AN ABSTRACT OF THE THESIS OF

Sumonthip Bunnag for the degree of Doctor of Philosophy in Horticulture presented on
August 7, 1995

Title: Somaclonal Variation, Regeneration and Transformation of Quince (Cydonia oblonga Mill.) and Pear (Pyrus communis L.)

Abstract approved: Dr. Machteld C. Mok

Quince (Cydonia oblonga Mill.) is widely used as a dwarfing rootstock for pear (Pyrus communis L.). The disadvantage of this rootstock is its inefficient uptake of Fe, resulting in leaf chlorosis. Application of tissue culture and gene transfer techniques may lead to production of quince clones with improved Fe efficiency. Pear may benefit from transformation by transfer of genes involved in disease resistance, insect resistance and delay of ripening. The objectives of this research were: 1) to determine the responses of two somaclonal variants, IE-1 and IE-2, to Fe-deficiency stress under greenhouse conditions; and 2) to establish transformation procedures for quince and pear.

Two somaclonal variants of quince, IE-1 and IE-2, with tolerance to low-Fe conditions were isolated previously (Dolcet-Sanjuan et al., 1992). These two variants were compared to the original Quince A clone under various conditions in the greenhouse in 1993 and 1994. Whereas Quince A was chlorotic under conditions with high pH (low Fe availability), the variants displayed only little chlorosis. Under those conditions, the chlorophyll and Fe²⁺ concentrations in the leaves were significantly higher in the variants than in Quince A.
Agrobacterium-mediated gene transfer protocols for quince and pear were devised using leaves as explants. Efforts were directed at the following: improving the regeneration system, selecting the best antibiotic to eliminate Agrobacterium while maintaining a high regeneration frequency, and identifying a suitable selectable marker.

Regeneration of Quince A was improved by replacement of agar with 1.6 g/l gelrite. Regeneration of the pear rootstock RV.113 was enhanced by cold treatment of the shoot cultures used as explants. The antibiotic timentin, which consists of ticarcillin and a β-lactamase inhibitor, was more effective in eliminating Agrobacterium and less inhibitory to regeneration than cefotaxime and carbenicillin. Vectors containing the bar gene (bialaphos resistance) were chosen for transformation experiments since bialaphos caused less damage to the leaf explants than kanamycin at effective concentrations for selection of transformants. GUS (β-glucorondase activity) assays showed that under the proper conditions, transformed tissues were obtained.
Somaclonal Variation, Regeneration and Transformation of Quince (*Cydonia oblonga* Mill.) and Pear (*Pyrus communis* L.)

by

Sumonthip Bunnag

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Completed August 7, 1995

Commencement June 1996
Doctor of Philosophy thesis of Sumonthip Bunnag presented on August 7, 1995

APPROVED:

________________________________________
Major Professor, representing Horticulture

________________________________________
Chair of Department of Horticulture

________________________________________
Dean of Graduate School

I understand that my thesis will become part of permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to reader upon request.

________________________________________
Sumonthip Bunnag (Author)
Acknowledgement

I would like to express my sincere appreciation to my major advisor, Prof. Dr. M. Mok, for her help, guidance, understanding and encouragement on my research and everything. Also I would like to thank Prof. Drs. David Mok, Tim Righetti, Pat Hays, Tom Savage and Bill Proesting for their help, suggestions, and comments on my Ph.D. thesis. I would like to thank Dr. Ken Rowe for his help with the statistical analyses. Also I would like to acknowledge USAID for its financial support throughout the year of my study, which gave me the opportunity to learn new technology in the United States. I hope to transfer this technology to my country. In addition I would like to thank all of my friends in the lab and in the Horticulture Department that gave me a valuable friendship. Finally I would like to give all my love to my parents for their understanding and caring throughout the years that I studied at Oregon State University. They gave me strength to carry out my dream.
# TABLE OF CONTENTS

## CHAPTER I: INTRODUCTION AND LITERATURE REVIEW

- Introduction ........................................................................... 1
- Literature Review .................................................................. 3
  - Pear and quince .................................................................. 3
  - Fe nutrition in plants .......................................................... 4
    - Importance of Fe ............................................................. 4
    - Strategies for iron uptake ................................................. 6
    - Lime-induced chlorosis .................................................... 8
  - Regeneration of fruit trees .................................................. 10
  - Somaclonal variation .......................................................... 13
    - Overview ............................................................................ 13
    - Somaclonal variation of fruit trees ..................................... 14
  - Plant transformation ............................................................ 16
    - Agrobacterium-mediated transformation ............................. 16
    - Direct transformation ...................................................... 19
    - Selectable and screenable markers ..................................... 20
    - Demonstration of stable integration of transferred DNA ...... 21
  - Transformation methods for fruit trees ................................. 23
- References .............................................................................. 26

