, it is improbable that foreign genes will be acceptable by consumers, and this type of transformed apple will not be commercialized in the near future.

Currently the apple genome is in the process of being sequenced; native apple resistance genes will be discovered and transferred into test cultivars, probably under the control of their own promoters, and, as some studies have reported, RNA interference technology will be able to block selected undesirable traits. Pathogen-derived genes inducing host resistance will be available. Native apple promoters expressing genes only where and when desired will become possible. With the "clean vector technology" (Krens *et al.* 2004; Malnoy *et al.* 2007b) allowing production of transformed plants without selectable markers, it will be possible to produce "intragenic" (Nielsen 2003), "all native" (Rommens 2004) or "cisgenic" (Schouten *et al.* 2006a, 2006b) plants for highly targeted genetic improvement. Many of the concerns about risks, whether mythical or scientifically based, will be obviated. However, until site specific transformation becomes available, the insertion site will not correspond to the original site of the gene and this could lead to epigenetic effects.

The apple cropping system based on artificial vegetative multiplication of the particular genotype and its planting over large areas has rendered this crop genetically vulnerable to many biotic and abiotic stresses. The possibility offered by recombinant DNA technology can be used to replace non-functional resistance alleles with functional resistance alleles (gene therapy). The benefits of GE apples resistant to various diseases will be real, not only for the owner of a patent but also for the consumer, and the environment. The reduction in use of fungicides, antibiotics and insecticides alone justifies all the efforts. It remains to be seen how long it will take until a broad acceptance by the public is achieved.

## *Pyrus*

### *1. Regeneration and transformation*

Development of a system for the production of genetically transformed fruit trees greatly depends on the establishment of a reliable and effective regeneration system. Adventitious shoot formation from leaf explants has been reported for a limited number of mostly commercial genotypes, including *Pyrus communis* cultivars (Chevreau *et al*. 1989; Predieri *et al*. 1989; Abu Qaoud 1991; Leblay *et al.* 1991; Chevreau *et al*. 1993, 1997), *P. pyrifolia* cultivars, *P. syriaca* Boiss. genotypes (Shibli *et al.* 2000), wild pear *P. communis* var. *pyraster* L. (Caboni *et al.* 1999), and quince rootstocks (Dolcet-Sanjuan *et al.* 1991). In all cases, leaves from *in vitro* grown plants have been used as explants and have been exposed to a sequence of dark (2 to 4 weeks) and light exposure. The optimum culture conditions for adventitious

regeneration are summarized in **Table 5**. These techniques involve a very limited callus phase at the site of wounding. Bud regeneration occured in 3 to 6 weeks.

Although sufficient regeneration levels were achieved in some of those genotypes, the regeneration of transformed plants remains a difficult task and seems to be strongly genotype dependent. The first successful gene transfer in the pear was in 1996 (Mourgues *et al.* 1996) some years after the first transformation in the apple (James *et al.* 1989), and to date, transformation of several pear cultivars has been reported (**Table 5**). The techniques of transformation used for these different pear genotypes are quite similar (**Table 6**). Due to the fact that all genotypes do not respond similarly to the same procedure of transformation, some variations have been reported.

**Genetic lines:** *P. communis* L. cultivars 'Conference', 'Doyenne du Comice', and 'Passe Crassane' (Mourgues *et al.* 1996) were the first pear cultivars to be transformed, with efficiency of 0.1 to 42% on a per explant basis. The genus *Pyrus* is comprised of over 25 species. Studies have been centered on development of transgenic procedures for *P. communis* cultivars (11) and rootstocks (3), *P pyraster* (1) and one *P. betulaefolia* (**Table 5**). However, the efficiency of transformation and regeneration capacity is highly dependent on the genetic background (Mourgues *et al.* 1996).

**Nature of explants:** In 75% of the procedures published, young expanded pear leaves were used as explants. The regeneration of transformed lines from leaves has proved to

**Table 5** Genetically transformed pear cultivars.

| Cultivars | Explants used | Hormones used for regeneration, nutrients and sugar | Antibiotic | Inoculation procedure | *Agrobacterium* strains | Selectable marker | Efficiency of transformation | References |
|---|---|---|---|---|---|---|---|---|
| ***Pyrus communis*** | | | | | | | | |
| 1) Ballade | Leaf | TDZ, NAA<br>Nitsch and Nitsch with sucrose | Cefotaxime 500 mg/L | Scalpel cut + immersion for 10-15 min | EHA 101<br>LBA4404 | Kan 30 mg/L | 0.9% | Matsuda *et al.* 2006<br>Wen *et al*. 2007 |
| 2) Bartlett | Cotyledon | NAA, TDZ<br>MS with sucrose | Cefotaxime 300 mg/L | Immersion for 15 min | EHA 101 | Kan 50 mg/L | 2.7%<br>5.2% | Gao *et al*. 2002 |
| | Leaf internode | nd | nd | nd | nd | Kan increasing from 15 then 50 mg/L | 0.2-6.3%<br>1.4-44% | Bommineri *et al*. 2000 |
| 3) Beurre Bosc | Leaf | TDZ, IBA<br>Modified Lepoivre with sucrose | Timentin 300 mg/L | Scalpel cut | EHA 101 | Kan 80 mg/L for 4 weeks only | 2-4% | Bell *et al*. 1999 |
| 4) Burakovka | Leaf | TDZ, NAA $Ga_3$<br>Nitsch and Nitsch with sucrose | Cefotaxime 250 mg/L | Scalpel cut + immersion for 40-50 min | CBE21 | Kan 25 mg/L | 25.4-59.4%<br>11.4-18.4%<br>1.2-1.5% | Lebedev *et al*. 2002a, 2002b |
| 5) Conference | Leaf | TDZ, NAA<br>Modified Lepoivre with sucrose | Cefotaxime 200 mg/L<br>Ticarcilin 100 mg/L | Scalpel cut | EHA 101 | Kan 100 mg/L | 12-42% | Mourgues *et al*. 1996 |
| 6) Doyenne du Comice | Leaf | TDZ, NAA<br>Modified Lepoivre with sucrose | Cefotaxime 200 mg/<br>Ticarcilin 100 mg/L | Scalpel cut | EHA 101 | Kan 100 mg/L | 12-42%<br>0.5%<br>1.3% | Mourgues *et al*. 1996 |
| 7) La France | Cotyledon | TDZ, NAA<br>MS with sucrose | Cefotaxime 300 mg/L | Immersion for 15 min | EHA 101 | Kan 50 mg/L | 2.7%<br>5.2% | Gao *et al*. 2002 |
| | Axillary shoot meristem | TDZ, NAA<br>Nitsch and Nitsch with sucrose | Cefotaxime 500 mg/L | Scalpel cut + immersion for 10-15 min | EHA 101 | Kan 30 mg/L | 1-3.2% | Matsuda *et al*. 2005, 2006 |
| 8) Passe Crassane | Leaf | TDZ, NAA<br>Modified Lepoivre with sucrose | Cefotaxime 200mg/<br>Ticarcilin 100 mg/L | Scalpel cut | EHA 101 | Kan 100 mg/L | 12-1.3% | Mourgues *et al*. 1996 |
| 9) Silver Bell | Leaf | TDZ, NAA<br>Nitsch and Nitsch with sucrose | Cefotaxime 500 mg/L | Scalpel cut + immersion for 10-15 min | EHA 101 | Kan 30 mg/L<br>Kan 5 mg/L | 3.2% | Matsuda *et al*. 2005 |
| 10) Spadona | Leaf | TDZ, NAA<br>MS with sucrose | Ticarcilin 300 mg/L | Scalpel cut + immersion for 20 min | EHA 105 | Kan 50 mg/L | 0.3-0.8%<br>3-4% | Yancheva *et al*. 2006 |
| 11) Vyzhintsa | Leaf | TDZ, NAA<br>MS with sucrose | Cefotaxime 500 mg/L | Immersion | A281 | Kan 50 mg/L | 1.8% | Merkulov *et al*. 1998 |
| **Rootstocks** | | | | | | | | |
| 1) BP10030 | Leaf | TDZ, NAA, BAP, IBA<br>Modified Lepoivre with sucrose | Neomycine 75 mg/L | Wounded with forceps + immersion | C58C1 | Kan decreasing from 100 to 50 mg/L | 0.33-1% | Zhu and Welander 2000b |
| 2) OHF333 | Leaf | TDZ, NAA, BAP, TIBA<br>Modified Lepoivre with sorbitol | Neomycine 75 mg/L | Wounded with forceps + immersion | C58C1 | Kan decreasing from 100 to 50 mg/L | 0.33-1% | Zhu and Welander 2000b |
| 3) GP217 | Leaf | TDZ, NAA $Ga_3$<br>Quorin and Lepoivre with sucrose | Cefotaxime 500 mg/L than 250 mg/L | Immersion | EHA 105 | Kan 25 mg/L<br>Hydromycin 5 mg/L | 0.4-3.1%<br>6.2-11.5% | Lebedev and Dolgov 2000 |
| ***P. pyraster*** | | | | | | | | |
| | Leaf | nd | nd | Scalpel cuts + 2 drops of inoculum added on the leaves | EHA 105<br>C58C1 | Kan 100 mg/L | nd | Caboni *et al*. 2002 |
| ***P. betulaefolia* Bunge** | | | | | | | | |
| | Cotyledon | BA, NAA<br>MS with sucrose | Cefotaxime 500 mg/L | Immersion | AKE10 | Kan 20 then 50 mg/L | 0.9-2.5% | Kaneyoshi *et al*. 2001 |

be enhanced by placing the leaf segment abaxial side up on the medium, possibly due to increased oxygen exchangeThe other explants used were the cotyledon, internode and axillary shoot meristem.

**PGRs:** Three different media have been used to produce the greatest shoot regeneration including the MS medium, Nitsch and Nitsch medium and Modified Lepoivre medium (**Table 5**). These different media were supplemented with 2-3% of sucrose and standard vitamin complements. The major difference from procedure to procedure is in the combination and concentration of phytohormones (auxin and cytokinin) used to induce callus and adventitious shoots.

The form of cytokinin used in most of the *Pyrus* regeneration and transformation procedures is TDZ at different concentrations (**Table 5**). Only one procedure reported the use of BA (Kaneyoshi *et al.* 2001). The type and concentration of auxin is more variable between procedures (**Table 5**). For the regeneration of the pear NAA was used in most of the regeneration protocols (**Table 5**), whereas IBA has been used in the regeneration of few cultivars (**Table 5**).

**Antibiotics:** To limit the development of *A. tumefaciens* during the process of transformation, explant tissues are commonly regenerated on a medium containing an antibiotic. Three antibiotics were used at different concentrations to control the proliferation of *A. tumefaciens* in pear: Carb, Cef and Tic. Cef has been the antibiotic most used in pear with a range of concentration of 200-500 mg/L, followed by Tic and Carb (**Table 5**). Some procedures used a combination of two antibiotics at low concentrations to inhibit the development of *A. tumefaciens*. The combination of Cef and Tic was used for several cultivars (Mourgues *et al.* 1996).

**Inoculation procedure and *Agrobacterium* strain:** Although many pear cultivars were transformed with different genes, almost all procedures (95%) relied on *Agrobacterium* mediated gene transfer, and were quite similar (**Table 5, Fig. 3**). Due to the fact that genotypes do not respond similarly to the same procedure of transformation, some modifications have been reported. The most common strain of *A. tumefaciens* used for pear transformation was EHA105, C58C1 (Zhu and Welander 2000b), A281 (Merkulov *et al.* 1998), CBE21 (Lebedev *et al.* 2002a, 2002b) and AK10 (Kaneyoshi *et al.* 2001).

Inoculation procedures were 1) wounding the pear leaves with a scalpel dipped in inoculum (Mourgues *et al.* 1996), or 2) bathing the leaves in inoculum for a few minutes (Gao *et al.* 2002).

In most cases, transformed shoots took at least 6 months to appear, although Gao *et al.* (2002) obtained transformed shoots within 1.5 months due to the use of juvenile explants. Using cotyledons, the authors concluded that transformants of woody fruit trees can be produced quickly when no specific cultivar is required. The method of Gao *et al.* (2002) involved two pieces of cotyledon from the same seed that were treated individually to produce transformed and non-transformed plants, thereby overcoming the disadvantage of transformants originating from different cotyledons that would differ both genetically and physiologically. However, this technique does not maintain the genetic background of the seed's parent cultivar and has limited potential for pear breeding.

**Selectable markers:** Regeneration of transgenic plants depends upon efficient incorporation of the transgenes and the ability to grow in the presence of the selectable agent. In 95% of cases, Kan has been used as selectable marker. Concentration of Kan used for the regeneration of transgenic pear varied with the cultivar. Depending on the cultivar the concentration of Kan varied from 15 to 100 mg/L. Based on the sensitivity of pear cultivars, selection has been performed for some pear cultivars on lower concentrations of this antibiotic after co-cultivation, followed by increased concentration in subsequent subculture (Bommineni *et al.*

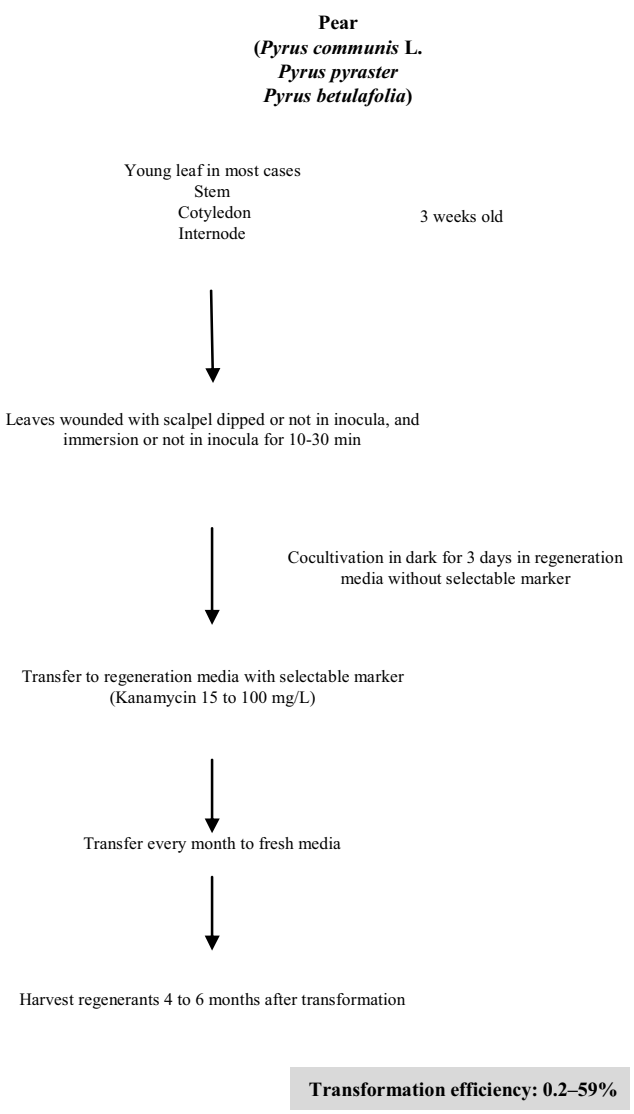


**Fig. 3 Representation of the pear (*Pyrus communis* L., *P. pyraster*, *P. betulafolia)* transformation procedure.**

2000; Kaneyoshi *et al.* 2001). The lower concentration of antibiotic permits a rapid growth of callus and early shoot initiation, while the eventual higher concentration eliminates non-transgenic shoots. The opposite procedure has been also reported, where Zhu and Welander (2000b) initiated the regeneration with a high concentration of Kan (100 mg/L) and after several weeks decreased the concentration to 50 mg/L. Another antibiotic tested as a selectable marker was Hyg (Lebedev *et al.* 2002a, 2002b).

Alternatively, transgenic pear can be produced by the use of marker genes that do not rely on antibiotic resistance but instead promote regeneration after transformation. Chevreau *et al.* (2007) reported a promising effect of the *Vr-ERE* gene (**Table 6**) for the selection of transgenic lines. BA, the substrate of Vr-ERE, is able to inhibit adventitious bud regeneration. BA may be usable instead of Kan but the efficiency of the selection pressure is not yet proven.

**Transformation efficiency:** Early procedures of transformation reported an efficiency of transformation of 0.2 to 60% (**Table 5**).

**Other modifications:** To increase the transformation efficiency, other modifications included the use of a plant phenolic compound (acetosyringone) to induce the expression of several virulence genes in *A. tumefaciens*, and changes in the gelling agent and the binary vector.

## *2. Application of these procedures for pear improvement*

Increased resistance to fire blight is one of the main objectives of pear GE (**Table 6**). Three types of strategies have been adopted : 1) a direct lytic action on the pathogen with transgenes encoding lytic peptides of insect origin (attacin, cecropin) or lysozyme isolated from the T4 bacteriophage (Mourgues *et al.* 1998); 2) a more specific inhibition of pathogenicity factors using a lactoferrin gene of bovine origin (for its possible competition with the bacterial siderophores), or a depolymerase gene (able to specifically degrade bacterial exopolysaccharides); and 3) induction of the pear defense reaction by expressing the Harpin N pathogenicity factor.