## CHAPTER II: RESPONSES OF TWO SOMACLONAL VARIANTS OF QUINCE (Cydonia oblonga Mill.) TO Fe-DEFICIENCY STRESS IN THE GREENHOUSE

- Abstract .................................................................................. 42
- Introduction ............................................................................. 43
- Materials and Methods .......................................................... 44
  - Plant Materials ..................................................................... 44
  - Design of Experiments ......................................................... 45
  - Chlorophyll Measurements .................................................... 46
  - Determination of Fe\(^{2+}\) Concentration ............................... 46
  - Data Analyses ....................................................................... 46
- Results ..................................................................................... 47
- Discussion ............................................................................... 55
- References .............................................................................. 58

## CHAPTER III: REGENERATION AND TRANSFORMATION OF QUINCE AND PEAR

- Abstract .................................................................................. 60
- Introduction ............................................................................. 61
- Materials and Methods .......................................................... 62
  - Regeneration of quince and pear ............................................ 62
| Selection of antibiotic for elimination of *Agrobacterium* after co-cultivation | 64 |
| Choice of selective agent, its concentration, and time of application | 64 |
| Transformation of quince and pear | 65 |
| GUS assays | 67 |
| Southern analyses | 71 |
| Results | 71 |
| Regeneration of quince and pear | 71 |
| Selection of antibiotic for elimination of *Agrobacterium* after co-cultivation | 77 |
| Selectable markers | 77 |
| Transformation experiments | 80 |
| Discussion | 87 |
| References | 91 |

| SUMMARY AND CONCLUSIONS | 95 |

| BIBLIOGRAPHY | 97 |
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>II-1. Chlorophyll concentrations of the upper leaves of Quince A and two somaclonal variants, IE-1 and IE-2, after 6 weeks in normal potting soil (A) and Fe-deficient soil (B-D) (1993 experiment)</td>
<td>48</td>
</tr>
<tr>
<td>II-2. Plant of IE-2 (left) and Quince A (right) after three months in soil D</td>
<td>49</td>
</tr>
<tr>
<td>II-3. Chlorophyll concentrations of the upper leaves of Quince A and two somaclonal variants, IE-1 and IE-2, in normal potting soil (A) and Fe-deficient soil (B-D) on June 15 (a), July 15 (b), and August 15 (c) (1994 experiment)</td>
<td>50</td>
</tr>
<tr>
<td>II-4. Chlorophyll concentrations of the upper leaves of Quince A and two somaclonal variants, IE-1 and IE-2, in soil E over the duration of the 1994 experiment</td>
<td>52</td>
</tr>
<tr>
<td>II-5. Fe$^{2+}$ concentrations of the upper leaves of Quince A and two somaclonal variants. IE-1 and IE-2, in normal potting soil (A) and Fe-deficient soil (B-E) on August 15 (1994 experiment)</td>
<td>53</td>
</tr>
<tr>
<td>III-1 Vectors used for transformation: A. pGSFR280; B. pGiPTV-BAR</td>
<td>66</td>
</tr>
<tr>
<td>III-2. Regeneration % and number of shoots per regenerating leaf as influenced by the type of quince explant after a culture period of 7 weeks</td>
<td>73</td>
</tr>
<tr>
<td>III-3. Regenerating shoots on quince leaves cultured on medium containing gelrite for 8 weeks</td>
<td>74</td>
</tr>
<tr>
<td>III-4. Regeneration % and number of shoots per regenerating leaf as influenced by storage of pear (RV.113) source material at 4°C for 2 weeks, followed by exposure to 25°C for 1-7 days</td>
<td>75</td>
</tr>
<tr>
<td>III-5. Regenerating shoots on pear (RV.113) leaves cultured for 8 weeks</td>
<td>76</td>
</tr>
<tr>
<td>III-6. Effects of timentin, cefotaxime, and carbenicillin on growth of <em>A. tumefaciens</em> EHA105 as measured by O.D. of the cultures after 24h.</td>
<td>78</td>
</tr>
<tr>
<td>III-7. Effects of timentin, cefotaxime, and carbenicillin on regeneration percentage (a) and number of shoots per regenerating leaf (b) after a culture period of 8 weeks of quince leaves on regeneration medium</td>
<td>79</td>
</tr>
</tbody>
</table>
LIST OF FIGURES (continued)