**Table 6** Traits expressed in transformed pear cultivars.

| Trait | Apple cultivars | Gene introduced | Gene origin | Promoter | Principal results | Reference |
|---|---|---|---|---|---|---|
| **Bacterial resistance** | | | | | | |
| Fire Blight resistance (*Erwinia amylovora)* | Passe Crassane | *Shiva SB37(+/- sp*) | Synthetic peptide | CaMV35S | Some transgenic lines with partial resistance *in vitro*. | Reynoird *et al* 1999b |
| | | *Attacin E* | *Hyalophora cecropia* (Giant Silk moth) | CaMV35S | Some transgenic lines showed partial resistance *in vitro*. No correlation with the level of attacin expression. | Reynoird *et al* 1999a |
| | | *Attacin E* | *Hyalophora cecropia* (giant silk moth) | Pin2 | Significant reduction of symptoms *in vitro* for the transgenic expression of the T4 Lysozyme. | Malnoy *et al*. 2000 |
| | | *T4 lysozyme* | *T4 Bacteriophage* | CaMV35S | | |
| | | *Lactoferrine* | Bovine | CaMV35S | Most of the transgenic clones demonstrated significant reduction in susceptibility to *E. amylovora in vitro* (17%) and in the greenhouse (60%). These transgenic clones also exhibited a significant reduction of symptoms when inoculated with two other bacterial pathogens of pear, *Pseudomonas syringae* pv. *syringae* and *A. tumefaciens*. | Malnoy *et al*. 2003a |
| | | *Dpo* | *Phage ϕEa1h* | CaMV35S | Only two of 15 transgenic clones consistently showed a decrease in fire blight susceptibility *in vitro* and in the greenhouse. | Malnoy *et al.* 2005a |
| | | *Hrp N* | *Erwinia amylovora* | CaMV35S | Most of the 17 transgenic clones displayed significant reduction of susceptibility to fire blight *in vitro* when inoculated by *E. amylovora*, which was positively correlated to their degree of expression of the transgene $hrpN_{ea}$. | Malnoy *et al.* 2005a |
| **Fungal resistance** | | | | | | |
| | Burokovka | Supersweet protein thaumatin II | *Thaumatococcus daniellii* | CaMV35S | Transgenic plants under evaluation. | Lebedev *et al*. 2002b |
| | | Defensin *Rs-AFP2* | Radis | CaMV35S | Transgenic plants under evaluation. | Lebedev *et al*. 2002a |
| **Insect resistance** | | | | | | |
| *Psylla resistance* | Bartlett | Synthetic lytic peptide *D5C1* | | CaMV35S | Four fold reduction in psylla population levels. | Putekrka *et al*. 2002 |
| **Modified metabolism** | | | | | | |
| Down regulation of ethylene production | La France | *Aminocyclopropane 1 carboxylic (ACC) oxydase sense and antisense* | Pear | CaMV35S | The gene co-suppression was found in almost all antisense lines and one sense line, while the over-expression was observed in other sense lines. The ethylene production in transgenic shoots was consistent with the expression of sense-strand. Ethylene production in *in vitro* shoots was reduced by 85% in an antisense line. *In vitro* flowering and abnormal rooting was found in some antisense shoots. | Gao *et al*. 2002, 2007 |
| **Abiotic stress toloerance** | | | | | | |
| | Ballad | *Spermidine synthase (SPDS)* | *Malus* | CaMV35S | Transgenic line with high expression of MdSPDS1 showed the strongest tolerance to salt (NaCl 150 mM), osmosis (300 mM mannitol) and heavy metal (500 µM $CuSO_4$).<br>Transgenic line showed a higher antioxidant enzyme activities, less malondialdehyde (MDA) and $H_2O_2$ than the wild, resulting in less injury. | Wen *et al*. 2008<br>He *et al*. 2008 |
| **Resistance to herbicide** | | | | | | |
| | GP217 | *Bar* | *Streptomyces* | CaMV35S | No damage of tree in greenhouse treated with 1% solution of the herbicide Basta. | Lebedev *et al* ??? |
| **Rooting ability / dwarfism** | | | | | | |
| | Beurre Bosc | *rolC* | *A. rhizogenes* | *RolC* promoter | Plant in greenhouse with reduction in height, number of nodes, and leaf area | Bell *et al*. 1999 |
| **Flowering time** | | | | | | |
| | BP10030 | *rolB* | *A. rhizogenes* | *RolB* promoter | *rol*B has increased the rooting ability of the dwarfing rootstock BP10030. Transgenic lines had a shortened stem length. | Zhu *et al*. 2003 |
| | La France Ballade | *FT* | | | *In vitro* flowering between 1 and 25 month after regeneration. | Matsuda *et al*. 2006 |
| **Promoter** | | | | | | |
| | Conference | | Tobacco | Sgd24<br>Str246<br>Hsr203J<br>CaMV35S | Sgd24 promoter was locally activated in response to pathogens but not in response to abiotic stress; Str246C promoter was systemically activated in response to pathogens and abiotic stress;<br>Hsr203J was not functional. | Malnoy *et al.* 2003b |
| **Selectable marker** | | | | | | |
| | Conference | *Vr-ERE* | *Vigna radiata* | CaMV35S | Benzaldehyde (BA) is able to inhibit adventitious bud regeneration. BA can be used instead of Kan but the efficiency of the selection pressure is not yet proven. | Chevreau *et al*. 2007 |

Transformation experiments with these genes have produced a total of more than 100 transgenic clones from the cultivar 'Passe Crassane'. Semi-quantitative RT-PCR revealed important differences among clones expressing the attacin E gene. These differences correlated very well with the differences of attacin E accumulation revealed by western blot analysis. However, no clear correlation between transgene expression and resistance to fire blight as determined by *in vitro* inoculation was established (Reynoird *et al.* 1999a). Among 16 transgenic diploid clones expressing the depolymerase gene, only two clones showed a slight reduction in fire blight symptoms in comparison to non-transformed plants. This partial resistance was correlated with a stronger expression of the transgene at transcriptional and translational levels. Very low depolymerase activity was detected in most transgenic clones (Malnoy *et al.* 2005a). Of eight transgenic clones expressing the lactoferrin gene, four expressed significantly less fire blight symptoms than the control. The increase of resistance was detected more strongly in the greenhouse than *in vitro*. A correlation with a mechanism of iron chelation was demonstrated by: 1) an increase of total iron content and ferric reductase activity in the transgenic plants and 2) an inhibitory activity of protein extracts from transgenic plants towards *E. amylovora* in conditions of iron deficiency (Malnoy *et al.* 2003a). Of the 17 transgenic clones of the very susceptible cultivar 'Passe Crassane' carrying the $hrpN_{ea}$ gene, most displayed significant reduction of susceptibility to fire blight in vitro when inoculated with *E. amylovora*, which was positively correlated to their degree of expression of the transgene (Malnoy *et al.* 2005b). In order to obtain a more efficient and targeted expression of these transgenes, inducible promoters were sought. GUS fusions have been transformed into the cultivar 'Conference' to analyze the expression of several pathogen-inducible promoters from tobacco (Malnoy *et al.* 2003b).

Transformation of the cultivar 'Bartlett' with another lytic peptide gene (*D5C1*) has been reported and its effect on a nontarget pest (pear psylla) was reported (Puterka *et al.* 2002).

A strategy to increase pear disease resistance, which is currently being tested by a Russian team, is the introduction of plant defensin genes from *Raphanus sativus* (Lebedev *et al.* 2002a). The same group is also evaluating herbicide resistance of transgenic pear rootstocks transformed with the phosphinotricin acetyl transferase (PAT) gene (Lebedev *et al.* 2002b), and the modification of fruit taste conferred by the supersweet gene thaumatin II (Lebedev *et al.* 2002c).

A recent strategy has shown that it is also possible to increase the tolerance to salt (NaCl 150 mM), osmosis (300 mM mannitol) and heavy metal (500 µM $CuSO_4$) by over-expressing the MdSPDS1 gene in pear (Wen *et al.* 2008).

The *rolC* gene from *Agrobacterium rhizogenes* has been introduced into the cultivar 'Beurre Bosc' under the control of its native promoter to induce a dwarfing effect (Bell *et al.* 1999). The first observations on self-rooted or budded plants in the greenhouse indicated reduced height, number of nodes and leaf area of three transgenic clones. A similar approach has been pursued by Zhu and Welander (2000b), who introduced the *rolB* gene in a dwarfing rootstock genotype (BP10030), which is very difficult to root. Recent results from cutting experiments in the greenhouse indicated a clear increase in rooting ability in the transgenic lines, together with a modification of root morphology and shortened stem length (Zhu *et al.* 2003).

The gene encoding S-adenosylmethionine hydrolase (*sam-k*) from bacteriophage T3 has been transferred into the cultivar 'Bartlett' in order to modify ethylene biosynthesis and improve post-harvest quality and shelf life (Bommineni *et al.* 2000).

Modification of the flowering time was achieved by over-expressing the *ft* gene in two pear cultivars (Matsuda *et al.* 2006). Their preliminary results showed flowering of the *in vitro* shoots between 1 and 25 months after regeneration.

It appears from these different reports that all the genes used to transform pear are genes from other organisms; only the work of Wen *et al.* (2007) used a gene from a related species (*Malus*).

According to the APHIS Field Test Releases Database, two groups have already released transgenic pears for field trials in the USA. The USDA-ARS in West Virginia has released transgenic pears carrying a cecropin gene for fire blight resistance, and the *rolC* gene for dwarfing. Agritope, in Oregon, released transgenic pears carrying the *sam-k* gene for delayed fruit ripening. In Europe field trials of transgenic pear expressing the *rol* gene are being undertaken in Sweden (Zhu *et al.* 2007).

## *Prunus*

### 1) *Prunus dulcis* (almond)

The first report of an *in vitro* regeneration and genetic transformation system for the cultivated *Prunus dulcis* was by Miguel and Oliveira (1999). In this first report, only two cultivars of almond were transformed (**Table 7**). Genotype and explant tissue are both important variables in the regeneration process.The four youngest, fully expanded leaves of almond seedlings were the tissue that gave the highest percentage of regeneration (Miguel and Oliveira 1999). Preculturing these leaves for 3 days in the dark on MS medium before inoculation increased the efficiency of transformation (Miguel and Olivereira 1999). The MS medium was also used as regeneration medium supplemented with 2-3% sucrose (**Table 7**), standard vitamins, and a growth regulator. The form of cytokin used in most of the almond regeneration and transformation procedures was TDZ (Miguel and Olivereira 1999; Costa *et al.* 2006) (**Table 7**). For one cultivar, BA was used (Ainsley *et al.* 2001; Ramesh *et al.* 2006). The type and concentration of auxin is variable among procedures (**Table 7**). For regeneration of 'Boa Casta' and 'Clone VII' IAA and 2,4-D were used, and for NE Plus Ultra, IBA was used (**Table 7**). Cef at different concentrations depending on the cultivars was also added to the regeneration medium to limit the development of *A. tumefaciens*.

Three almond cultivars were transformed with procedures that rely on *Agrobacterium* mediated gene transfer, and are quite similar (**Table 7, Fig. 4A**). *A. tumefaciens* EHA105 was the strain used in 75% of the transformation reports. Strain LBA4404 was also used. Two modes of infection with *Agrobacterium* were used, 1) wounding the explants with a scalpel, or 2) co-cultivation for 1h. Inoculation was followed by 3 days in darkness on varied selective media. The selectable marker used was Kan. Based on the sensitivity of the almond tissue, selection was generally performed at lower concentrations (15 mg/L) of Kan after co-cultivation, followed by an increased concentration in subsequent subculture (Costa *et al.* 2006). Alternatively, transgenic almond can be produced by the use of marker genes that do not rely on antibiotic or herbicide resistance but instead promote regeneration after transformation. Ramesh *et al.* (2006) have shown a promising effect of the *pmi* gene for the selection of transgenic lines. The positive (mannose/*pmi*) and the Kan selection protocols used in this study have greatly improved transformation efficiency in almond. Indeed, the efficiency in this study was a little increased from 5.6 to 6.8%. However, the efficiency was not comparable to the efficiency of 12% described by Costa *et al.* (2006).

No application of the transformation procedure has yet been reported in *Prunus dulcis* to study the function of any genes.

### 2) *Prunus armeniaca* (apricot)

Laimer da Camara Machado *et al.* (1992) published the first report of *in vitro* regeneration and genetic transformation systems for the cultivated *Prunus armeniaca* cultivar 'Kecskemeter'. However, at this date, only two other reports have been published about the transformation of another apricot

cultivar 'Helena' (**Table 7**). The transformation procedures for the two apricot cultivars were completely different (**Table 7, Fig. 4B**). The tissue chosen for regeneration was the youngest fully expanded leaves (Petri *et al.* 2004; Lopez-Noguera *et al.* 2006) and the immature embryo (Laimer da Camara Machado *et al.* 1992). Two different media

**Table 7** *Prunus* cultivars transformed genetically.

| Cultivars | Explants used | Hormones used for regeneration, nutrients and sugar | Antibiotic | Inoculation procedure | *Agrobacterium* strains | Selectable marker | Efficiency of transformation | References |
|---|---|---|---|---|---|---|---|---|
| ***Prunus dulcis* (almond)** | | | | | | | | |
| 1) Bao Casta | Leaf pre-cultured for 3 days in MS medium | TDZ, IAA, 2,4-D MS with sucrose | Cefotaxime 200 mg/L | Leaf wounded by 4-5 cuts perpendicular to midrib with scalpel dipped in *Agrobacterium*. | LBA4404 EHA105 | Kan 15 mg/L | 0.1% | Miguel and Olivereira 1999 |
| 2) Clone VII | Leaf pre-cultured for 3 days, in dark, in MS medium | TDZ, IAA, 2,4-D MS with sucrose | Cefotaxime 300 mg/L | Leaf wounded by 4-5 cuts perpendicular to midrib with scalpel dipped in *Agrobacterium*. | EHA105 | Kan 15 mg/L then 30 and 50 mg/.L | 12.3% | Costa *et al*. 2006 |
| 3) Ne Plus ultra | Leaf pre-cultured for 3 days in MS medium | IBA, BAP MS with sucrose and mannose | Cefotaxime 630 µM | Leaf co-cultivated with *Agrobacterium* for 1 h at 28°C. | EHA105 | Mannose Kan 15 mg/L | 6.8% 5.6% | Ainsley *et al*. 2001 Ramesh *et al*. 2006 |
| ***P. armeniaca* (apricot)** | | | | | | | | |
| 1) Kecskemeter | Immature embryo | 2,4-D, BA MS with sucrose | Carb 250 mg/L | Embryos immerged 5-10 sec in *Agrobacterium*. | LBA4404 | nd | nd | Lamier da Camara Machado *et al*. 1992 |
| 2) Helena | Leaf | TDZ, NAA Quoirin and lepoivre with sucrose | Cefotaxime 0.63 mM and Vancomycin0 .13 mM. | Leaf incubated 10 min in *Agrobacterium* suspension and after cut in 3-4 pieces with scalpel. | EHA105 | Kan 22.7 µM | 2.1-8.5% | Petri *et al*. 2004; Lopez-Noguera *et al*. 2006 |
| ***Prunus cerasus* L. (cherry)** | | | | | | | | |
| 1) Montmorency | Leaf pretreated with TDZ 0.1 mg/L | IBA, BA, NAA MS with sucrose | Cefotaxime 250 mg/L | Leaf wounded 3-4 times with scalpel perpendicular to midrib and immersed in *Agrobacterium* for 30 min. | EHA105 | Kan 50 mg/L Kan 25 mg/L then 50 mg/L | 3.1% 7.6% | Song and Sink 2005, 2006, 2007 |
| 2) Gisela (*P. cerasus x P. canescens*) | Leaf | TDZ, IBA, MS with sucrose | TImentin 500 mg/L | Leaf wounded 3-4 times with scalpel perpendicular to midrib and immersed in *Agrobacterium* for 30 min. | EHA105 | Kan 50 mg/L Kan 25 mg/L then 50 mg/L | 3.9% 9.7% | Song and Sink 2006, 2007 |
| 3) Colt (*P. avium)* Mazzard F12 | Shoot | NAA, BAP MS with sucrose | Cefotaxime 200 mg/L | Shoot wounded in the middle with scalpel dipped in *Agrobacterium*. | *A. rhizogenes* | | | Gutierrez-pesce *et al*. 1998 ; Gutierrez-pesce and Rugini 2004 |
| ***P. subhirtella* (ornamental cherry)** | | | | | | | | |
| | Embryo | NAA, IBA, 2-4D MS with sucrose | Carb 250 mg/L | Embryos immersed 5-10 sec in *Agrobacterium*. | LBA4404 | Kan 100 mg//L | nd | Da Camara *et al*. 1995; Maghuly *et al*. 2007 |
| ***P. incise x serrula* Inmil (rootstock)** | | | | | | | | |
| | Shoot | NAA, BAP MS with sucrose | nd | Shoot wounded in the middle with scalpel dipped in *Agrobacterium*. | *A. rhizogenes* | | nd | Druart and Gruselle 2007 |
| ***Prunus persica* L. (peach)** | | | | | | | | |
| *Transient expression* | | | | | | | | |
| 1) Akatsuki | Protoplast from mesocarp | | | Electroporate plasmid in to protoplast | | | | Honda *et al*. 2006 |
| *Stable expression* | | | | | | | % of explant with GFP expression | |
| 1) VBailey, 2) Lady Nancy, 3) Harrow Beauty, 4) KV930465, 5) KV930408, 6) KV930303, 7) KV930455, 8) KV930478, 9) KV930311 | Internode Cotyledon | NAA, BA MS then Quoirin and lepoivre with sucrose | Timentin 300 mg/L | Explants were immersed in resuspended Agrobacterium for 20 min, blotted briefly on sterile paper and placed on cocultivation medium. | EHA105 | Kan 80 mg.L | 6-12 9-18 0-3.9 2.8-3.2 2.7-28 7.5-26 4-12 0-5 3.6-9.5 | Padilla *et al*. 2006 |
| 10) Miraflores | Embryo section | TDZ, IAA Quoirin and Lepoivre with sucrose 3% | Cefotaxime 500 mg/L | Embryo immersed 15 min in suspension of *A. tumefaciens* with gentle shaking. | C58 | Kan 40 mg.L | 77.6 Efficiency transformation 3.6% | Perez-Clement *et al*. 2004 |
| 11) Redhaven | Embryo | 2,4 D, NAA, BA MS with sucrose | nd | Embryo wounded with forceps cocultivated overnight with *Agrobacterium*. | Mutant shoot strain tms328::Tn5 | nd | 4.1% | Smigochi and Hammerschlag 1991 |

**Table 7 (Cont.)** *Prunus* cultivars transformed genetically.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ***Prunus domestica* L. (plum)** | | | | | | | | |
| 1) B70146 | Hypocotyl | TDZ, IBA MS with sucrose | Carb 500 mg/L Cefotaxime 200 mg/L | Hypocotyl co-cultivated with *Agrobacterium* | EHA101 | Kan 75 mg./L | | Mante *et al*. 1991 |
| 2) B69158 | Hypocotyl | TDZ, IBA MS with sucrose | Carb 500 mg/L Cefotaxime 200 mg/L | Hypocotyl immersed in *Agrobacterium* for 10-20 min | EHA101 | Kan 75 mg./L | 4.8% | Scorza *et al*. 1994 |
| 3) Stanley Bluebyrd | Hypocotyl | TDZ, IBA MS with sucrose | Timentin 300 mg/L | Hypocotyl immersed in *A. tumefaciens* for 20 min | EHA105 | Kan 80 mg/L | 0.4-4.2% | Padilla *et al*. 2003 |
| | | | | | EHA105 GV3101 | | 4-42% | Petri *et al*. 2008 |

have been used to produce the greatest shoot regeneration, including MS, and Quorin and Lepoivre medium (**Table 7**). These media were supplemented with 2-3% sucrose, standard vitamin complements, auxin and cytokinin, and antibiotics (**Table 7**).

The two apricot cultivars were transformed with a procedure that relies on *Agrobacterium* mediated gene transfer (**Table 7**). The strains of *A. tumefaciens* used in the transformation protocol were EHA105 and LBA440. One mode of infection with *Agrobacterium* was the immersion of the explants in the inoculum for a few seconds to 10 min. The selectable marker used was Kan. The efficiency of transformation obtained with this procedure was up to 8.5%.

No application of the transformation procedure has yet been reported in *Prunus armeniaca* to study gene function.

### 3) *Prunus cerasus* L. and *P. subhirtella* (cherry)

Only three reports of transformation of cultivated cherry, one ornamental cherry, and one rootstock have been published (**Table 7**). The transformation procedures for these cherry cultivars were quite similar (**Table 7, Fig. 4C**). Genotype and explant tissue are important variables in the regeneration process. Many cherry shoots have been successfully regenerated from several tissues, including leaves (Song and Sink 2005, 2006), shoots (Gutierrez-Pesce *et al.* 1998; Gutierrez-Pesce and Rugini 2004) and embryos (Da Camara *et al.* 1995) (**Table 7**). MS is the most commonly used regeneration medium for the regeneration of transformed cherry. It was supplemented with 2-3% sucrose (**Table 7**), standard vitamins, and PGRs (**Table 7**).

Three of five cherry cultivars were transformed with a procedure using *A. tumefaciens* mediated gene transfer. Two other protocols used a strain of *A. rhizogenes* (**Table 7**). The strains of *A. tumefaciens* used were EHA105 and LBA440. One mode of infection with *Agrobacterium* was the immersion of the explants in the inoculum for a few seconds to 30 min. The selectable marker used was Kan at different concentrations, depending on the cultivar. Recently, Song and Sink (2007) modified the transformation system for cherry by the addition of a two step selection system, whereby the selection of the transformed shoot was carried out on selection medium starting with a low concentration of Kan (25 mg/L) for 4 weeks, followed by an increased concentration (50 mg/L). Compared with the one step selection using 50 mg/L of Kan, the two step selection minimized necrosis of inoculated leaf explants, and the transformation frequency was increased from 3.1 to 7.6% with the cultivar 'Montmorency' and from 3.3 to 9.7% with 'Gisela' (Song and Sink 2007).