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-8. Effects of bialaphos (a) and kanamycin (b) on regeneration of quince leaves</td>
<td>81</td>
</tr>
<tr>
<td>III-9. Effect of 2 mg/l bialaphos, given at the start (0) and after 1, 2, and 3 weeks of culturing, on regeneration percentage and number of shoots per regenerating leaf after a culture period of 8 weeks of quince leaves on regeneration medium</td>
<td>82</td>
</tr>
<tr>
<td>III-10. Leaf of quince (experiment 2-1) showing GUS activity 6 days after incubation with <em>A. tumefaciens</em> EHA105/pGPTV-BAR</td>
<td>84</td>
</tr>
<tr>
<td>III-11. Leaf of quince (experiment 2-2) showing GUS activity 6 days after incubation with <em>A. tumefaciens</em> EHA105/pGPTV-BAR</td>
<td>85</td>
</tr>
<tr>
<td>III-12. Callus on quince leaves showing GUS activity after a culture period of 6 weeks following incubation with <em>A. tumefaciens</em> EHA105/pGPTV-BAR.</td>
<td>86</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>II-1. Analysis of variance of chlorophyll concentration in leaves as influenced by soil, genotype, and date (1994 experiment)</td>
<td>51</td>
</tr>
<tr>
<td>II-2. Average soil pH at the start of the experiment (April 15), and at the three dates of chlorophyll analyses (June 15, July 15, August 15)</td>
<td>51</td>
</tr>
<tr>
<td>III-1. Conditions and results of the first series of transformation experiments with quince leaves and EHA105/pGiPTV-BAR</td>
<td>68</td>
</tr>
<tr>
<td>III-2. Conditions and results of the second series of transformation experiments with quince leaves and EHA105/pGiPTV-BAR</td>
<td>69</td>
</tr>
<tr>
<td>III-3. Conditions and results of transformation experiments with pear leaves and EHA105/pGiPTV-BAR</td>
<td>70</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Plant breeding has led to significant improvements in the yield, disease and pest resistance, stress tolerance, and nutritional quality of food crops. However, since conventional plant breeding depends on the genetic variation available in a crop species, further improvements will be increasingly difficult to attain and may require generation of additional genetic variation or input of genes from unrelated organisms. Biotechnology can provide such new avenues. Two approaches that have been useful in this respect are generation of genetic variation by tissue culture (somaclonal variation) and manipulation of genes by vector-mediated transformation or direct gene transfer. Application of these techniques may be particularly valuable for vegetatively propagated crops, such as fruit trees, since they can bring about single genetic changes without disturbing the rest of the genome of a cultivar. This should be of great assistance to fruit tree breeding, which is a complicated and lengthy process due to high heterozygosity and long juvenile period.

Somaclonal variation has been reported for an array of plant species (Larkin and Scowcroft, 1981; Larkin, 1987; Van den Bulk, 1991; Hammerschlag, 1992). Variation in a number of traits has been observed, including agronomically important traits such as disease resistance, herbicide resistance, salt tolerance, drought tolerance, and growth
habit (Ahloowalia, 1986; Evans and Sharp, 1986; Van den Bulk, 1991; Bouharmont, 1994). Somaclonal variation may be caused by chromosomal changes, point mutations, amplification, or methylation of DNA (Karp and Bright, 1985; Lee and Phillips, 1988; Peschke and Phillips, 1992). However, the exact genetic changes causing a variant phenotype are often not known; moreover, newly acquired traits sometimes display instability, indicating that changes in gene expression, rather than genetic changes, are involved (Hammerschlag, 1992; Skirvin et al., 1993).

Transformation has been successful for many plant species (Fisk and Dandekar, 1993) and crops with resistance to herbicides, prolonged shelf life, and resistance to virus will soon be commercially available. Besides its potential in crop improvement, transformation can be used to study the function and expression of isolated genes. However, there are still a number limitations, including the lack of adequate regeneration procedures for some species and the failure to recover transformed plants (Potrykus, 1990; Schueman and Dandekar, 1993).