Since trees are particularly suited for long-term evaluations of the impact of transformation, *P. subhirtella* 'Autumno Rosa' was chosen by Maghuly *et al.* (2007) to study the long term stability of the *GUS* reporter gene under the control of the CaMV35S promoter. Using Southern blotting and GUS fluorometric techniques, they compared transgene copy numbers and observed stability of transgene expression levels in 34 different transgenic plants, grown under *in vitro*, greenhouse and screenhouse conditions, over a period of 9 years. An influence of grafting on gene expression was not observed. No silenced transgenic plant was detected. Overall, these results suggest that transgene expression in perennial species, such as fruit trees, remains stable in time and space, over extended periods and in different organs. While the *Agrobacterium*-derived *Prunus* transformants contained one to two copies of the transgenes, 91% of the transgenic events also contained various lengths of the bacterial plasmid backbone, confirming the need to screen against read-through events.

### 4) *Prunus persica* L. (peach)

Four reports on transformation of cultivated peach have been published (**Table 7**), but only two of these reported the regeneration of transformed lines (Smigochi and Hammerschlag 1991; Perez-Clement *et al.* 2004). Peach transformation has been successfully achieved from several tissues, including embryo (Smigochi and Hammerschlag 1991; Perez-Clement *et al.* 2004), cotyledon (Padilla *et al.* 2006) and protoplast (Honda *et al.* 2006) (**Table 7, Fig. 4D**). The MS and Quorin and Poivre media have been used for the regeneration of transformed peach, supplemented with 2-3% sucrose (**Table 7**), standard vitamins, and PGRs (**Table 7**). Most of the peach transformation protocols relied on *A. tumefaciens*-mediated gene transfer (strains EHA and C58); one protocol used a strain of *A. rhizogenes* (**Table 7**). Two modes of infection with *Agrobacterium* were used: 1) immersion of the explants in the inoculum for 15 min, and 2) wounding of the explants with a scalpel. Apart from *Agrobacterium*-mediated transformation, other inoculation procedures have been described, such as protoplast electroporation (Honda *et al.* 2006). The author reported only a transient expression of the *GUS* reporter gene in the transformed cell but no regeneration of transformed lines from the transformed protoplast.

The selectable marker used was Kan at different concentrations, depending on the cultivar. The efficiency of transformation obtained with this procedure was up to 4%.

### 5) *Prunus domestica* L. (plum)

Three different plum cultivars have been transformed (**Table 7**), with similar transformation procedures using hypocotyl explants. MS was the most frequently used regeneration medium, supplemented with 2-3% sucrose (**Table 7**), standard vitamins, antibiotics and IBA and TDZ as PGRs (**Table 7**). This transformation procedure relied on *A. tumefaciens*-mediated gene transfer *via* immersion of the hypocotyl in *A. tumefaciens* strain EHA105 inoculum for 10 to 20 min (**Fig. 4C**). Gonzalez-Padilla *et al.* (2003) modified the procedure by placing the regenerants on medium with (early selection) or without (late selection) Kan for 2 weeks, after 2 days of co-cultivation. After 2 weeks, the explants were transferred to a Kan containing medium. With this change in the protocol they demonstrated that the late selection increased the efficiency of transformation by 1.7-fold,

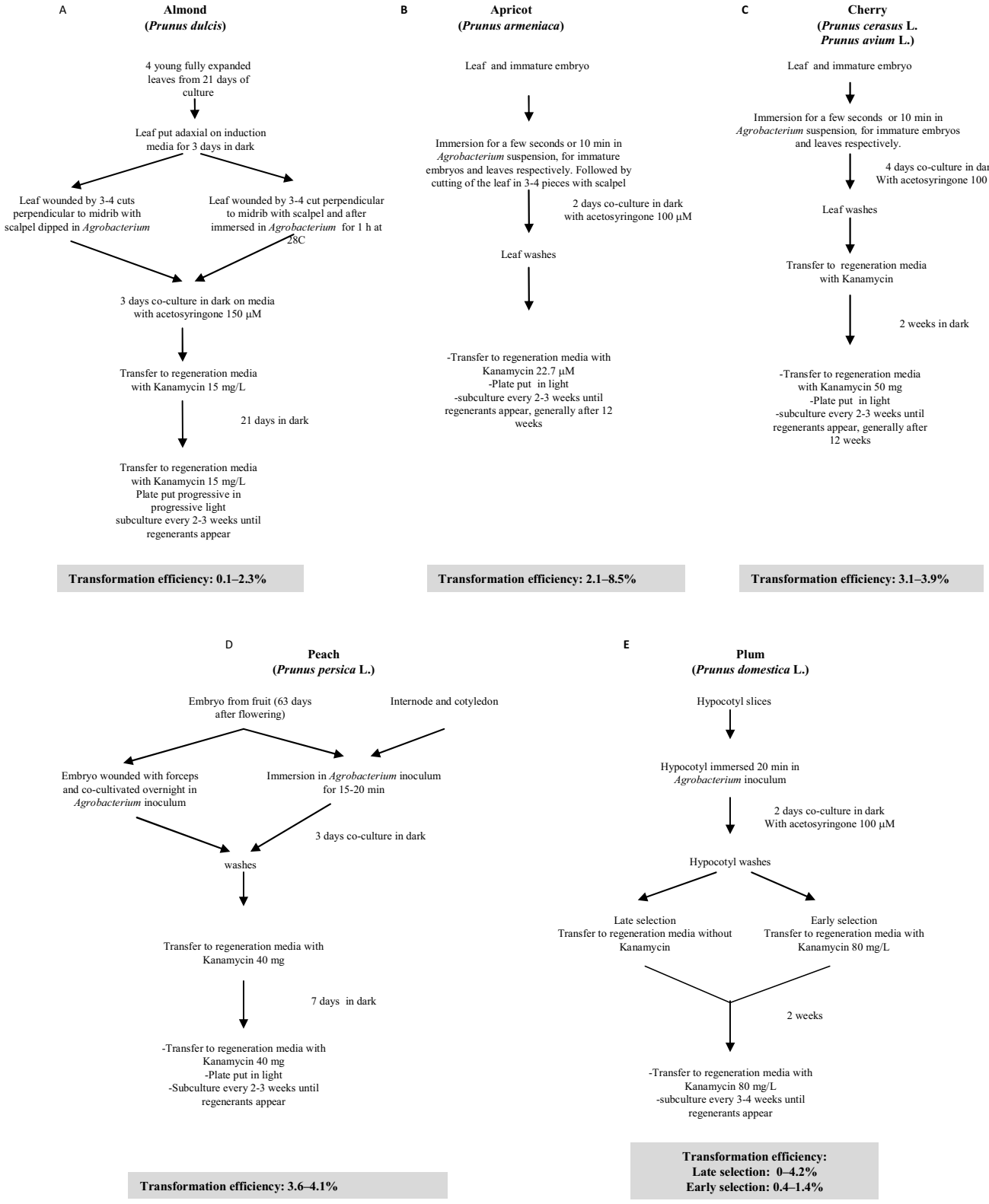


**Fig. 4 Representation of the *Prunus* transformation procedure.** (**A**) Almond (*Prunus dulcis*); (**B**) apricot (*P. armeniaca*); (**C**) cherry (*P.s cerasus* L., *P.s avium*); (**D**) peach (*P.s persica* L.); (**E**) plum (*P. domestica* L.).

compared to early selection. Mante *et al.* (1991) reported that plum hypocotyl slices produced one to five putatively transformed shoots per slice. In Scorza *et al.* (1994), only one regenerated shoot was selected from each hypocotyl slice because in the case of multiple shoot production it was not clear whether these shoots were derived from the same or different transformation events. In late selection, Gonzalez-Padilla *et al.* (2003) also observed multiple shoot formation from the hypocotyl slices, but not in early selection, where only one shoot per slice was the common regeneration pattern. Their results obtained with pGA482GGi/antisenseACO1 showed that only three out of eight of the shoots that formed from the same explant were from unique transformation events. Thus, in the case of late selection it appears that choosing one shoot per explant is warranted. The efficiency of transformation obtained with this procedure was 5 to 42%.

*Plum pox virus* (PPV, genus *Potyvirus*), causing sharka disease of *Prunus*, is responsible for extensive economic losses (Németh 1994; Roy and Smith 1994). Control of PPV has been chiefly through quarantine and eradication of infected trees. These measures have been insufficient to

control the spread of PPV within and between countries and continents. The use of resistant cultivars is a critical control strategy that remains to be implemented. There are two approaches to the development of resistant cultivars. Natural PPV resistance may be exploited by the identification of resistant genotypes, followed by the transfer of resistance genes into new germplasm through hybridization. To assure the durability of resistance and high levels of fruit quality necessary for the market, promising resistant selections must then be extensively field-tested before being released to growers. Genomic studies and genetic markers can speed the selection of putative resistant seedlings and this approach is currently being pursued (Abernathy *et al.* 2004). Even then, the process from hybridization to cultivar release can take many years. Alternatively, resistant cultivars can be obtained by use of transformation with genes for PPV resistance.

In the early 1990s, the successful transformation of *Prunus* (Mante *et al.* 1991) led to efforts to control PPV through transgene-based resistance. A modified PPV coat protein (CP) gene construct designed to express CP was developed (Ravelonandro *et al.* 1992). The results of challenging transformed herbaceous plants demonstrated both a recovery reaction and the apparent immunity of transgenic plants (Da Câmara Machado *et al*. 1992; Ravelonandro *et al.* 1993, 1994; Jacquet *et al.* 1998). These reactions were associated with a down-regulation of the transgene products (Ravelonandro *et al.* 1994; Jacquet *et al.* 1998) suggesting RNA-mediated sense suppression as the basis for resistance. Following the successful development of PPV-CP transgenic PPV-resistant herbaceous plants, PPV-CP gene constructs were transferred into *Prunus* species (Laimer da Câmara Machado *et al.* 1992; Scorza *et al.* 1994). Transgenic plums containing the PPV-CP transgene insert demonstrated various levels of resistance with the highest level shown by clone C5 (today named 'HoneySweet') which contained a multicopy insert and produced low levels of PPV-CP mRNA and no detectable PPV-CP (Scorza *et al.* 1994; Ravelonandro *et al.* 1997). Further molecular analyses of C5 demonstrated that this clone contained aberrant copies of the transgene insert, and that the PPV-CP gene was methylated and silenced. Post transcriptional gene silencing (PTGS) was indicated as the mechanism of resistance (Scorza *et al.* 2001a, 2001b).

To examine the stability and durability of the PTGS-based PPV resistance of C5, plants were tested in the field against infection by bud-grafting with infected buds and by aphid vectors under natural orchard conditions. These field tests were initiated in Europe, under the appropriate permits in Bistrita (RO), Skierniewice (PL) and Valencia (ES) in 1995, 1996, and 1997, respectively. After 10, 9, and 8 years, respectively, these field tests have shown that no C5 plants have been infected by natural aphid vectors. Trees inoculated by infected chip buds or rootstocks have shown only a very low level of infection and with transient symptoms on only a few leaves on a tree. These graft inoculated trees appear to recover and it is difficult to either find symptoms or positive molecular indications of infection (Hily *et al.* 2004; Malinowski *et al.* 2006).

Currently, 'HoneySweet' plum is being evaluated for deregulation by U.S. regulatory agencies so that it may possibly be released in the future to breeders and growers who are concerned about the threat of PPV to U.S. stone fruit production (Scorza *et al.* 2007). Bringing 'HoneySweet' plum to the point of deregulation was based on field test data and research into the resistance mechanism that took approximately 12 years from the time that the first transformations with the PPV-CP gene were initiated. Pre-submission consultations with U.S. regulatory agencies [USDA Animal and Plant Health Inspection Service (APHIS), the U.S. Food and Drug Administration (FDA) and the U.S. Environmental Protection Agency (EPA)] began in 2003. In the U.S., APHIS has general jurisdiction over the planting of GE plants. FDA has jurisdiction over GE plants used as food, and EPA regulates GE crop plantings of over ten acres and regulates GE plants that produce molecules that protect plants against pests (protection against PPV in the case of 'HoneySweet'). An application for determination of non-regulatory status was submitted to APHIS in April 2006 and was approved in 2008. Data packages have also been submitted to FDA and EPA (Scorza *et al.* 2007).

## *Rosa* (rose)

### *1. Regeneration and transformation*

Successful development of regeneration systems for a number of rose species has been reported (reviewed by Rout *et al*. 1999; Khosh-Khui and Teixeira da Silva 2006; Pati *et al.* 2006), but there are only a few published reports on *Agrobacterium*- (Firoozabady *et al*. 1994; Van der Salm *et al.* 1997, 1998; Dohm *et al.* 2001; Kim *et al.* 2004; Li *et al.* 2002, 2003) and bombardment-mediated transformation of roses (Marchant *et al*. 1998a, 1998b) (**Table 8**).

Firoozabady *et al.* (1994) produced transgenic plants by transforming friable embryogenic tissues of rose, induced from filament cultures, using both *A. tumefaciens* and *A. rhizogenes*. Van der Salm *et al.* (1997) obtained transgenic plants from roots derived from stem sections of the rootstock *Rosa hybrida* cv. 'Moneyway' following cocultivation with *A. tumefaciens* strain GV3101 containing an *nptII* gene and a *rol* gene from *A. rhizogenes*. Of these two procedures (**Table 8, Fig. 5**), the procedure of Firoozabody was the one chosen with modification to transform 5 other rose cultivars (**Table 8**). These modifications concerned 1) the nature and concentration of the growth regulator and antibiotic used for limiting the concentration of *A. tumefaciens*, 2) the strain of *A. tumefaciens*, and 3) the concentration of selectable marker which was Kan for all procedures (**Table 8**). Firoozabady *et al.* (1994) have used Carb to eliminate *Agrobacterium* from transformed friable embryogenic tissues in *R. hybrida* cv. 'Royalty'. Derks *et al.* (1995) used Cef to eliminate *Agrobacterium* from transformed somatic embryos in *R. hybrida* cv. 'Sonia'. Van der Salm *et al.* (1997) have also used cef to eliminate *Agrobacterium* from transformed stem sections of *R. hybrida* cv. 'Moneyway'. Li *et al.* (2002) found that adding Carb at any concentration to any of the tissues used, including leaf, undifferentiated callus, and embryogenic callus completely inhibited shoot regeneration of *R. hybrida* cv. 'Carefree Beauty'. Using Cef at both 250 and 500 mg/L had a negative effect on callus induction from leaf tissue of 'Carefree Beauty'. After callus had been induced from leaf tissue, adding Cef at 250 mg/L did not inhibit somatic embryogenesis; but increasing the concentration to 500 mg/L resulted in inhibition of callus differentiation. In addition, Cef at 250 mg/L did not inhibit the induction of secondary somatic embryos, and their subsequent regeneration. Firoozabady *et al.* (1994) kept transformed friable embryogenic tissues on a selection medium containing Carb for 2 months. Thus, the effect of Carb appears to be genotype-dependent.

A concentration of 50 mg/L Kan was used for selection of transformed leaf tissue, and a concentration of 60 to 100 mg/L Kan was used for selection of both transformed embryogenic callus and secondary somatic embryos (**Table 8**). Both Firoozabady *et al.* (1994) and Derks *et al.* (1995) have used 300 mg/L Kan for selection of putative transformed embryogenic callus of rose via *Agrobacterium*-mediated transformation. Marchant *et al.* (1998a) used a concentration of 250 mg/L Kan for selection of putative transformed embryogenic callus of *R. hybrida* following biolistic bombardment. However, van der Salm *et al.* (1997) used only 5 mg/L Kan for selection of root formation from transformed stem sections already undergoing early stages of somatic embryogenesis. From all the above studies, Kan appeared to have a higher inhibitory effect on regeneration during early rather than later stages of cell differentiation. An efficiency of transformation of up to 5% was obtained with this procedure.

**Table 8** *Rosa* varieties transformed genetically.

| Cultivars | Explants used | Hormones used for regeneration, nutrients and sugar | Antibiotic | Inoculation procedure | *Agrobacterium* strains | Selectable marker | Efficiency of transformation | References |
|---|---|---|---|---|---|---|---|---|
| 1) Carefree Beauty | Embryogenic callus | TDZ, $GA_3$, BA MS with sucrose | Cefotaxime 500 mg/L | Embryogenic calli injured with multi-prong needle and immersed 30 min in *Agrobacterium* | GV3101 | Kan 100 mg/L | 9% | Li *et al*. 2002, 2003 |
| 2) Gad Tidings | Embryogenic callus | 2,4-D, $GA_3$ MS with sucrose | none | Biolistic | | Kan 250 mg/L | 15.6% | Marchand *et al.* 1998a, 1998b, 1996 |
| 3) Heckenzauber 4) Pariser charme | Embryogenic callus | IBA, BAP, $GA_3$ MS with sucrose | Cefotaxime 200 mg/L Carb 50 mg/L then Timentin 500 mg/L | Embryogenic calli immerged 1h in *Agrobacterium* | EHA105 GV2260 | Kan 60 mg/L | 2-3% | Dohm *et al*. 2001 |
| 5) Moneyway | Stem slices | BAP, IBA MS with sucrose | Cefotaxime 200 mg/L Vancomycin 400 mg/L | Stem slice co-cultivated with *Agrobacterium* for regeneration transformed root, followed by regeneration of plant from these roots via somatic embryogenesis | GV3101 *A. rhizogenes* | Kan 5 mg/L | 2-3% | Van der Salm *et al*. 1997, 1998 |
| 6) Tineke | Embryogenic callus | NAA, BA MS with Kinetin, and dicamba, charcoal and sucrose | Clavomox 200 mg/L | Embryogenic calli immersed 30 min in *Agrobacterium* | LBA4404 | Kan 60 mg/L | 6.6-12.6 callus with *gfp* expression | Kim *et al*. 2004 |
| 7) Sonia | | No hormone MS with sucrose | Cefotaxime 200 mg/L Vancomycin 400 mg/L | Embryogenic calli immersed 15 min in *Agrobacterium* | AGL0 C58C1 | Kan 300 mg/L | Somatic embryo with *Gus* expression but not transformed plant regenerated | Derks *et al*. 1995 |
| 8) Royalty | | 2,4-D, BAP, IBA MS with sucrose | nd | Friable embryogenic calli immersed 30min in *Agrobacterium* | LBA4404 *A. rhizogenes* (15834) | Kan 300 mg/L | | Firoozabady *et al*. 1994 |

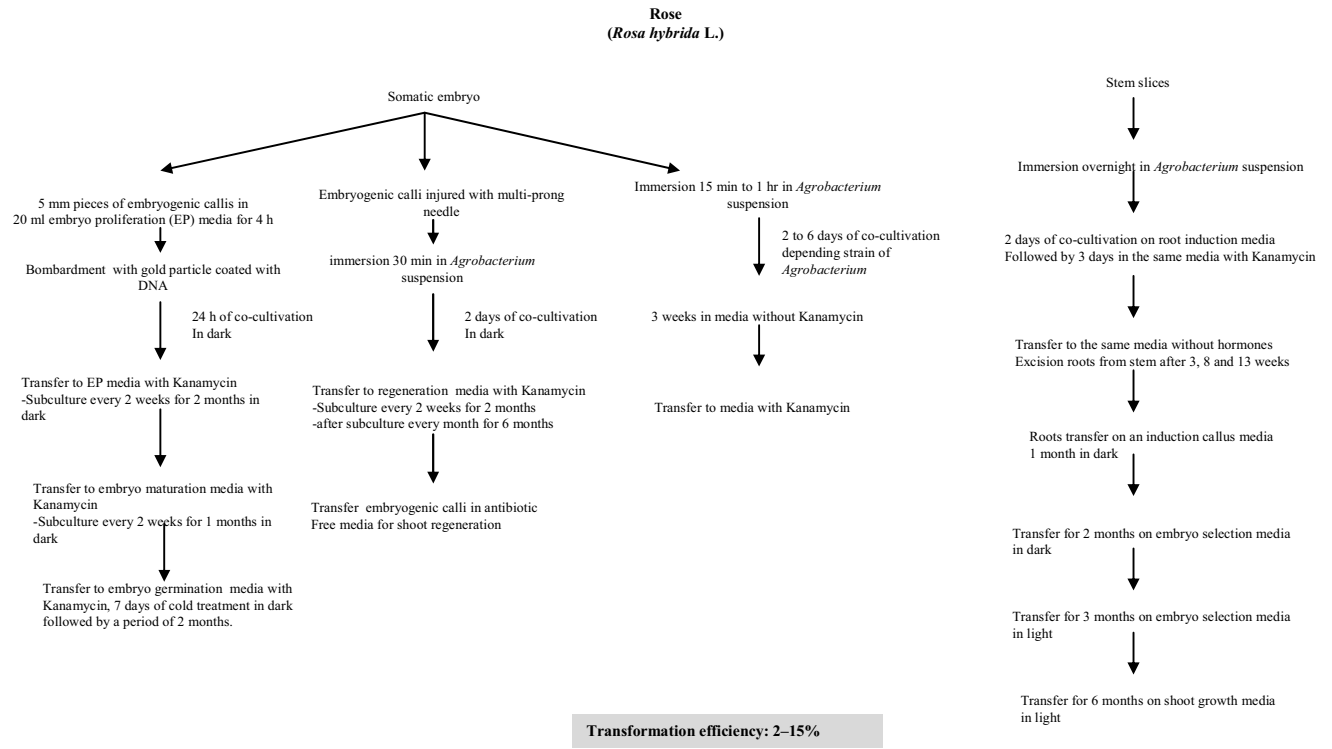


**Fig. 5 Representation of the rose (*Rosa hybrida* L.) transformation procedure.**

## *2. Application of these procedures for rose improvement*

Derks *et al.* (1995) reported obtaining transgenic somatic embryos via *Agrobacterium*-mediated transformation, but no transgenic plants were recovered. Marchant *et al.* (1996, 1998a) regenerated transgenic plants from embryogenic callus of *R. hybrida* following biolistic bombardment. Subsequently, Marchant *et al.* (1998b) successfully applied their procedure to introduce a chitinase gene into *R. hybrida* cv. 'Glad Tidings', and reported that expression of the chitinase transgene reduced the severity of black spot disease development by 13-43%. Testing three different genes (a Chitinase class II, T4-Lysozyme and barley RIP), Dohm *et al.* (2001) were able to reduce more significantly the severity of black spot disease development by 60% in case of the RIP gene. The increased resistance to another fungal disease, caused by *Sphaerotheca pannosa*, was also assessed in the transgenic *R. hybrida* cv. 'Carefree Beauty', expressing the antimicrobial protein gene, *Ace-AMP1*. Transgenic rose lines inoculated with conidial spores showed enhanced resistance to powdery mildew, using both a detached-leaf assay and an *in vivo* greenhouse whole-plant assay (Li *et al.* 2003). Using GE to develop roses with resistance to fungal disease appears to be a feasible approach.