Both pear and quince could benefit from the application of biotechnology, by improvement of characteristics which now limit production. Such traits for pear include insect resistance, disease (fireblight) resistance, dwarfing, and delay of fruit ripening. Quince, which suffers from inefficient Fe uptake in calcareous soils (Lombard and Westwood, 1987; Duron et al., 1989; Viti and Cinelli, 1989, 1993), may become a more effective rootstock if its ability to utilize the available Fe is increased. Progress towards this goal has already been made. Two somaclonal variants of quince, IE-1 and IE-2, were selected from about 2,000 regenerated clones, showing less chlorosis than the
original Quince A clone under Fe-limiting conditions \textit{in vitro} (Dolcet-Sanjuan, 1991; Dolcet-Sanjuan \textit{et al.}, 1991, 1992). The variants displayed adaptive responses typical of Strategy I plants: their ability to reduce Fe(III) and to acidify the medium was significantly higher than that of the unmodified quince A (Dolcet-Sanjuan \textit{et al.}, 1992). However, the stability of these variant phenotypes \textit{ex vitro} has not been determined. As to transformation, protocols for pear and quince have not been reported, although regeneration procedures have been established (Chevreau \textit{et al.}, 1989; Predieri \textit{et al.}, 1989; Stimart and Harbage, 1989; Dolcet-Sanjuan \textit{et al.}, 1991).

The objectives of the research reported in this thesis were: 1) to determine the responses of the two somaclonal variants, IE-1 and IE-2, to Fe-deficiency stress under greenhouse conditions (Chapter II); and 2) to improve regeneration of quince and pear from leaf discs and to establish transformation procedures for quince and pear (Chapter III). The following literature review will give an overview of published research relevant to these two studies.

\textbf{Literature Review}

\textbf{Pear and quince}

Pear (\textit{Pyrus communis} L.) and quince (\textit{Cydonia oblonga} Mill.) belong to the Rosaceae family. They are self-incompatible diploids, with a chromosome number of \(2n=2x=34\) (Chevreau and Skirvin, 1992). Pear is widely cultivated throughout the world and is, after apple, the most important deciduous fruit tree in temperate zones (Zhao and Gu, 1990). Pear production is centered in areas with dry and warm summers,
which are important for fruit set and fruit quality, while reducing problems with diseases (Lombard and Westwood, 1987). Leading countries in pear production are China, the USA, the former USSR, Italy and Spain (Zhao and Gu, 1990). Pear scions are usually grafted on rootstocks. A number of pear species, *P. communis*, *P. betulaefolia*, and *P. calleryana*, as well as quince, can serve as suitable rootstocks (Zhao and Gu, 1990; Chevreau and Skirvin, 1992). The choice of rootstock depends on the soil type, climate, and desired growth habit (Stebbins, 1989). Moreover, the rootstock influences the severity of diseases in pear trees, including fire blight, pear decline, and bacterial blight (Stebbins, 1989).

Quince is a deciduous shrub or small tree. Quince populations were first classified by Hatton at East Malling in 1920's; seven groups were identified and designated A to G. Two of these selection have been commercially used, quince A and quince C (Duron *et al.*, 1989). Quince is a widely used dwarfing rootstock for pear scions. It is particularly prevalent in Europe, not only because of its dwarfing capacity, but also because of its precocity and high yield in intensive orchards (Stebbins, 1989). Its use in the Pacific Northwest is limited to the pear-growing region in the vicinity of Medford, Oregon.

**Fe nutrition in plants**

**Importance of Fe**

Fe is a micronutrient involved in many metabolic processes. It forms part of several important proteins involved in photosynthesis and respiration (Marschner, 1986;
Romheld and Marschner, 1986; Barton and Hemming, 1993). Fe functions as an electron carrier, since it can be oxidized and reduced (conversion between the Fe$^{3+}$ and Fe$^{2+}$ forms) (Marschner, 1986; Barton and Hemming, 1993; Manthey et al., 1994). Deficiency of Fe results in decreased synthesis of Fe-containing proteins, reduced production of chlorophyll, and lower photosynthetic capacity. The first visible symptom of iron deficiency is interveinal chlorosis in the youngest leaves; the older leaves generally remain green. Interverinal chlorosis is sometimes followed by chlorosis of the vein, and in severe cases the young leaves become white with necrotic lesions (Marschner, 1986; Salisbury and Ross, 1992). Fe deficiency occurs commonly at Fe levels below 50 $\mu$g/g dry weight. Up to 80 % of Fe in green plants is located in the chloroplasts, stored in the stroma as Fe$^{3+}$ in phytoferritin (Seckbach, 1982; Smith, 1984; Salisbury and Ross, 1992). Fe$^{3+}$ is covered with a protein coat (apoferritin), which allows storing iron at high concentrations, which are otherwise toxic (Seckbach, 1982).