**Table 9** *Rubus* variety transformed genetically.

| Cultivars | Explants used | Hormones used for regeneration, nutrients and sugar | Antibiotic | Inoculation procedure | *Agrobacterium* strains | Selectable marker | Efficiency of transformation | References |
|---|---|---|---|---|---|---|---|---|
| **Nectarberry (*Rubus arcticus* L.)** | | | | | | | | |
| 1) Pima | Internodal stem | BAP, NAA MS with sucrose | Cefotaxime 50 mg/L | 0.5 to 2h co-culture with *Agrobacterium* suspension | EHA101 | Kan 50 mg/L | 1.5-7.6% | Kokko and Karenlampi 1998 |
| **Blackberry** | | | | | | | | |
| 1) Dirksen Thornless | Leaf and cotyledon | TDZ, BA MS with sucrose | Cefotaxime 1 mM | 10 or -60 min iummersion in suspension of *Agrobacterium* respectively for leaf and cotyledon | C58 | Kan 10 mg/L | 0.25% | Hassan *et al*. 1993 |
| 2) Loch Ness | Leaf and stem | BAP, 2,4-D for stem IBA, BAP for leaf MS with sucrose | Carb 400 mg/L | 20 min immersion in suspension of *Agrobacterium* | LBA4404 | Kan 50 mg/L | | Graham *et al*. 1990 |
| **Raspberry (*Rubus ideaus* L.)** | | | | | | | | |
| 1) Comet | Leaf discs | nd | Cefotaxime 500 mg/L | 20 min immersion in suspension of *Agrobacterium* | LBA4404 | Kan 40 mg/L | 0.37% | De Faria *et al*. 1997 |
| 2) Candy | Petiole | IBA, TDZ | Carb 300 | 30-60 min immersion in suspension | EHA105 | Hygro- | 8.1% | Mathews *et al*. |
| 3) Chilliwak | | Silver nitrate | mg/L | of *Agrobacterium* | | mycin | 0.9% | 1995b |
| 4) Meeker | | MS with sucrose | | | | 10 mg/L | 49.6% | |
| 5) SCRI8242E6 | Leaf and stem | BAP, 2,4-D for stem IBA, BAP for leaf MS with sucrose | Carb 400 mg/L | 20 min immersion in suspension of *Agrobacterium* | LBA4404 | Kan 50 mg/L | | Graham *et al*. 1990 |
| **Blackberry x raspberry hybrids** | | | | | | | | |
| 1) Sunberry | Leaf and stem | BAP, 2,4-D for stem IBA, BAP for leaf MS with sucrose | Carb 400 mg/L | 20 min immersion in suspension of *Agrobacterium* | LBA4404 | Kan 50 mg/L | | Graham *et al*. 1990 |
| 2) Tayberry | | | | | | | | |

The *rolA*, *B*, and *C* genes from *A. rhizogenes* have been introduced into the cultivar 'Moneyway' under the control of its native promoter to improve yield in stem production (van der Salm *et al.* 1998). One of four transgenic lines expressing *rolA+B+C*, showing good growth and clearly decreased apical dominance, was selected for a grafting experiment with the cut rose cultivar 'Madelon' as scion. Grafting on this *rolA+B+C* transformed rootstock resulted in a stimulation of both root development of the rootstock and axillary bud release of the non-transformed scion. Axillary bud release increased from 0.1 to 0.6 and from 0.3 to 1.3 basal shoots per plant at 15 and 20°C, respectively. Since basal shoots form flowers, this altered plant architecture will presumably lead to enhanced flower production (van der Salm *et al.* 1998).

## *Rubus*

*Rubus* is among the most complicated taxonomic groups known for its propensity for interspecific hybridization, polyploidy, and apomixis. This genus is divided into 12 subgenera but, most of the species are classified in the raspberry and blackberry subgenera, *ideaus* and *ursinus*, respectively. Few reports have indicated the regeneration and transformation of 3 of the 12 subgenera, *viz*., *Rubus ideaus*, *R. ursinus* and *R. articus*, respectively raspberry, blackberry and nectarberry (**Table 9**). One report also described the transformation of a hybrid between raspberry and blackberry (Graham *et al.* 1990).

### 1) *Rubus articus*

Only one report mentions a procedure of transformation of nectacberry. This protocol is based on *Agrobacterium*-mediated transformation (**Table 9, Fig. 6A**) (Kokko and Karenlampi 1998).

### 2) *Rubus ursinus*

Successful development of regeneration and transformation systems was reported for two blackberry cultivars (**Table 9**). The procedure used for the regeneration and transformation of these cultivars is very similar (**Table 9, Fig. 6B**). The differences are 1) time of incubation of the leaf explant in the *Agrobacterium* inoculum, and 2) PGRs, antibiotics, and strain of *Agrobacterium* used in the procedure.

### *3) Rubus ideaus*

Five raspberry cultivars have been successfully transformed with efficiency of transformation ranging from 0.4 to 50%. The transformation procedures were based on *Agrobacterium*-mediated transformation. Transformation has been successful from many tissues, including leaf (De Faria *et al.* 1997), petiole (Mathews *et al.* 1995b) and stem (Graham *et al.* 1990) (**Table 9, Fig. 6C**). MS medium was used for regeneration of transformed raspberry, supplemented with 2-3% sucrose (**Table 9**), standard vitamins, and PGRs (**Table 9**). All the raspberry transformation protocols relied on the use of *Agrobacterium* strains EHA105 or LBA4044. One mode of infection with *Agrobacterium* was used, consisting of the immersion of the explants in the inoculum for 20 to 60 min, depending on the protocol (**Table 9**). Two different selectable markers were used, Kan (40 and 50 mg/L) and Hyg (10 mg/L) depending upon the cultivar. With this procedure transformation efficiency of up to 50% was obtained.

## SIMILARITIES AND DIFFERENCES IN THE TRANSFORMATION SYSTEMS FOR DIFFERENT ROSACEAE GENERA

Comprehensive analyses of Rosaceae genomes combined with comparative and functional genomics will provide the basis for understanding the complex biological processes underlying the array of diverse and unique traits seen in this family. This initiative will maximize knowledge transferability among the breadth of rosaceous crops, while concurrently developing key crop specific resources. The current sequencing of apple (*Malus*), strawberry (*Fragaria*) and peach (*Prunus*) genomes will give us resources for gene and regulatory element discovery. Following this sequencing, the development of resources and tool optimization to study gene function, transcriptome analysis and epigenetic influence will be necessary. Forward and reverse genetic approaches will be useful to validate the function of a gene of interest or to determine which genes in a cluster are responsible for a phenotype. For example, the resistance to apple scab is determined by the *Vf* cluster, composed of 4 RGAs, of which 3 are full-length *R* genes, and only two of

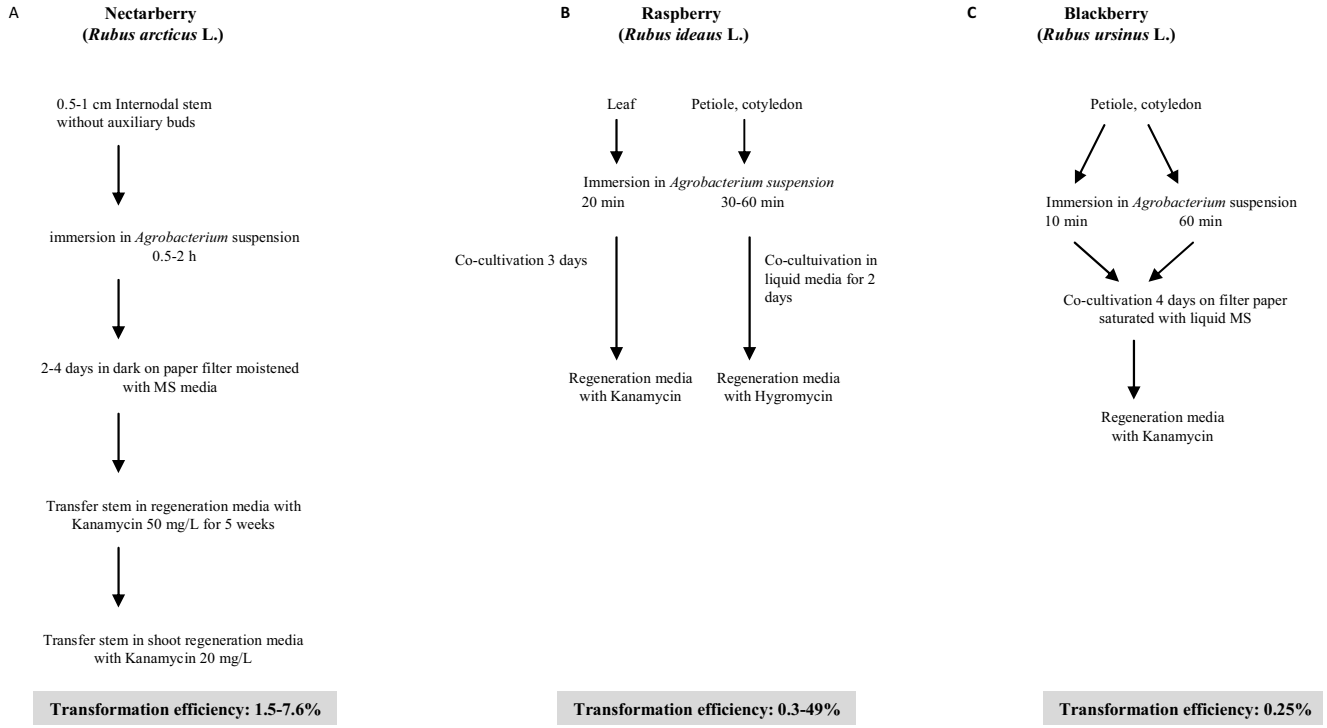


**Fig. 6 Representation of the *Rubus* transformation procedure.** (**A**) Nectarberry (*Rubus arcticus* L.); (**B**) raspberry (*R. ideaus* L.); (**C**) blackberry (R. *ursinus* L.).

these were found to be responsible for the resistance phenotype by reverse genetics (Malnoy *et al*. 2008). To take full advantage of these approaches, transformation protocols have been developed for a range of Rosaceae species and are described in the section on plant regeneration and transgenesis. However, for some of these species, transformation procedures will need to be made more efficient to achieve reverse genetics. Instead of spending time and money to improve the transformation protocols for the validation of genes of interest in all of these Rosaceae genera, one or two rosaceous genera could be selected as model systems for gene validation.

The Rosaceae family consists of various genera with different cultural, physiological and genomic characteristics as shown in **Table 10**. All of these differences make each member of this family unique, and it is for this reason that, for each rosaceous crop, a transformation procedure has been or is currently being developed for the purpose of improving some characteristic of each specific crop. However, when the purpose is gene function determination, one or two Rosaceae species can be selected as a model system for the whole family. In this section, we will describe the similarity and differences among the different transformation protocols of each Roseaceae crop and propose one or two species as models for gene validation in the Rosaceae family.

Almost all transformation systems, described in paragraph 2, (excluding the few specially mentioned exceptions), are based on *Agrobacterium*-mediated transformation, and relied on the selection gene *nptII*, with a non-Rosaceous promoter (CaMV35S among others). Often, the regeneratiom media that have produced the greatest shoot regeneration included MS medium supplemented with 2-3% sucrose and standard vitamin complements. For some Rosaceae genera such as *Pyrus*, *Prunus* and *Malus*, other regeneration media such as Nitsch and Nitsch, and Quorin Lepoivre have been used. Also, the carbon source was modified from sucrose to glucose or sorbitol for a few *Fragaria*, *Malus* and *Pyrus* species. However, the major differences among all the transformation protocols are the combination and concentration of PGRs used to induce callus and adventitious shoots. The nature of the explant varied among the differents cultivars of each species, but in general, leaf, stem and cotyledon are the explants with greatest regeneration capacity for all the rosaceous crops, except for *Rosa* where somatic embryos were used (**Table 10**). The mode and time of inoculation also varied depending on the cultivars transformed. The efficiency of transformation for each genus depended on the cultivars studied, and ranged from 0.5 to 100% (**Table 10**). *Fragaria* and *Malus* are the two members of the Rosaceae family with the reported highest transformation efficiency, 100 and 80%, respectively.

Based on the different transformation procedures and transformation efficiencies we propose to use *Fragaria* and *Malus* as transformation model systems to study reverse genetics of Rosaceae. *Fragaria* can be useful in creating T-DNA insertional mutant lines and high-throughput gene validation. Transformation rates were especially high in the 'Hawaii-4' line, as 100% of explants produced transgenic shoots. Almost 400 rooted plants were produced from 38 explants, demonstrating that these lines and protocols may be useful in generating a reverse-genetic population of diploid strawberry. Based on the small genome of diploid strawberry, it would take 225,000 reverse-genetic lines to mutagenize a population to affect any one gene with 95% certainty (Oosumi *et al.* 2005). The efficiency of these reverse genetic systems encourages their exploitation, as thousands of explants can be easily generated for transformation. Another advantage of *Fragaria* is the rapid transformation procedure, whereby one transgenic line can be produced and transferred to soil in 2 months (Folta et al. 2006). This rapid regeneration and the short juvenile period of the *Fragaria* genus compared to long-juvenility of tree crops such as apple, and peach, make *Fragaria* a useful translational genomic platform, as strawberry may be used to quickly test gene function in a tractable background. However, genes related to tree architecture and some diseases will be difficult to study in *Fragaria* because this crop is herbaceous. *Malus* appears to be the best available complementary genus to strawberry as a Rosaceae transformation model system for translational study.

**Table 10** Plant species for Rosaceae genetics and genomic research.

| | *Malus* | *Pyrus* | *Prunus* | | | | | *F. vesca* | *Rubus* | *Rosa* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Almond | Apricot | Cherry | Plum | Peach | | Blackberry, Nectaberry Raspberry | |
| Genome size (Mbp/C) | 750 | 540 | 300 | | 770 | 280 | 280 | 206 | 294 | 628 |
| Chromosome number (2n) | 34 | 34 | 16 | | | | | 14 diploid 56 octoploid | 14 | 14 |
| Diploid | Yes ( several cvs. triploid) | Yes (several cvs. triploid) | yes | yes | yes | yes | yes | yes (5 species) | yes | yes |
| | | | | | | | | | | |
| Generation time (seed to seed) | 5-7 years | 5-7 years | | | | | 3-5 years | 10-16 weeks | | |
| Juvenile period | 3-4 y | 3-4 y | | | | | 1-2 y | none | none | none |
| Vegetative propagation | yes, hard and softwood cuttings | | | | | | | yes, runners an crown divisions | | |
| Self-compatible | no | no | | | | | yes | yes | yes | yes |
| | | | | | | | | | | |
| ESTs | > 263,000 | >2,400 | >4,900 | >16,000 | >2,000 | >4,000 | >100,000 | >21,000 | >2,200 | >10,000 |
| Linkage maps | **yes** | | | | | | | | | |
| Physical map | **yes** | no | no | no | no | no | **yes** | **yes** in progress | no | no |
| Full genome sequence | **yes** in progress | no | no | no | no | no | **yes** in progress | yes in progress | no | no |
| | | | | | | | | | | |
| Nature of explants used for transformation | Tissue culture (leaf, cotyledon, internod, hypocotyl) protoplast | | | | | | protoplast | protoplast | | Somatic embyogenesis |
| No. of cultivars transformed | 40 | 17 | 3 | 2 | 3 | 2 | 2 | 40 | 6 | 8 |
| Transformation efficiency (max.) reported | 80% | 60% | 12.3% | 8.5% | 9.7% | 4.8% | 4.1% | 100% | 49% | 15% |
| Regeneration media and sugar | MS and N6 medium. Sucrose, sorbitol | MS, Quoirin Lepoivre, Nitsch and Nitsch medium. Sucrose and sorbitol | MS medium sucrose | MS and Quoirin Lepoivre medium sucrose | MS medium sucrose | MS and Quoirin Lepoivre medium sucrose | MS medium sucrose | MS medium Sucrose glucose | MS medium sucrose | MS medium sucrose |
| Selection agent | Kan Hygromycin | Kan Hygromycin | Kan | Kan | Kan | Kan | Kan | Kan Hygromycin | Kan Hygromycin | Kan |
| Transient transformation system Fruit | **yes** | **yes** | no | no | no | no | **yes** | **yes** | no | no |
| Leaf | **yes** | **yes** | no | no | no | no | no | **yes** | no | no |
| High-throughput transformation system | Yes in development | no | no | no | no | no | no | **yes** | no | no |
| Markerless transformed plants Cre-lox, Mat | **yes** (Cre-lox) | no | no | **yes** (mannose) | no | no | no | **yes** (Cre-lox)) | no | no |
| Other sources of carbohydrate | **yes** (mannose,, VreRE) | **yes** (VreRE) | no | no | no | no | no | no | no | no |
| Without any selectable amrker | **yes** | no | no | no | no | no | no | no | no | no |
| | | | | | | | | | | |
| Field trial | **yes** | **yes** | no | no | no | **yes** | no | **yes** | no | no |
| Varieties transgenic commercial | no | no | no | no | no | **deregulated** | no | no | no | no |

## FUTURE PERSPECTIVES

Genetic research in Rosaceae crops is commonly directed at understanding the genetic control of important agronomic characteristics in order to improve these crops. Genomic knowledge is increasing day by day and provides tremendous information that can be used for genetic improvement of cultivars through breeding or GE. From all of this information, narrowing down the vast array of data resulting from genomic research to specific genes responsible for a specific trait is fundamental for crop improvement. Till now, the candidate gene approach attempted to utilize knowledge generated by structural, functional and comparative genomics. As the total genomic sequences become available in the near future for three representative members of the Rosaceae, *Malus*, *Prunus* and *Fragaria* (Sosinski *et al.* 2009), it will be possible to scan coding regions of those genomes within established QTLs for identification of potential candidate genes. Validation of these candidate genes will require efficient transformation systems as described above for strawberry and apple.

The high cost of developing transgenic over-expressing or silenced lines limits their usefulness to the genomic analysis of a limited number of important candidate genes. A low cost and rapid alternative to produce stable transgenic lines for determining gene function could be transient RNAi expression by agroinfiltration as demonstrated by Hoffman *et al.* (2006) in strawberry.

Before transformed Rosaceae crop species are made commercially available, evaluation of their potential risk for the environment (gene flow and gene impact on other micro-organisms) and for their benefits to agricultural practices (reduced inputs, improved yield, increased nutritional value) must be done. The concerns of regulatory agencies and consumers about the environmental safety and public health impact of GE varieties of rosaceous fruits must be convincingly addressed. We believe that the recent advances in technology reported here will provide adequate answers to these concerns.

## REFERENCES


**Abernathy D, Zhebentyayeva T, Abbott AG, Salava J, Polák J, Vilanova S, Badenes ML, Krska B, Damsteegt VD** (2004) Molecular genetic mapping of the *Plum pox virus* resistance genes in apricot. *Acta Horticulturae* **657**, 283-288

**Abdul-Kader AM, Bauer DW, Beer SV, Norelli JL, Aldwinckle HS** (1999) Evaluation of the *hrpN* gene for increasing resistance to fire blight in transgenic apple. *Acta Horticulturae* **489**, 247-250

**Abu-Qaoud H. Skirvin RM, Below FE** (1991) Influence of nitrogen form and $NH_4^+$-N/$NO_3$-N ratios on adventitious shoot formation from pear (*Pyrus communis*) leaf explants *in vitro*. *Plant Cell, Tissue and Organ Culture* **27**, 315-319

**Agius F, Amaya I, Botella MA, Valpuesta V** (2005) Functional analysis of homologous and heterologous promoters in strawberry fruits using transient expression. *Journal of Experimental Botany* **56**, 37-46

**Ainsley PJ, Collins GG, Sedgley M** (2001). Factors affceting *Agrobacterium*-mediated gene transfer and the selection of transgenic calli in paper shell almond (*Prunus dulcis* Mill.). *Journal of Horticultural Science and Biotechnology* **76**, 522-528

**Aldwinckle H, Ko K, Norelli JL, Brown SK, During K** (1998) Enhanced resistance to fire blight of apple lines transgenic for attacin E and T4 lysozyme genes. In: *7th International Congress of Plant Pathology* (Vol 3), ICPP, Edinburgh, Scotland, p. 5.3.17

**Aldwinckle HS, Borejsza-Wysocka EE, Malnoy M, Brown SK, Norelli JL, Beer SV, Meng X, He SY, Jin QL** (2003) Development of fire blight resistant apple cultivars by genetic engineering. *Acta Horticulturae* **622**, 105-111

**Alsheikh MK, Suso HP, Obson M, Nattey NH** (2002) appropriate choice of antibiotic and *Agrobacterium* strain improves transformation of antibiotic sensitive *Fragaria vesca* and *F. v. semperflorens*. *Plant Cell Reports* **20**, 1173-1180

**Amstrong J, Carter N** (2008) A new addition to the buffet. *1st International Symposium on Biotechnoology of Fruit Species*, September 1-5, 2008, Dresden, Germany

**Atkinson RG, Schroder R, Hallet IC, Cohen D, MacRae EA** (2002) Overexpression of polygalacturonase in transgenic apple trees leads to a range of novel phenotypes involved in cell adhesion. *Plant Physiology* **129**, 122-133

**Artlip T, Wisniewski M, Norelli JL, Cui M, Fuchigami L** (2007) Using biotechnology to improve resistance to environmental stress in fruit crops: The importance of understanding physiology. *Acta Horticulturae* **738**, 145-156

**Baker BS, Bhatia SK** (1993) Factors affecting shoot regeneration from leaf explants of quince (*Cydonia oblonga*). *Plant Cell, Tissue and Organ Culture* **35**, 273-277

**Barbieri C, Morini S** (1987) *In vitro* regeneration from somatic tissues and seed explants of apple. *Advances in Horticultural Science* **1**, 8-10

**Barbieri M, Belfanti E, Tartarini S, Vinatzer BA, Sansavini S, Silverberg-Dilworth E, Gianfranceschi L, Hermann D, Patocchi A, Gessler C** (2003) Progress of map based cloning of the *Vf* resistance gene and functional verification: preliminary results from expression studies in transformed apple. *HortScience* **38**, 329-331

**Barcelo M, El-Mansouri I, Mercado JA, Quesada MA, Pliego F** (1998) Regeneration and transformation via *Agrobacterium tumefaciens* of the strawberry cultivar Chandler. *Plant Cell Tissue and Organ Culture* **54**, 29-36

**Belfanti E, Silverberg-Dilworth E, Tartarini S, Patocchi A, Barbieri M, Zhu J, Vinatzer BA, Gianfranceschi L, Gessler C, Sansavini S** (2004) The *HcrVf2* gene from a wild apple confers scab resistance to a transgenic cultivated variety. *Proceedings of the National Academy of Sciences USA* **101**, 886-890

**Bell RL, Scorza R, Srinivasan C, Webb K** (1999) Transformation of 'Beurre Bosc' pear with the *rolC* gene. *Journal of the American Society of Horticultural Science* **124**, 570-574

**Bolar JP, Brown SK, Norelli JL, Aldwinckle HS** (1999) Factors affecting the transformation of 'Marshall McIntosh' apple by *Agrobacterium tumefaciens*. *Plant Cell, Tissue and Organ Culture* **55**, 31-38

**Bolar JP, Norelli JL, Harman GE, Brown SK, Aldwinckle HS** (2001) Synergistic activity of endochitinase and exochitinase from *Trichoderma atroviride* (*T. harzianum*) against the pathogenic fungus (*Venturia inaequalis*) in transgenic apple plants. *Transgenic Research* **10**, 533-543

**Bolar JP, Norelli JL, Wong K-W, Hayes CK, Harman GE, Aldwinckle HS** (2000) Expression of endochitinase from *Trichoderma harzianum* in transgenic apple increases resistance to apple scab and reduces vigor. *Phytopathology* **90**, 72-77

**Bommineni VR, Mathews H, Clendennen SK, Samuel SB, Bristol N, Wagoner W, Kellogg J, Phan C, Schuster D, Kramer M, Wagner DR** (2000) Development of an enhanced transformation method in pear (*Pyrus communis* L.) cv. Bartlett. Abstract. *VIII International Symposium on Pear*, 4-9 September, Ferrara-Bologna, Italy, p 38

**Borejsza-Wysocka EE, Malnoy M, Kim WS, Geider K, Beer S, Aldwinckle HS** (2007) Expression of Phi-Ea1h phage depolymerase gene with constitutive and inducible promoters, translation enhancer and signal sequence in transgenic apple plants increases resistance to fire blight. *Acta Horticulturae* **738**, 273-276

**Borejsza-Wysocka EE, Malnoy M, Meng X, Bonasera JM, Nissinen RM, Kim JF, Beer SV, Aldwinckle HS** (2006) The fire blight resistance of apple clones in which DspE-interacting proteins are silenced. *Acta Horticulturae* **704**, 509-513

**Broothaerts W, Van Nerum I, Keulemans J** (2004a) Update on and review of the incompatibility (*S*-) genotypes of apple cultivars. *HortScience* **39**, 943-946

**Broothaerts W, Keulemans J, Van Nerum I** (2004b) Self-fertile apple resulting from S-Rnase gene silencing. *Plant Cell Reports* **22**, 497-501

**Broothaerts W, De Cubber K, Zaman S, Coppens S, Keulemans J** (2000) The feasibility of fungal disease resistance in apple by expression of antimicrobial peptide genes. *Acta Horticulturae* **521**, 91-94

**Bulley SM, Wilson FM, Hedden P, Phillips AL, Croker S, James D** (2005) Modification of gibberellin biosynthesis in the grafted apple scion allows control of tree height independent of the rootstock. *Plant Biotechnology Journal* **3**, 215-223

**Caboni E, Tonelli MG, Lauri P, D'Angeli S, Damiano C** (1999) *In vitro* shoot regeneration from leaves of wild pear. *Plant Cell, Tissue and Organ Culture* **59**, 1-7

**Caboni E, Lauri P, Watillon B** (2002) Factors affecting *Agrobacterium*-mediated transformation efficiency of wild pear (*Pyrus communis*, var *Pyraster*). *Acta Horticulturae* **596**, 203-206

**Cao H, Bowling SA, Gordon SA, Dong X** (1994) Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* **6**, 1583-1592

**Cao H, Gladzebrook J, Clarke JD, Volko S, Dong X** (1997) The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* **88**, 57-63

**Chalavi V, Tabaeizadeh Z** (2003) Enhanced resistance to *Verticillium dahliae* in transgenic strawberry plants expressing a *Lycopersicon chilense* chitinase gene. *Journal American Society Horticultural Science* **128**, 747-753

**Cheng L, Zhou R, Reidel EJ. Sharkey TD, Dandekar AM** (2005) Antisense inhibition of sorbitol synthesis leads to up regulation of starch synthesis without altering $CO_2$ assimilation in apple leaves. *Planta* **220**, 767-776

**Chevreau E, Leblay C** (1993) The effect of mother plant pretreatment and explant choice on regeneration from in vitro pear leaves. *Acta Horticulturae* **336**, 263-268

**Chevreau E, Mourgues F, Neveu M, Chevalier M** (1997) Effect of gelling agents and antibiotics on adventitious bud regeneration from *in vitro* leaves of pear. *In Vitro Cellular and Developmental Biology - Plant* **33**, 173-179

**Chevreau E, Skirvin RM, Abu-Qaoud HA, Korban SS, Sullivan JG** (1989) Adventitious shoot regeneration from leaf tissue of three pear (*Pyrus* sp.) cultivars *in vitro*. *Plant Cell Reports* **7**, 688-691

**Chevreau E, Taglioni JP, Cesbron C, Dupuis F, Sourice S, Loridon K** (2007) Feasibility of alternative selection methods for transgenic apple and pear using the detoxification gene *Vr-ERE*. *Acta Horticulturae* **738**, 277-281

**Chung EY, Kim BH, Lee MK, Yun YP, Lee SH, Min KR, Kim Y** (2003) Anti-inflammatory effect of the oligomeric stilben alpha-Viniferin and its mode of the action through inhibition of cyclooxygenase-2 and inducible nitric axide synthase *Planta Medica* **69**, 710-714

**Condero de Mesa M, Santiago-Domenech N, Pliego-Alfaro F, Quesada MA, Mercado JA** (2004) The CaMV35S promoter is highly active on floral organs and pollen of transgenic strawberry plants. *Plant Cell Reports* **23**, 32-38

**Condero de Mesa M, Jimenez-Bermudez S, Pliego-Alfaro F, Quesada MA, Mercado JA** (2000) *Agrobacterium* cells as microprojectile coating a novel approach to enhance stable transformation rates in strawberry. *Australian Journal of Plant Physiol*ogy **27**, 1093-1100

**Costa MS, Miguel C, Oliveira MM** (2006) An improved selection strategy and the use of acetosyringone in shoot induction medium increase almond transformation efficiency by 100-fold. *Plant Cell, Tissue and Organ Culture* **85**, 205-209

**Da Camara Machado A, Puschmann M, Puhringer H, Kremen R, Katinger H, Laimer da Camara Machado M** (1995) Somatic embryogenesis of *Prunus subhirtella autumno rosa* and regeneration of transgenic plants after *Agrobacterium*-mediated transformation. *Plant Cell Reports* **14**, 335-340

**Dandekar AM** (1992) Transformation. In: Hammerschlag FA, Litz RE (Eds) *Biotechnology of Perennial Fruits Crops*, CAB International, Wallingford, UK, pp 141-168

**Dandekar AM, Teo G, Defilippi BG, Uratsu SL, Passey AJ, Kader AA, Stow JR, Colgan RJ, James DJ** (2004) Effect of down regulation of ethylene biosynthesis on fruit flavor complex in apple fruit. *Transgenic Research* **13**, 373-384

**De Bondt A, Eggermont K, Druart P, De Vil M, Goderis I, Vanderleyden J, Broekaert WF** (1994) *Agrobacterium* mediated transformation of apple (*Malus X domestica* Borkh.): an assessment of factors affecting gene transfer efficiency during early transformation steps. *Plant Cell Reports* **13**, 587-593

**De Bondt A, Eggermont K, Penninckx I, Goderis I, Broekaert WF** (1996) *Agrobacterium* mediated transformation of apple (*Malus X domestica* Borkh.): an assessment of factors affecting regeneration of transgenic plants. *Plant Cell Reports* **15**, 549-554

**De Bondt A, Zaman S, Broekaert W, Cammue B, Keulemans J** (1999) Genetic transformation of apple (*Malus pumila* Mill.) for increased fungal resistance: *in vitro* antifungal activity in protein extracts of transgenic apple expressing *RS-AFP2* or *ACE-AMP1*. *Acta Horticulturae* **484**, 565-570

**De la Fuente JI, Amaya I, Castillojo C, Sanchez-Sevilla JF, Quesada MA, Botella MA, Valpuesta V** (2006) The strawberry gene *FaGAST* affects plant growth through inhibition of cell elongation. *Journal of Experimental Botany* **57**, 2401-2411

**Defilippi B, Dandekar AM, Kader AA** (2004) Impact of suppression of ethylene action or biosynthesis on flavor metabolites in apple (*Malus X domestica* Borkh.) fruit. *Journal of Agricultural and Food Chemistry* **52**, 5694-5701

**Defilippi B, Dandekar AM, Kader AA** (2005a) Relationship of ethylene biosynthesis to volatile production, related enzymes, and precursor availability in apple peel and flesh tissues. *Journal of Agricultural and Food Chemistry* **53**, 3133-3141

**Defilippi B, Dandekar AM, Kader AA** (2005b) Apple aroma: alcohol acyltransferase, a rate limiting step for ester biosynthesis, is regulated by ethylene. *Plant Science* **168**, 199-1210

**de Faria MJSS, Donnelly DJ, Cousineau JC** (1997) Adventitious shoot regeneration and *Agrobacterium*-mediated transformation of red raspberry. *Arquivos de Biologia e Tecnologia* **40**, 518-529

**de Mesa MC, Jimenez-Bermudez S, Pliego-Alfaro F, Quesada MA, Mercado JA** (2000) *Agrobacterium* cells as microprojectile coating: a novel approach to enhance stable transformation rates in strawberry. *Australian Journal of Plant Physiology* **27**, 1093-1100

**Degenhardt J, Poppe A, Montag J, Szankowski I** (2006) The use of the phosphomannose-isomerase/mannose selection system to recover transgenic apple plants. *Plant Cell Reports* **25**, 1149-1156

**Derks FHM, van Dijk AJ, Hänisch ten Cate CH, Florack DEA, Dubois LAM, de Vries DP** (1995) Prolongation of vase life of cut roses via introduction of genes coding for antibacterial activity, somatic embryogenesis and *Agrobacterium*-mediated transformation. *Acta Horticulturae* **405**, 205-209

**Dirlewanger E, Graziano E, Joobeur T, Garriga-Caldere F, Cosson P, Howad W, Arus P** (2004) Comparative mapping and marker-assisted selection in Rosaceae fruit crops. *Proceedings of the National Academy Sciences USA* **101**, 9891-9896

**Dohm A, Ludwig C, Schilling D, Debener T** (2001) Transformation of roses with genes for antifungal proteins. *Acta Horticulturae* **547**, 27-33

**Dolcet-Sanjuan, R, Mok DWS, Mok MC** (1991) Plantlet regeneration from cultured leaves of *Cydonia oblonga* L. (quince). *Plant Cell Reports* **10**, 240-242

**Dolgov SV, Skryabin KG** (2004) Transgenic apple clonal rootsock resistant to Basta herbicide. *Acta Horticulturae* **663**, 499-502

**Dolgov SV, Schestibratov KA, Morishnichenko DN** (2004) Apple transformation with the gene of supersweet protein thaumatin II. *Acta Horticulturae* **663**, 507-510

**Dolgov SV, Morishnichenko DN, Schestibratov KA** (2000) *Agrobacterium* transformation of apple cultivar and rootstock. *Acta Horticulturae* **538**, 619-624

**Duffy B, Dandekar AM** (2008) Sorbitol has no role in fire blight as demonstrated using transgenic apple with constitutively altered content. *Acta Horticulturae* **783**, 279-283

**Du Plessis HJ, Brand RJ** (1997) Efficient genetic transformation of strawberry (*Fragaria X anannassa* Duch.) cultivars selekta. *Acta Horticulturae* **447**, 289-293

**Druart P, Gruselle R** (2007) Behaviours of *Agrobacterium rhizogenes*-mediated transformants of Inmil (*P. incise x serrula*) cherry rootstock. *Acta Horticulturae* **738**, 589-592

**Ebner C, Hirschwehr R, Bauer L, Breiteneder H, Valenta R, Ebner H** (1995) Identification of allergens in fruits and vegetables: IgE crossreactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *Journal of Allergy and Clinical Immunology* **95**, 962-969

**Eichholzer M, Luthy J, Gutzwiller F, Stahelin HB** (2001) The role of folate, antioxidant vitamins and other constitutents in fruit and vegetables in the prevention of cardiovascular disease: The epidemiological evidence. *International Journal for Vitamin and Nutrition Research* **71**, 5-17

**El Mansouri I, Mercado JA, Valpuesta V, Lopez-Aranda JM, Pliego-Alfaro F, Quesada MA** (1996) Shoot regeneration and *Agrobacterium*-mediated transformation of *Fragaria vesca* L. *Plant Cell Reports* **15**, 642-646

**Espley RV, Hellens RP, Putterill J, Stevenson DE, Kutty-Amma S, Allan AC** (2007) Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. *The Plant Journal* **49**, 414-427

**Etienne H, Berthouly M** (2002) Temporary immersion systems in plant micropropagation. *Plant Cell, Tissue and Organ Culture,* **69**, 215-231

**Faize M, Malnoy M, Dupuis F, Chevalier M, Parisi L, Chevreau E** (2003) Chitinases of *Trichoderma atroviridae* induce scab resistance and some metabolic changes in two cultivars of apple. *Phytopathology* **93**, 1496-1504

**Faize M, Sourice S, Dupuis F, Parisi L, Gautier MF, Chevreau E** (2004) Expression of wheat puroindoline b reduces scab susceptibility in transgenic apple (*Malus X domestica* Borkh.). *Plant Science* **167**, 347-354

**Fasolo F, Zimmermann RH, Fordham I** (1989) Adventitious shoot formation on excised leaves of *in vitro* grown shoots of apple cultivars. *Plant Cell, Tissue and Organ Culture* **16**, 75-87

**Firoozabady E, Moy Y, Courtney-Gutterson N, Robinson K** (1994) Regeneration of transgenic rose (*Rosa hybrida*) plants from embryogenic tissue. *Bio/Technology* **12**, 609-613

**Flachowsky H, Birk T, Hanke V** (2004) Preliminary results to establish an alternative selection system for apple transformation. *Acta Horticulturae* **663**, 425-430

**Flachowsky H, Richter K, Kim W-S, Geider K, Hanke V** (2008) Transgenic expression of a viral EPS-depolymerase is potentially useful to induce fire blight resistance in apple. *Annals of Applied Biology* **153**, 345-355

**Flachowsky H, Peil A, Sopanen T, Elo A, Hanke MV** (2007) Overexpression of BpMADS4 from silver birch (*Betula pendula* Roth.) induces early-flowering in apple (*Malus domestica* Borkh.). *Plant Breeding* **126**, 137-145

**Folta KM, Dhingra A** (2006) Transformation of strawberry: the basis for translational genomics in Rosaceae. *In Vitro Cellular and Developmental Biology – Plant* **42**, 482-490

**Folta KM, Dhingra A, Howard L, Stewart PJ, Chandler CK** (2006) Characterization of LF9 an octoploid strawberry genotype selected for rapid regeneration and transformation. *Planta* **224**, 1058-1067

**Gao M, Murayama H, Matsuda N, Isuzugawa K, Dandekar A, Nakano H** (2002) Development of *Agrobacterium* mediated transformation of pear (*Pyrus communis* L.) with cotyledon explants and production of transgenic pears using ACC oxidase cDNA. *Plant Biotechnology* **19**, 319-327

**Gao M, Murayama H, Murayama H, Toyomasu T, Mitsuhashi W, Dandekar A, Tao R, Nishimura K** (2007) Gene expression and ethylene production in transgenic pear (Pyrus communis cv. 'La France') with sense or antisense cDNA encoding ACC oxidase. *Plant Science* **173**, 32-42

**Gilissen LJWJ, Bolhaar STHP, Matos CI, Rouwendal GJA, Boone MJ, Krens FA, Zuidmeer L, Van Leeuwen A, Akkerdaas J, Hoffmann-Sommergruber K, Knulst AC, Bosch D, Van de Weg WE, Van Ree R** (2004) Silencing the major apple allergen Mal d1 by using the RNA interference approach. *Journal of Allergy and Clinical Immunology* **115**, 364-369

**Gittins JR, Hiles ER, Pellny TK, Biricolti S, James DJ** (2001) The *Brassica napus* extA promoter: A novel alternative promoter to *CaMV*35S for directing transgene expression to young stem tissues and load bearing regions of transgenic apple trees (*Malus pumila* Mill). *Molecular Breeding* **7**, 51-62

**Gittins JR, Pellny TK, Biricolti S, Hiles ER, Passey AJ, James DJ** (2003) Transgene expression in the vegetative tissues of apple driven by the vascular specific rolC and CoYMV promoters. *Transgenic Research* **12**, 91-402

**Gittins JR, Pellny TK, Hiles ER, Rosa C, Biricolti S, James DJ** (2000) Transgene expression driven by heterologous ribulose-1,5-bisphosphate carboxylase/oxygenase small-subunit gene promoters in the vegetative tissues of apple (*Malus pumila* Mill.). *Planta* **210**, 232-240

**Gonzalez Padilla IM, Webb K, Scorza R** (2003) Early antibiotic selection and efficient rooting and acclimatization improve the production of transgenic plum plants (*Prunus domestica* L.). *Plant Cell Reports* **22**, 38-45

**Graham J, McNicol RJ, Kumar A** (1990) Use of the *Gus* gene as a selectable marker for *Agrobacterium*-mediated transformation of *Rubus*. *Plant Cell, Tissue and Organ Culture* **20**, 35-39

**Graham J, McNicol RJ, Kumar A** (1995) *Agrobacterium*-mediated transformation of soft fruit *Rubus*, *Ribes* and *Fragaria*. *Agrobacterium* Protocols. *Methods in Molecular Biology* **13**, 1-12

**Graham J, Gordon SC, McNicol RJ** (1997) The effect of the CpTi gene in strawberry against attack by vine weevil (*Otiorhynchus sulcatus* F. Coleoptera: Curculionidae). *Annals of Applied Biology* **131**, 133-139

**Graham J, Gordon SC, Smith K, McNicol RJ, McNicol JW** (2002) The effect of the cowpea trypsin inhibitor in strawberry on damage by vine weevil under field conditions. *Journal of Horticultural Science and Biotechnology* **77**, 33-40

**Griesser M, Hoffmann T, Bellido ML, Rosatri C, Fink B, Kurtzer R, Aharoni A, Munoz-Blanco J, Schwab W** (2008) Redirection of flavonoid biosynthesis through the down regulation of an anthocyanindin glucosyltransferase in ripening strawberry fruit. P*lant Physiology* **146**, 1528-1539

**Gruchala A, Korbin M, Zurawicz E** (2004) Conditions of transformation and regeneration of Induka and Elisata strawberry plants. *Plant Cell, Tissue and Organ Culture* **79**, 153-160

**Gutierrez P, Rugini E** (2004) Influence of plant growth regulators, carbon sources and iron on the cyclic secondary somatic embryogenesis and plant regeneration of transgenic cherry rootstock 'Colt' (*Prunus avium x P. pseudocerasus*). *Plant Cell, Tissue and Organ Culture* **79**, 223-232

**Gutierrez P, Taylor K, Muleo R, Rugini E** (1998) Somatic embryogenesis and shoot regeneration from transgenic roots of the cherry rootstock 'Colt' (*Prunus avium x P. pseudocerasus*) mediated by pRi 1855 T-DNA of *Agrobacterium rhizogenes*. *Plant Cell Reports* **17**, 574-580

**Hammerschlag FA, Liu Q, Zimmerman RH, Gercheva P** (2000) Generating apple transformants free of *Agrobacterium tumefaciens* by vacuum infiltrating explants with an acidified medium and without antibiotics. *Acta Horticulturae* **530**, 103-111

**Hanhineva KJ, Karenlampi SO** (2007) Production of transgenic strawberries by temporal immersion bioreactor system and verification by TAIL-PCR. *BMC Biotechnology* **7**, 11

**Hanke V, Geider K, Richter K** (2003) Transgenic apple plants expressing viral EPS-depolymerase: evaluation of resistance to the phytopathogenic bacterium *Erwinia amylovora*. In: Vasil IK (Ed) Plant Biotechnology*: 2002 and Beyond*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 153-157

**Hanke V, Kim WS, Geider K** (2002) Plant transformation for induction of fire blight resistance: transgenic apples expressing viral EPS depolymerase. *Acta*

*Horticulturae* **590**, 393-395

**Hanke V, Hiller I, Klotzsche G, Richter K, Norelli JL, Aldwinckle HS** (2000) Transformation in apple for increased disease resistance. *Acta Horticulturae* **538**, 611-616

**Hassan MA, Swartz HJ, Inamine G, Mullineaux P** (1993) *Agrobacterium tumefaciens* mediated transformation of several *Rubus* genotypes and recovery of transformed plants. *Plant Cell, Tissue and Organ Culture* **33**, 9-17

**Haymes KM, Davis TM** (1998) *Agrobacterium*-mediated transformation of Alpine *Fragaria vesca*, and transmission of transgenes to R1 progeny. *Plant Cell Reports* **17**, 279-283

**He L, Ban Y, Inoue H, Matsuda N, Liu J, Moriguchi T** (2008) Enhancement of spermidine content and antioxidant capacity in transgenic pear shoots overexpressing apple *spermidine synthase* in response to salinity and hyperosmosis. *Phytochemistry* **69**, 2133-2141

**Hily JM, Scorza R, Malinowski T, Zawadzka B, Ravelonandro M** (2004) Stability of gene silencing based resistance to plum pox virus in transgenic plum (*Prunus domestica* L.) under field conditions. *Transgenic Research* **13**, 427-436

**Hoffmann T, Kalinowski G, Schwab** W (2006) RNAi-induced silencing of gene expression in strawberry fruit (*Fragaria x ananassa*) by agroinfiltration: a rapid assay for gene function analysis. *The Plant Journal* **48**, 818-826

**Holefors A, Xue ZT, Zhu LH, Welander M** (2000) The *Arabidopsis* phytochrome B gene influences growth of the apple rootstock M26. *Plant Cell Reports* **19**, 1049-1056

**Holefors A, Xue ZT, Welander M** (1998) Transformation of the apple rootstock M26 with rolA gene and its influence on growth. *Plant Science* **136**, 69-78

**Honda C, Moriguchi T** (2006) High Gus expression in protoplasts isolated from immature peach fruits. *Scientia Horticulturae* **109**, 244-247

**Houde M, Dallaire S, N'Dong D, Sarhan F** (2004) Overexpression of the acidic dehydrin WCOR410 improves freezing tolerance in transgenic strawberry leaves. *Plant Biotechnology Journal* **2**, 381-387

**Hrazdina G, Kiss E, Galli Z, Rosenfield C, Norelli JL, Aldwinckle HS** (2003) Down regulation of ethylene production in Royal Gala apples. *Acta Horticulturae* **628**, 239-251

**Huetteman CA, Preece JE** (1993) Thidiazuron – a potent cytokinin for woody plant-tissue culture. *Plant Cell, Tissue and Organ Culure* **33**, 105-119

**Husaini AM, Abdin MZ** (2008) Development of transgenic strawberry (*Fragaria x ananassa* Duch.) plants tolerant to salt stress. *Plant Science* **174**, 446-455

**Hyung NI, Lee CH, Kim SB** (1995) Foreign gene transfer using electroporation and transient expression in apple (*Malus domestica* Borkh). *Acta Horticulturae* **392**, 179-185

**Igarashi M, Ogasawara H, Hatsuyama Y, Saito A, Suzuki M** (2002) Introduction of rolc into Marubakaidou (*Malus prunifolia* Borkh. Var. ringo Asami Mo 84-A) apple rootstock via *Agrobacterium tumefaciens*. *Plant Science* **163**, 463-473

**James DJ, Passey AJ, Barbara DJ** (1990) *Agrobacterium*-mediated transformation of the cultivars strawberry (*Fragaria x anannassa* Duch.) using disarmed binary vectors. *Plant Science* **69**, 79-94

**James DJ, Passey AJ, Baker SA** (1994) Stable gene expression in transgenic apple tree tissues and segregation of transgenes in the progeny – preliminary evidence. *Euphytica* **77**, 119-121

**James DJ, Passey AJ, Barbara DJ, Bevan MV** (1989) Genetic transformation of apple (*Malus pumila* Mill.) using disarmed Ti-binary vector. *Plant Cell Reports* **7**, 658-666

**James DJ, Passey AJ, Webster AD, Barbara DJ, Viss P, Dandekar AM, Uratsu S** (1993) Transgenic apples and strawberries: Advances in transformation, introduction of genes for insect resistance and field studies of tissue cultured plants. *Acta Horticulturae* **336**, 179-184

**Jacquet C, Ravelonandro M, Bachlier JC, Dunez J** (1998) High resistance to plum pox virus (PPV) in transgenic plants containing modified and truncated forms of PPV coat protein gene. *Transgenic Research* **7**, 29-39

**Janse J, Schaart JG, Puite KJ, Florack DEA, Groenwold R, Pelgrom K, Krens FA** (2002) Enhanced resistance to *Venturia inaequalis* in transgenic apple by a gene coding for hordothionin. *10th IAPTC&B Congress, Plant Biotechnology 2002 and Beyond*, June 23-28, 2002, Orlando, Florida, USA

**Jimenez-Bermudez S, Redondo-Nevado J, Munoz-Blanco J, Cabellero JL, Lopez-Aranda JM, Valpuesta V, Pliego-Afaro F, Quesada MA, Mercado JA** (2002) Manipulation of strawberry fruit softening by antisense expression of a pectate lyase gene. *Plant Physiology* **128**, 751-759

**Khosh-Khui M, Teixeira da Silva JA** (2006) *In vitro* culture of *Rosa* species. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol II), Global Science Books, Isleworth, UK, pp 514-526

**Kanamaru N, Ito Y, Komori S, Saito M, Kato H, Takahashi S, Omura, M, Soejima J, Shiratake K, Yamada K, Yamaki S** (2004) Transgenic apple transformed by sorbitol-6-phosphate dehydrogenase switch between sorbitol and sucrose supply due to its gene expression. *Plant Science* **167**, 55-61

**Kaneyoshi J, Wabiko H, Kobayashi S, Tsuchiya T** (2001) *Agrobacterium tumefaciens* AKE10-mediated transformation of an Asian pea pear, *Pyrus betulaefolia* Bunge: host specificity of bacterial strains. *Plant Cell Reports* **20**, 622-628

**Kim JH, Song KJ, Dong-Hee L, Cha JE, Woo JG** (2006) *Agrobacterium* mediated transformation using disease resistance gene, *Rab* in 'McIntosh Wijcik' apple. *Proceedings of the 1st Symposium on Biotechnology of Temperate Fruit Crops and Tropical Species*

**Kim CK, Chung JD, Park SH, Burrell AM, Kamo KK, Byrne DH** (2004) *Agrobacterium tumefaciens*-mediated transformation of *Rosa hybrida* using the green fluorescent protein (GFP) gene. *Plant Cell, Tissue and Organ Culture* **78**, 107-111

**Klee HJ, Rogers SG** (1989) Plant genetic vectors and transformation: plant transformation systems based on the use of *Agrobacterium tumefaciens*. In: Shell J, Vasil IK (Eds) *Cell Culture and Somatic Cell Genetics of Plants (Vol 6) Molecular Biology of Plant Nuclear Genes*, Academic Press, San Diego, CA, USA, pp 2-25

**Ko K, Brown S, Norelli JL, Aldwinckle HS** (1998) Alteration in *nptII* and *gus* expression following micropropagation of transgenic M.7 apple rootstock lines. *Journal of the American Society of Horticultural Science* **123**, 11-18

**Ko K, Norelli JL, Reynoird JP, Boresjza-Wysocka EE, Brown S, Aldwinckle HS** (2000) Effect of untranslated leader sequence of AMV RNA 4 and signal peptide of pathogenesis-related protein 1b on attacin gene expression, and resistance to fire blight in transgenic apple. *Biotechnology Letters* **22**, 373-381

**Ko K, Norelli JL, Reynoird JP, Aldwinckle HS, Brown SK** (2002) T4 lysozyme and attacin genes enhance resistance of transgenic Galaxy apple against *Erwinia amylovora*. *Journal of the American Society of Horticultural Science* **127**, 515-519

**Kokko HI, Karenlampi SO** (1998) Transformation of artic bramble (*Rubus articus* L.) by *Agrobacterium tumefaciens*. *Plant Cell reports* **17**, 822-826

**Kondrak M, van der Meer I, Banfalvi Z** (2006) Generation of marker and backbone-free transgenic potatoes by site specific recombination and a bi functional marker gene in a non regular one border *Agrobacterium* transformation vector. *Transgenic Research* **15**, 729-737

**Kotoda N, Iwanami H, Takahash, S, Abe K** (2006) Antisense expression of *MdTFL1*, a *TFL1* like gene, reduces the juvenile phase in apple. *Journal of the American Society of Horticultural Science* **131**, 74-81

**Korban SS, O'Connor PA, Elobeidy A** (1992) Effects of thidiazuron, naphthaleneacetic acid, dark incubation and genotype on shoot organogenesis from *Malus* leaves. *Journal of the American Society of Horticultural Science* **67**, 341-349

**Krens FA, Pelgrom KTB, Schaart JG, Den Nijs APM, Rouwendal GJA** (2004) Clean vector technology for marker-free transgenic fruit crops. *Acta Horticulturae* **663**, 431-435

**Laimer da Camara Machado M, da Camara Machado A, Hanzer V, Weiss H, Regner F, Steinkellner H, Mattanovich D, Plail R, Knapp E, Kalthoff B, Katinger H** (1992) Regeneration of transgenic plants of *Prunus armeniaca* containing the coat protein gene of plum pox virux. *Plant Cell Reports* **11**, 25-29

**Landi L, Mezzetti B** (2006) TDZ, auxin and genotype effects on leaf organogenesis in *Fragaria*. *Plant Cell Reports* **25**, 281-288

**Lebedev VG, Dolgov SV** (2000) The effect of selective agent and a plant intron on tranformation efficiency and expression of heterologous genes in pear, *Pyrus communis* L. *Russian Journal of Genetics* **36**, 792-798

**Lebedev VG, Dolgov SV** (2002) Influence of various factors on adventitious shoot formation from pear leaf explants. *Acta Horticulturae* **596**, 469-472

**Lebedev VG, Skryabin KG, Dolgov SV** (2002a) Transgenic pear rootstocks resistant to herbicide "Basta". *Acta Horticulturae* **596**, 193-197

**Lebedev VG, Taran SA, Shmatchenko VV, Dolgov SV** (2002b) Pear transformation with the gene for supersweet protein thaumatin II. *Acta Horticulturae* **596**, 199-202

**Lebedev VG, Lavrova N, Lunin VG, Naroditski BS, Dolgov SV** (2002c) Plant-defensin genes introduction for improvement of pear phytopathogenic resistance. *Acta Horticulturae* **596**, 167-171

**Leblay C, Chevreau E, Raboin LM** (1991) Adventitious shoot regeneration from *in vitro* leaves of several pear cultivars (*Pyrus communis* L.). *Plant Cell Reports* **25**, 99-105

**Lee KK, Lee HJ, Lee CY** (2005) Antioxidant and antitumor promoting activities of apple phenolics. In: Shahidi F, Ho CT (Eds) *Phenolic Compounds in Foods and Natural Health Products*, American Chemical Society Symposium Series Book 909. American Chemical Society, Oxford University Press, Washington, DC., pp 254-270

**Li X, Krasnyanski S, Korban SS** (2002) Optimization of the *uidA* gene transfer into somatic embryos of rose via *Agrobacterium tumefaciens*. *Plant Physiology and Biochemistry* **40**, 453-459

**Li X, Gasic K, Cammue B, Broekaert W, Korban SS** (2003) Transgenic rose lines harboring an antimicrobial protein gene, *Ace-Amp1*, demonstrate enhanced resistance to powdery mildew (*Sphaerotheca pannosa*). *Planta* **218**, 226-232

**Li H, Flachowsky H,·Fischer TC, Hanke MV, Forkmann G, Treutter D, Schwab W, Hoffmann T, Szankowski I** (2007) Maize *Lc* transcription factor enhances biosynthesis of anthocyanins, distinct proanthocyanidins and phenylpropanoids in apple (*Malus domestica* Borkh.) *Planta* **226**, 1243-1254

**Lin JJ, Assad-Garcia N, Kuo J** (1995) Plant hormone effect of antibiotics on the transformation efficiency of plant tissues by *Agrobacterium tumefaciens* cells. *Plant Sci*ence **109**, 171-177

**Liu Q, Ingersoll J, Owens L, Salih S, Meng R, Hammerschlag FA** (2001) Response of transgenic Royal Gala apple (*Malus X domestica* Borkh.) shoots carrying a modified cecropin MB39 gene, to *Erwinia amylovora. Plant Cell Reports* **20**, 306-312

**Liu Q, Salih S, Hammerschlag F** (1998) Etiolation of 'Royal Gala' apple (*Malus × domestica* Borkh.) shoots promotes high frequency shoot organogenesis and enhanced β-glucuronidase expression from stem internodes. *Plant Cell Reports* **18**, 32-36

**Liu ZR, Sanford JC** (1988) Plant-regeneration by organogenesis from strawberry leaf and runner tissue. *HortScience* **23**, 1057-1059

**Liu JR, Sink KC Dennis FG Jr.** (1983a) Adventive embryogenesis from leaf explants of apple seedling. *HortScience* **18**, 871-873

**Liu JR, Sink KC Dennis FG Jr.** (1983b) Plant regeneration from apple seedling explants of apple and callus cultures. *Plant Cell, Tissue and Organ Culture* **2**, 293-304

**Lopez-Noguera S, Petri C, Burgos L** (2006) Production of marker-free transgenic plants after transformation of apricot cultivars. *Acta Horticultureae* **717**, 225-227

**Lunkenbein S, Coiner H, Ric de Vos CH, Schaart JG, Boone MJ, Krens FA, Schwab W, Salentijm EMJ** (2006a) Molecular characterization of a stable antisense chalcone synthase phenotype in strawberry (*Fragaria x ananassa). Journal of Agricultural and Food Chemistry* **54**, 2145-2153

**Lunkenbein S, Bellido M, Aharoni A. Salentijm EMJ, Kaldenhoff R, Coiner HA, Munoz-Blanco J, Schwab W** (2006b) Cinnamate metabolism in ripening fruit. Characterization of a UDP-Glucose:cinnamate glucosyltransferase from strawberry. *Plant Physiology* **140**, 1047-1058

**Lunkenbein S, Salentijm EMJ, Coiner H, Boone MJ, Krens FA, Schwab W** (2006c) Up- and down-regulation of *Fragaria x ananassa O*-methyltransferase: impacts on furanone and phenulpropanoid metabolism. *Journal of Experimental Botany* **57**, 2445-2453

**Macheix JJ, Sapis JC, Fleuriet A** (1991) Phenolic compounds and polyphenoloxidase in relation to browning in grapes and wines. *Critical Reviews in Food Science and Nutrition* **30**, 441-486

**Maheswaran G, Pridmore L, Franz P, Anderson MA** (2007) A proteinase inhibitor from *Nicotiana alata* inhibits the normal development of light-brown apple moth, *Epiphyas postvittana* in transgenic apple plants. *Plant Cell Reports* **26**, 773-782

**Maghuly F, da Camara Machado LS, Khan MA, Katinger H, Laimer M** (2007) Long-term stability of marker expression in *Prunus subhirtella*: a model fruit tree species. *Journal of Biotechnology* **127**, 310-321

**Malinowski T, Cambra M, Capote N, Zawadzka B, Gorris MT, Scorza R, Ravelonandro M** (2006) Field trials of plum clones transformed with the *plum pox virus* coat protein (PPV-CP) gene. *Plant Disease* **90**, 1012-1018

**Malnoy M, Venisse JS, Chevreau E** (2005b) Expression of a bacterial effector, Harpin N, causes increased resistance to fire blight in *Pyrus communis* L. *Tree Genetics and Genomes* **1**, 41-49

**Malnoy M, Reynoird JP, Chevreau E** (2000) Preliminary evaluation of new gene transfer strategies for resistance to fire blight in pear. *Acta Horticulturae* **538**, 635-638

**Malnoy M, Venisse JS, Reynoird JP, Chevreau E** (2003a) Activation of three pathogen-inducible promoters of tobacco in transgenic pear after abiotic and biotic elicitation. *Planta* **216**, 802-814

**Malnoy M, Venisse JS, Brisset MN, Chevreau E** (2003b) Expression of bovine lactoferrin cDNA confers resistance to *Erwinia amylovora* in transgenic pear. *Molecular Breeding* **12**, 231-244

**Malnoy M, Reynoird JP, Borejsza-Wysocka E, Aldwinckle HS** (2006) Activation of the pathogen-inducible *Gst1* promoter of potato after elicitation by *Venturia inaequalis* and *Erwinia amylovora* in transgenic apple (*Malus × domestica*). *Transgenic Research* **15**, 83-93

**Malnoy M, Jin Q, Borejsza-Wysocka EE, He SY, Aldwinckle HS** (2007) Over-expression of the apple gene MpNPR1 causes increased disease resistance in *Malus* X *domestica*. *Molecuar Plant-Microbe Interactions* **20**, 1568-1580

**Malnoy M, Xu M, Borejsza-Wysocka EE, Korban SS, Aldwinckle HS** (2008) Two receptor-like genes, *Vfa1* and *Vfa2*, confer resistance to the fungal pathogen *Venturia inaequalis* inciting apple scab disease *Molecuar Plant Microbe Interaction* **21**, 448-458

**Malnoy M, Faize M, Venisse JS, Geider K, Chevreau E** (2005a) Expression of viral EPS-depolymerase reduces fire blight susceptibility in transgenic pear. *Plant Cell Reports* **23**, 632-638

**Malnoy M, Borejsza-Wysocka E, Abbott P, Lewis S, Norelli J, Flaishman M, Gidoni D Aldwinckle HS** (2007b) Genetic transformation of apple without use of a selectable marker. *Acta Horticulturae* **738**, 319-322

**Mante S, Morgens PH, Scorza R, Cordts JM, Callahan AM** (1991) *Agrobacterium*-mediated transformation of plum (*Prunus domestica* L.) hypocotyl slices and regeneration of transgenic plants. *Bio/Technology* **9**, 853-857

**Marchant R, Power JB, Lucas JA, Davey MR** (1998a) Biolistic transformation of rose (*Rosa hybrida* L.). *Annals of Botany* **81**, 109-114

**Marchant R, Davey MR, Lucas JA, Lamb CJ, Dixon RA, Power JB** (1998b) Expression of a chitinase transgene in rose (*Rosa hybrida* L.) reduces development of black spot disease (*Diplocarpon rosae* Wolf). *Molecular Breeding* **4**, 187-194

**Marchant R, Davey MR, Lucas JA, Power JB** (1996) Somatic embryogenesis and plant regeneration in Floribunda rose (*Rosa hybrida* L.) cvz. Trumpeter and Glad Tidings. *Plant Science* **120**, 95-105

**Markwick NP, Docherty LC, Phung MM, Lester MT, Murray C, Yao JL, Mitra DS, Cohen D, Beuning LL, Kutty-Amma S, Christeller JT** (2003) Transgenic tobacco and apple plants expressing biotin-binding proteins are resistant to two cosmopolitan insect pests, potato tuber moth and lightbrown apple moth, respectively. *Transgenic Research* **12**, 671-681

**Mathews H, Dewey V, Wagoner W, Bestwick RK** (1998) Molecular cellular evidence of chimaeric tissues in primary transgenics and elimination of chimaerism through improved selection protocols. *Transgenic Research* **7**, 123-129

**Mathews H, Wagoner W, Kellogg J, Bestwick R** (1995a) Genetic-transformation of strawberry – stable integration of a gene to control biosynthesis of ethylene. *In Vitro Cellular and Developmental Bioloogy – Plant* **31**, 36-43

**Mathews H, Wagoner W, Cohen C, Kellog J, Bestwick R** (1995b) Efficient genetic transformation of red raspberry, *Rubus ideaus* L. *Plant Cell Reports* **14**, 471-476

**Matsuda N, Ikeda K, Kurosaka M, Isuzugawa K, Endo T, Omura M, Takashina T** (2006) *In vitro* flowering of transgenic pears (*Pyrus communis* L.) expressing *CiFT*, a *Citrus* ortholog of the *Arabidopsis FT* gene. *3rd International Rosaceae Genomics Conference*, Napier, New Zealand

**Matsuda N, Gao M, Isuzugawa K, Takashina T, Nishimura K** (2005) Development of an *Agrobacterium*-mediated transformation method for pear (*Pyrus communis* L.) with leaf-section and axillary shoot-meristem explants. *Plant Cell Reports* **24**, 45-51

**Maximova SN, Dandekar AM, Guiltinan MJ** (1998) Investigation of *Agrobacterium* mediated transformation of apple using green fluorescent protein: high transient expression and low stable transformation suggest that factors other than T-DNA transfer are rate-limiting. *Plant Molecular Biology* **37**, 549-559

**Mehli L, Schaart JG, Kjellsen TD, Tran DH, Salentijn EMJ, Schouten HJ, Iversen TH** (2004) A gene encoding a polygalacturonase-inhibiting protein (PGIP) shows developmental regulation and pathogen-induced expression in strawberry *New Phytologist* **163**, 99-110

**Merkulov SM, Bartish IV, Dolgov SV, Pasternak TP, McHugen A** (1998) The genetic transformation of the pear *Pyrus communis* L. mediated by *Agrobacterium tumefaciens*. *Plant Genetics* **34**, 289-293

**Mezzetti B, Landi L, Pandolfini T, Spena A** (2004) The *defH9-iaaM* auxin-synthesizing gene increases plant fecundity and fruit production in strawberry and raspberry. *BMC Biotechnology* **4**: 4

**Miguel CM, Oliveira MM** (1999) Transgenic almond (*Prunus dulcis* Mills) plants obtained by *Agrobacterium*-mediated transformation of leaf explants. *Plant Cell Reports* **18**, 387-393

**Mooney PA, Goodwin PB** (1989) Presumptive transformation of apple by co-cultivation with *Agrobacterium tumefaciens*. *Acta Horticulturae* **240**, 5961

**Morgan A, Baker CM, Lee K, Crandall BA, Jose L** (2002) Production of herbicide tolerant strawberry through genetic engineering. *Acta Horticulturae* **567**, 113-116

**Mourgues F, Brisset MN, Chevreau E** (1998) Activity of different antibacterial peptides on *Erwinia amylovora* growth, and evaluation of the phytotoxicity and stability of cecropins. *Plant Science* **139**, 83-91

**Mourgues F, Chevreau E, Lambert C, De Bondt A** (1996) Efficient *Agrobacterium*-mediated transformation and recovery of transgenic plants from pear (*Pyrus communis* L.). *Plant Cell Reports* **34**, 373-378

**Murashige T, Skoog F** (1962) A revised method for rapid growth and bioassays with tobacco tissue cultures. *Physioloiay Plantarum* **15**, 472-497

**Murata M, Nishimura M, Murai N, Haruta M, Homma S, Itoh Y** (2001) A transgenic apple callus showing reduced polyphenol oxidase activity and lower browning potential. *Bioscience, Biotechnology and Biochemistry* **65**, 383-388

**Murata M, Haruta M, Murai N, Tanikawa N, Nishimura M, Homma S, Itoh Y** (2000) Transgenic apple (*Malus X domestica*) shoot showing low browning potentail. *Journal of Agricultural and Food Chemistry* **48**, 5243-5248

**Nehra NS, Chibbar RN, Kartha KK, Datla RSS, Crosby WL, Stushnoff C** (1990a) Agrobacterium-mediated transformation of strawberry calli and recovery of transgenic plants. *Plant Cell Rep*orts **9**, 10-13

**Nehra NS, Chibbar RN, Kartha KK, Dalta RSS, Crosby WL, Stushnoff C** (1990b) Genetic transformation of strawberry by *Agrobacterium tumefaciens* using a leaf disk regeneration system. *Plant Cell Reports* **9**, 293-298

**Németh M** (1994) History and importance of plum pox in stone-fruit production. *OEPP/EPPO Bulletin* **24**, 525-536

**Nielsen KM** (2003) Transgenic organisms: time for a conceptual change. *Nature Biotechnology* **21**, 227-228

**Norelli JL, Mills J, Aldwinckle HS** (1996) Leaf wounding increases efficiency of *Agrobacterium* mediated transformation of apple. *HortScience* **31**, 1026-1027

**Norelli JL. Aldwinckle HS, Destefano Beltran L, Jaynes JM** (1994) Transgenic 'Malling 26' apple expressing the attacin E gene has increased resistance to *Erwinia amylovora*. *Euphytica* **77**, 123-128

**Norelli JL Mills JZ, Momol MT, Aldwinckle HS** (1999) Effect of cecropin-like transgenes on fire blight resistance of apple. *Acta Horticulturae* **489**, 273-278

**Norelli JL, Basset CL, Artlip TS, Aldwinckle HS, Malnoy M, Boresjza-Wysocka EE, Gidoni D, Flaishman MA** (2007) Inducible DNA promoters for use in apple. *Acta Horticulturae* **738**, 329-334

**Nyman M, Wallin A** (1992) Transient expression in strawberry (*Fragaria ananassa* Duch.) protoplasts and the recovery of transgenic plants. *Plant Cell Reports* **11**, 105-108

**Oh CS, Beer SV** (2007) AtHIPM, an ortholog of the apple HrpN-interacting protein, is a negative regulator of plant growth and mediates the growth-enhancing effect of HrpN in Arabidopsis. *Molecuar Plant-Microbe Interactions* **145**, 426-436

**Oosumi T, Gruszewski HA, Blischak LA, Baxter AJ, Wadl PA, Shuman JL, Veilleux RE, Shuleav V** (2006) High-efficiency transformation of the diploid strawberry (*Fragaria vesca*) for functional genomics. *Planta* **223**, 1219-1230

**Osorio S, Castellejo C, Quesada MA, Medina-Escobar N, Brownsey GJ, Suau R, Heredia A, Botella MA, Valpuesta V** (2008) Partial demethylation of oligogalacturonides by pectin methyl esterase 1 is required for eliciting defence responses in wild strawberry (*Fragaria vesca*). *The Plant Journal* **54**, 43-55

**Ortolani C, Ispano M, Pastorello E, Bigi A, Ansaloni R** (1988) The oral allergy syndrome. *Annals Allergy* **61**, 47-52

**Owens CL, Thomashow MF, Hancock JF, Iezzoni AF** (2002) *CBF1* orthologs in sour cherry and strawberry and the heterologous expression of *CBF1* in strawberry. *Journal of American Society for Horticultural Science* **127**, 489-494

**Padilla IMG, Golis A, Gentile A, Damiano C, Scorza R** (2006) Evalutation of transformation in peach *Prunus persica* explants using green fluorescent protein (GFP) and beta-glucuronidase (GUS) reporter genes. *Plant Cell, Tissue and Organ Culture* **84**, 309-314

**Palomer X, Llop-Tous I, Vendrell M, Krens FA, Schaart JG, Boone MJ, van der Valk H, Salentijn EMJ** (2006) Antisense down-regulation of strawberry endo-β-(1,4)-glucanase genes does not prevent fruit softening during ripening. *Plant Science* **171**, 640-646

**Pati PK, Rath SP, Sharma M, Sood A, Ahuja PS** (2006) *In vitro* propagation of rose – a review. *Biotechnology Advances* **24**, 94-114

**Paris R, Cova V, Pagliarani G, Tartarini S, Komjanc M, Sansavini S** (2009) Expression profiling in *Hcrvf2*-transfromed apple plants in response to *Venturia inaequalis*. *Tree Genetics and Genomes* **5**, 81-91

**Park JI, Lee YK, Chung WI, Lee IH, Choi JH, Lee WM, Ezura H, Lee SP, Kim IJ** (2006) Modification of sugar composition in strawberry fruit by antisense suppression of an ADP-glucose pyrophosphorylase. *Molecular Breeding* **17**, 269-279

**Pasqualis G, Biricolti S, Locatelli F, Baldoni E, Mattana M** (2008) *Osmyb4* expression improves adaptive responses to drought and cold stress in transgenic apples. *Plant Cell Reports* **27**, 1677-1686

**Passey AJ, Barrett K, James DJ** (2003) Adventitious shoot regeneration from seven commercial strawberry cultivars (*Fragaria x ananassa* Duch.) using a range of explant types. *Plant Cell Reports* **21**, 397-401

**Pawlicki-Jullian N, Sedira M, Welander M** (2002) The use of *Agrobacterium rhizogenes* transformed roots to obtain transgenic shoots of apple rootstock *Jock9. Plant Cell, Tissue and Organ Culture* **70**, 163-171

**Petri C, Webb K, Hily JM, Dardick C, Scorza R** (2008) High transformation efficiency in plum (*Prunus domestica* L.) a new tool for functional genomics studies in *Prunus* spp. *Mol Breeding* **22**, 581-591

**Petri C, Albuquerque N, Garcia-Castillo S, Egea J, Burgos L** (2004) Factors affecting gene transfer efficiency to apricot leaves during early *Agrobacterium*-mediated transformations steps. *Journal of Horticultural Science and Biotechnology* **79**, 704-712

**Perez-Clemente RM, Perez-Sanjuan A, Garcia-Ferriz L, Beltran JP, Canas A** (2004) Transgenic peach plants (*Prunus persica* L.) produced by genetic transformation of embryo sections using green fluorescent protein (GFP) as an *in vitro* marker. *Molecular Breeding* **14**, 419-417

**Predieri S, Fasolo F, Malavasi F, Passey AJ, Ridout MS, James DJ** (1989) Regeneration from *in vitro* leaves of Conference and other pear cultivars (*Pyrus communis*). *Journal of Horticultural Science* **64**, 553-559

**Preininger E, Gyurjan I, Boka K, Ponyi T, Zatyko J, Koranyi P** (2003) A new approach for the biolistic method: bombardment of living nitrogen-fixing bacteria into plant tissues. *In Vitro Cellular and Develpmental Biology – Plant* **39**, 443-449

**Puterka GJ, Bocchetti C, Dang P, Bell RL, Scorza R** (2002) Pear transformed with a lytic peptide gene for disease control affects nontarget organism, pear psylla (*Homoptera: Psyllidea*). *Journal of Economic Entomology* **94**, 797-802

**Puite KJ, Schaart, JG** (1996) Genetic modification of the commercial apple cultivars Gala, Golden Delicious and Elstar via an *Agrobacterium tumefaciens*-mediated transformation method. *Plant Science* **119**, 125-133

**Puhringer H, Moll D, Hoffmann-Sommergruber K, Watillon B, Katinger H, Machado MLD** (2000) The promoter of an apple *Ypr10* gene, encoding the major allergen Mal d 1, is stress- and pathogen-inducible. *Plant Science* **152**, 35-50

**Qin Y, Texeira da Silva JA, Zhang L, Zhang S** (2008) Transgenic strawberry: State of the art for improved traits. *Biotechnology Advances* **26**, 219-232

**Qu SC, Huang XD, Zhang Z, Yao Q-H, Tao J-M., Qiao YS, Zhang JY** (2005) *Agrobacterium* mediated transformation of *Malus robusta* with tomato iron transporter gene. *Journal of Plant Physiology and Molecular Biology* **31**, 235-240

**Radchuck VV, Korkhovoy VI** (2005) The *rolB* gene promotes rooting *in vitro* and increases fresh root weight *in vivo* of transformed apple scion cultivar 'Florina'. *Plant Cell, Tissue and Organ Culture* **81**, 203-212

**Ramesh SA, Kaiser BN, Francks T, Collins G, Sedgley M** (2006) Improved methods in *Agrobacterium*-mediated transformation of almond using positive (mannose/*pmi*) or negative (Kan resistance) selection based protocols. *Plant Cell Reports* **25**, 821-828

**Ratnasiri M, Fung, RMW, Weir I, Ding H, Simons JL, Allan AC** (2002) Efficient transient transformation of suspension culture-derived apple protoplasts. *Plant Cell, Tissue and Organ Culture* **70**, 77-82

**Ravelonandro M, Monsion M, Delbos R, Dunez J** (1993) Variable resistance to plum pox virus and potato virus Y infection in transgenic *Nicotiana* plants expressing plum pox virus coat protein. *Plant Science* **91**, 157-169

**Ravelonandro M, Monsion M, Teycheney PY, Delbos R, Dunez J** (1992) Construction of a chimeric viral gene expressing plum pox coat protein. *Gene* **120**, 167-173

**Ravelonandro M, Scorza R, Bachelier JC, Labonne G, Levy L, Damsteegt V, Callahan AM, Dunez J** (1997) Resistance of transgenic *Prunus domestica* to plum pox virus infection. *Plant Disease* **81**, 1231-1235

**Ravelonandro M, Scorza R, Callahan A, Cordts J, Monsion M, Fuchs M, Delbos R, Bachelier J, Gonsalves D, Dunez J** (1994) Production of plants of *Nicotiana benthamiana* and *Prunus domestica* transgenic for plum pox potyvirus coat protein, and demonstration of PPV resistance in transformed *N. benthamiana*. *Bulletin OEPP/EPPO Bulletin* **24**, 713-719

**Reynoird JP, Mourgues F, Norelli JL, Aldwinckle HS, Brisset MN, Chevreau E** (1999a) First evidence for improved resistance to fire blight among transgenic pears expressing *attacin E* gene from *Hyalophora cecropia*. *Plant Science* **149**, 23-31

**Reynoird JP, Mourgues F, Chevreau E, Brisset MN, Aldwinckle HS** (1999b) Expression of *SB-37* gene in transgenic pears enhanced resistance to fire blight. *Acta Horticulturae* **489**, 243-244

**Ricardo VG, Coll Y, Castagnaro A, Ricci JCD** (2003) Transformation of a strawberry cultivar using a modified regeneration medium. *HortScience* **38**, 277-280

**Rice-Evans CA, Sampson J, Bramley PM, Holloway DE** (1997) Why do we expect carotenoids to be antioxidants *in vivo*? *Free Radical Research* **26**, 381-398

**Rommens CM** (2004) All-native DNA transformation: a new approach to plant genetic engineering. *Trends in Plant Science* **9**, 457-464

**Rosin FM, Aharoni A, Salentinj EMJ, Schaart JG, Boone MJ, Hannapel DJ** (2003) Expression patterns of a putative homolog of *AGAMOUS, STAG1* from strawberry. *Plant Science* **165**, 959-968

**Rout GR, Samantaray S, Mottey J, Das P** (1999) Biotechnology of the rose: a review of recent progress, *Science Horticultural* **81**, 201-228

**Roy AS, Smith IM** (1994) Plum pox situation in Europe. *EPPO Bulletin* **24**, 515-523

**Rudolf JR, Resurreccion AV** (2005) Elicitation of resveratrol in peanut kernels by application of abiotic stresses. *Journal of Agricultural and Food Chemistry* **53**, 10186-10192

**Rugini E, Orlando R** (1992) High-efficiency shoot regeneration from calluses of strawberry (*Fragaria x ananassa*-Duch) stipules of *in-vitro* shoot cultures. *Journal of Horticultural Science* **67**, 577-582

**Ruhmann S, Treutter D, Fristsche S, Briviba K, Szankowski I** (2006) Picied (resveratol glucoside) synthesis in stilbene synthase transgenic apple fruit. *Journal of Agricultural and Food Chemistry* **54**, 4633-4640

**Santigo-Domenech N, Jimenez-Bemudez S, Matas AJ, Rose JKC, Munoz-Blanco J, Mercado JA, Quesada MA** (2008) Antisense inhibition of a pectate lyase gene supports a role for pectin depolymerizaation in strawberry fruit softening. *Journal of Experimental Botany* **59**, 2769-2779

**Salentijn EMJ, Aharoni A, Schaart JG, Boone MJ, Krens FA** (2003) Differential gene expression analysis of strawberry cultivars that differ in fruit-firmness. *Physiologia Plantarum* **118,** 571-578

**Schaart JG, Salentijn EMJ, Krens FA** (2002) Tissue-specific expression of the α-glucuronidase reporter gene in transgenic strawberry (*Fragaria x ananassa*) plants. *Plant Cell Reports* **21**, 313-319

**Schaart JG, Puite KJ, Kolova L, Pogrebnyak N** (1995) Some methodological aspects of apple transformation by *Agrobacterium*. *Euphytica* **85**, 131-134

**Schaart JG, Krens FA, Pelgrom KTB, Mendes O, Rouwendal GJA** (2004) Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. *Plant Biotechnology Journal* **2**, 233-240

**Schaffer RJ, Friel EN, Souleyere EJF, Bolitho K, Thodey K, Ledger S, Bowen JH, Ma JH, Naim B, Cohen D, Gleave AP, Crowhurst RN, Janssen BJ, Yao JL, Newcomb RD** (2007) A genomics approach reveals that aroma production in apple is controlled by ethylene predominantly at the final step in each biosynthetic pathway. *Plant Physiology* **144**, 1899-1912

**Schestibratov KA, Dolgov SV** (2005) Transgenic strawberry plants expressing a thaumatin II gene demonstrate enhanced resistance to *Botrytis cinerea*. *Scientia Horticulturae* **106**, 177-189

**Schieber A, Stintzing FC, Carle R** (2001) By-products of plant food processing as a source of functional compounds – recent developments. *Trends in*

*Food Science and Technology* **12**, 401-407

**Schouten HJ, Krens FA, Jacobsen E** (2006a) Do cisgenic plants warrant less stringent oversight? *Nature Biotechnology* **24**, 753

**Schouten HJ, Krens FA, Jacobsen E** (2006b) Cisgenic plants are similar to traditionally bred plants. *EMBO Reports* **7**, 750-753

**Schulze K, Screiber L, Szankowski I** (2005) Inhibiting effects of resveratrol and its glucoside piceid against Venturia inaequalis, the causal agent of apple scab. *Journal of Agricultural and Food Chemistry* **53**, 356-362

**Scorza R, Callahan A, Levy L, Damsteegt V, Ravelonandro M** (2001a) Resistance to plum pox potyvirus in a transgenic woody perennial fruit tree, European plum (*Prunus domestica*) results from post-transcriptional gene silencing. *Acta Horticulturae* **550**, 425-430

**Scorza R, Callahan A, Levy L, Damsteegt V, Webb K, Ravelonandro M** (2001b) Post-transcriptional gene silencing in plum pox resistant transgenic European plum containing the plum pox potyvirus coat protein gene. *Transgenic Research* **10**, 201-209

**Scorza R, Ravelonandro M, Callahan A, Cordts JM, Fuchs M, Dunez J, Gonsalves D** (1994) Transgenic plums (*Prunus domestica* L.) express the plum pox virus coat protein gene. *Plant Cell Reports* **14**, 18-22

**Scorza R, Hily JM, Callahan A, Malinowski T, Cambra M, Capote N, Zagra I, Damsteegt V, Briard P, Ravelonandro M** (2007) Deregulation of plum pox resistant transgenic plum 'HoneySweet'. *Acta Horticulturae* **738**, 669-673

**Sedira M, Butler E, Gallagher T, Welander M** (2005) Verification of auxin induced gene expression during adventitious rooting in *rolB* transformed and untransformed apple Jork 9. *Plant Science* **168**, 1193-1198

**Sedira M, Holefors A, Welander M** (2001) Protocol for transformation of the apple rootstock Jork9 with the *rolB* gene and its influence on rooting. *Plant Cell Reports* **20**, 517-524

**Seong ES, Song KS, Jegal S, Yu CY, Chung IM** (2005) Silver nitrate and aminoethoxyvinylglycine affect *Agrobacterium*-mediated apple transformation. *Plant Growth Regulation* **45**, 75-82

**Sesmero R, Quesada MA, Mercado JA** (2007) Antisense inhibition of pectate lyase gene expression in strawberry fruit: characteristics of fruits processed into jam. *Journal of Food Engineering* **79**, 194-199

**Shibli RA, Ajlouni MM, Obeidat A** (2000) Direct regeneration from wild pear (*Pyrus syriaca*) leaf explants. *Advances in Horticulturae Science* **14**, 12-18

**Silfverberg-Dilworth E, Patocchi A, Belfanti E, Tartarini S, Sansavini S, Gessler C** (2005) *Hcrvf2* introduced into Gala confers race-specific apple scab resistance. In: *Plant & Animal Genome XIII Conference*, January 15-19, 2005, San Diego, CA, USA

**Smigocki AC, Hammerschlag FA** (1991) Regeneration of plants from peach embryo cells infected with a shoot mutant strain of *Agrobacterium*. *Journal of the American Society for Horticultural Science* **116**, 1092-1097

**Song GQ, Sink KC** (2007) Transformation of cherry: *Prunus cerasus* L. Montmorency and *P. cerasus x P. canescens* Gisela 6 mediated by *Agrobacterium tumefaciens* and a two-step selection system. *Acta Horticulturae* **738**, 683-689

**Song GQ, Sink KC** (2006) Transformation of Montmorency sour cherry (*Prunus cerasus* L.) and Gisela 6 (*P. cerasus x P. canescens*) cherry rootstock mediated by *Agrobacterium tumefaciens*. *Plant Cell Reports* **25**, 117-123

**Song GQ, Sink KC** (2005) Optmizing shoot regeneration and transient expression factors for *Agrobacterium tumefaciens* transformation of sour cherry (*Prunus cerasus* L.) cultivar Montmorency. *Scientia Horticulturae* **106**, 60-69

**Spolaore S, Trainotti L, Casadoro G** (2001) A simple protocol for transient gene expression in ripe fleshy fruit mediated by *Agrobacterium*. *Journal of Experimental Botany* **52**, 845-850

**Spolaore S, Trainotti L, Pavanello A, Casadoro G** (2003) Isolation and promoter analysis of two genes encoding different endo-β-(1-4)glucanases in the non-climacteric strawberry. *Journal of Experimental Botany* **54**, 271-277

**Sriskandarajah S, Goodwin PB** (1998) Conditionong promotes regeneration and transformation in apple leaf explants. *Plant Cell, Tissue and Organ Culture* **53**, 1-11

**Sriskandarajah S, Goodwin PB, Speirs J** (1994) Genetic transformation of the apple scion cultivar 'Delicious' via *Agrobacterium tumefaciens*. *Plant Cell, Tissue and Organ Culture* **36**, 317-329

**Sriskandarajah S, Skirvin RM, Abu Qaoud H, Korban SS** (1990) Factors involved in elongation and growth of adventitious shoots from tree apple scion cultivars *in vitro*. *Journal of Horticultural Science* **65**, 113-121

**Sule S, Kim WS, Geider K** (2002) Transformation of SR1 tobacco and JTE-H apple rootstock with the EPS depolymerase gene from *Erwinia amylovora* phage. *Acta Horticulturae* **590**, 407-409

**Swartz HJ, Bors R, Mohamed F, Naess SK** (1990) The effect of *in vitro* pretreatments on subsequent shoot organogenesis from excised *Rubus* and *Malus* leaves. *Plant Cell, Tissue and Organ Culture* **21**, 179-184

**Swietlik D, Vann C, Wisniewski M, Artlip T, Norelli JL, Kochian L** (2007) The effect of transporter genes on zinc stress in apple (*Malus x domestica* Borkh.). *Acta Horticulturae* **738**, 345-349

**Szankowski I, Degenhardt J** (2007) Alternative selection for apple transformation. *Acta Horticulturae* **738**, 287-292

**Szankowski I, Lubke A, Schoenherr J, Jacobsen HJ** (2001) Influence of sonication on regeneration and transformation efficiencies in apple. *Acta Horticulturae* **560**, 505-508

**Szankowski I, Briviba K, Fleschhut J, Schoenherr J, Jacobsen HJ, Kiesecker H** (2003) Transformation of apple (*Malus domestica* Borkh.) with the stilbene synthase gene from grapevine (*Vitis vinifera L.*) and a *PGIP* gene from kiwi (*Actinidia deliciosa*). *Plant Cell Reports* **22**, 41-149

**Szankowski I, Flachowsky H, Li H, Halbwirth H, Treutter D, Regos I, Hanke MV, Stich K, Fischer TC** (2009) Shift in polyphenol profile and sublethal phenotype caused by silencing of anthocyanidin synthase in apple (*Malus* sp.). *Planta* **229**, 681-692

**van der Salm TPM, van der Toorn CJG, Bouwer R, Don HJM** (1997) Production of *Rol* gene transformed plants of *Rosa hybrida* L. and characterization of their rooting ability, *Molecular Breeding* **3**, 39-47

**van der Salm TPM, Bouwer R, van Dijk AJ, Keizer LCP, Hanish Ten Cate CH, Van Der Plas LHW, Dons JJM** (1998) Stimulation of scion bud release by *rol* gene transformed rootstocks of *Rosa hybrida* L. *Journal of Experimental Botany* **49**, 847-852

**Vanek-Krebitz M, Hoffmann-Sommergruber K, Machado MLD, Susani M, Ebner C, Kraft D, Scheiner O, Breiteneder H** (1995) Cloning and sequencing of Mal d 1, the major allergen from apple (*Malus domestica*), and its immunological relationship to Bet v 1, the major birch pollen allergen. *Biochemical and Biophysical Research Communications* **214**, 538-551

**Vaughan SP, James DJ, Lindsey K, Massiah AJ** (2006) Characterization of *FaRB7*, a near root-specific gene from strawberry (*Fragaria x ananassa* Duch.) and promoter activity analysis in homologous and heterologous hosts. *Journal of Experimental Botany* **57**, 3901-3910

**Vellicce GR, Coll Y, Castagnaro AP, Diaz Ricci JC** (2003) Transformation of a strawberry cultivar using a modified regeneration medium. *Hortscience* **38**, 277-280

**Vellicce GR, Diaz Ricci JC, Hernandez L, Castagnaro AP** (2006) Enhanced resistance to *Botrytis cinerea* mediated by the transgenic expression of the chitinase gene *ch5B* in strawberry. *Transgenic Research* **15**, 57-68

**Vinatzer BA, Patocchi A, Gianfranceschi L, Tartarini S, Zhang HB. Gessler C, Sansavini S** (2001) Apple contains receptor-like genes homologous to the *Cladosporium fulvum* resistance gene family of tomato with a cluster of genes cosegregating with *Vf* apple scab resistance. *Molecular Plant-Microbe Interactions* **14**, 508-514

**Viss WJ, Pitrak J, Humann J, Cook M, Driver J, Ream W** (2003) Crown gall resistance transgenic trees that silence *Agrobacterium tumefaciens* oncogenes. *Molecular Breeding* **12**, 283-295

**Wang J, Ge H, Peng S, Zhang H, Chen P, Xu J** (2004) Transformation of strawberry (*Fragaria ananassa* Duch.) with late embryogenesis abundant protein gene. *Journal of Horticultural Science and Biotechnology* **79**, 735-738

**Wawzynczak D, Michalczuk L, Sowik I** (2005) Modification in indole-3-acetic acid metabolism, growth and development of strawberry through transformation with maize IAA-glucose synthase gene (*iaglu*). *Acta Physiologiae Plantarum* **27**, 19-27

**Wawzynczak D, Sowik I, Michalczuk L** (2003) *In vitro* proliferation and rhizogenesis of transgenic strawberry carrying maize IAA glucose synthetase gene (*iaglu*). *Acta Horticulturae* **616**, 449-452

**Welander M** (1988) Plant regeneration from leaf and stem segments of shoots raised *in vitro* from mature apple trees. *Journal of Plant Physiology* **132**, 738-744

**Welander M, Pawlicki N, Holefors A, Wilson F** (1998) Genetic transform-tion of apple rootstock M26 with *rolB* gene and its influence on rooting. *Journal of Plant Physiology* **53**, 371-380

**Welander M, Zhu LH, Li XY** (2004) Transformation of dwarfing apple and pear rootsocks aith the *rolB* gene and its influence on rooting and growth. *Acta Horticulturae* **663**, 437-442

**Wen XP, Pang XM, Matsuda N, Kita M, Inoue H, Hao YJ, Honda C, Moriguchi T** (2008) Over-expression of the apple spermidine synthase gene in pear confers multiple abiotic stress tolerance by altering polyamine titers. *Transgenic Research* **17**, 251-263

**Wilson FM, James DJ** (2003) Regeneration and transformation of the premier UK apple (*Malus x pumila* Mill.) cultivar Queen Cox. *Journal of Horticultural Science and Biotechnology* **78**, 656-662

**Woolley LC, James DJ, Manning K** (2001) Purification and properties of an endo-β-1,4-glucanase from strawberry and down-regulation of the corresponding gene, *cel1*. *Planta* **214**, 11-21

**Xue ZT, Holefors A, Welander M** (2008) Intron splicing in 5′ untranslated region of the *rol*A transcript in transgenic apple. *Journal of Plant Physiology* **165**, 544-552

**Xu ML, Korban SS** (2002) A cluster of four receptor-like genes resides in the *Vf* locus that confers resistance to apple scab disease. *Genetics* **162**, 1995-2006

**Yamashita H, Daimon H, Akasaka-Kennedy Y, Masuda T** (2004) Plant regeneration from hairy roots of apple rootstock, *Malus prunifolia* Borkh. Var. *ringo* Asami, strain Nagano No. 1, transformed by *Agrobacterium rhizogenes*. *Japanese Society of Horticultural Science* **73**, 505-510

**Yancheva SD, Shlizerman LA, Golubowicz S, Yabloviz Z, Perl A, Hanania U, Flaishman MA** (2006) The use of green fluorescent protein (GFP) improves *Agrobacterium* mediated transformation of Spadona pear (*Pyrus communis* L.). *Plant Cell Reports* **25**, 183-189

**Yao JL, Cohen D, Atkinson R, Richardson K, Morris B** (1995) Regeneration of transgenic plants from the commercial apple cultivar 'Royal Gala'. *Plant Cell Reports* **14**, 407-412

**Yau MH, Che CT, Liang SM, Kong YC, Fong WP** (2002) An aqueous extract of *Rubus chingii* fruits protects primary rat hepatocytes against *tert*-butyl hydroperoxide induced oxidative stress. *Life Science* **72**, 329-338

**Yepes LM, Aldwinckle HS** (1994) Factors that affect leaf regeneration efficiency in apple, and effect of antibiotics in morphogenesis. *Plant Cell, Tissue and Organ Culture* **37**, 257-269

**Zhang Z, Sun A, Cong Y, Sheng B, Yao Q, Cheng MZ** (2006) Agrobacterium-mediated transformation of the apple rootstock *Malus micromalus* makino with the *rol*C gene. *In Vitro Cellular and Developmental Biology – Plant* **42**, 491-497

**Zhao Y, Liu Q, Davis RE** (2004) Transgene expression in strawberries driven by a heterologous phloem-specific promoter. *Plant Cell Reports* **23**, 224-230

**Zhu LH, Welander M** (2000a) Growth characteristics of apple cultivar Gravenstein plants grafted onto the transformed rootstock M.26 with *rolA* and *rolB* under non-limiting nutrient conditions. *Plant Science* **147**, 75-80

**Zhu LH, Welander M** (2000b) Adventitious shoot regeneration of two dwarfing pear rootstocks and the development of a transformation protocol. *Journal of Horticultural Science and Biotechnology* **75**, 754-752

**Zhu LH, Li XY, Welander M** (2008) Overexpression of the *Arabidopsis gai* gene in apple significantly reduces plant size. *Plant Cell Reports* **27**, 289-296

**Zhu LH, Li XY, Welander M** (2005) Optimisation of growing conditions for the apple rootstock M26 grown in RITA containers using temporary immersion principle. *Plant Cell, Tissue and Organ Culture* **81**, 313-318

**Zhu LH, Ahlman A, Li XY, Welander M** (2001a) Intergration of the *rolA* gene into the genome of the vigorous apple rootstock A2 reduced plant height and shortened internodes. *Journal of Horticultural Science and Biotechnology* **76**, 758-763

**Zhu LH, Li XY, Ahlman A, Xue ZT, Welander M** (2004) The use of mannose as a selection agent in transformation of the apple rootstock M.26 via *Agrobacterium tumefaciens*. *Acta Horticulturae* **663**, 503-506

**Zhu LH, Li XY, Ahlman A, Welander M** (2003) The rooting ability of the dwarfing pear rootstock BP10030 (*Pyrus communis*) was significantly increased by introduction of the *rolB* gene. *Plant Science* **165**, 829-835

**Zhu LH, Li XY, Nyqvist M, Welander M** (2007) Improvement of rooting and reduction in plant height in apple and pear through gene transfer. *Acta Horticulturae* **738**, 353-360

**Zhu LH, Holefors A, Ahlman A, Xue ZT, Welander M** (2001b) Transformation of the apple rootstock M.9/29 with the *rolB* gene and its influence on rooting and growth. *Plant Science* **160**, 433-439