Fe is essential for chlorophyll biosynthesis. It is required for formation of delta-aminolevulenic acid (ALA), a precursor of heme and chlorophyll (Miller et al., 1984). Fe is also present in coproporphyrinogen oxidase which catalyzes biosynthesis of chlorophyllides and chlorophylls from Mg-protoporphyrin IX (Smith, 1984; Marschner, 1986). Fe deficiency leads to disorganization of the chloroplast structure, inhibition of grana stack formation, and parallel aggregation of lamellae (Marschner, 1986). In the bundle sheath chloroplasts of C$_4$ plants, which are devoid of grana, Fe deficiency leads to swelling of thylacoids (Marschner, 1986).
An important group of Fe-containing proteins are the hemoproteins, to which cytochromes, catalases, peroxidases, and leghemoglobin belong (Marschner, 1986). Cytochromes are electron carriers with Fe-porphyrin as prosthetic group and are of importance in the Photosystem II of chloroplasts and in the mitochondrial respiratory chain (Smith 1984; Marschner, 1986). Catalase mediates conversion of $H_2O_2$ to $H_2O$ and $O_2$, while peroxidase is involved in oxidation of organic hydrogen donors, leading to formation of $H_2O$ (Marschner, 1986). Leghemoglobin is found in nodules of plants that are infected with *Rhizobium* and maintains anaerobic conditions by its ability to bind $O_2$ (Tang et al., 1990). Of the non-heme Fe proteins, Fe-S proteins take an important place. The most prominent of these is ferredoxin; other examples are Fe-S e\textsuperscript{-} carrier and aconitase (Smith, 1984; Marschner, 1986). Ferredoxin serves as a redox protein in Photosystem I, nitrate reduction, and $N_2$ reduction. Aconitase plays an important role in the tricarboxylic acid cycle, catalyzing the isomerization of citrate to isocitrate in mitochondria. Inhibition of this enzyme by Fe deficiency causes accumulation of organic acids, such as malic and citric acid, in the roots (Bienfait, 1988a).

**Strategies for iron uptake**

There are two different strategies for Fe uptake in plants: Strategy I, utilized by dicots and non-gramineous monocots, and Strategy II, used by the gramineous monocots (Marschner, 1986; Romheld, 1987; Bienfait, 1988a; Guerinot and Yi, 1994; Manthey et al., 1994). The most important features of Strategy I include Fe reduction by plasma membrane reductases and acidification of the rhizosphere, while excretion of phenolics with reducing or chelating ability also plays a role (Chaney et al., 1972; Romheld and...
Marchner, 1984a; Marschner, 1986; Guerinot and Yi, 1994). Essential for iron uptake in Strategy II plants is the excretion of phytosiderophores, small molecules excreted into the rhizosphere which are able to mobilize iron for uptake by the roots (Marchner, 1986; Romheld and Marschner, 1986; Chaney et al., 1989). Examples of such compounds are mugeneic acid, avenic acid, and ditichonic acid A (Marschner, 1986; Salisbury and Ross, 1992). Since pear and quince are dicots and utilize Strategy I, the following description will center on the mechanisms involved in Strategy I.

Fe in the soil occurs in the oxidized (Fe$^{3+}$) form and needs to be reduced to the Fe$^{2+}$ form before absorption into root cells, as first suggested by Chaney et al. (1972). A small region, generally less than 5 mm located about 1-2 cm from the root tip, has high reductive activity due to the presence of Fe reductases in the plasma membranes of the root cells (Bienfait et al., 1982; Barrett-Lennard et al., 1983; Romheld and Marschner, 1983; Bell et al., 1988). Fe-reducing activity is present at low level when Fe availability is high and increases when Fe availability decreases. Bienfait (1985, 1988a) first suggested the occurrence of two types of reductases, a constitutive reductase and inducible or "turbo" reductase. Since that time several reductases have been isolated from plasma membranes (Bruggeman et al., 1990; Holden et al., 1991; Moog and Bruggeman, 1992; Holden et al., 1994). However, the current findings can not support the induction of separate reductases under Fe-deficient conditions, but indicate an increase in the reductases that are already expressed at low levels under Fe-sufficient conditions (Holden et al., 1991; Bruggemann et al., 1993).
Fe-stressed Strategy I plants show increased release of H\(^+\) ions. This ATPase-driven proton pumping lowers the pH of the apoplast and rhizosphere (Romheld and Marschner, 1984\textit{a}; Romheld \textit{et al.}, 1984\textit{b}; Welkie and Miller, 1993). The decrease in pH enhances solubility of Fe\(^{3+}\), resulting in higher Fe uptake. However, in highly alkaline soil, the acidification is often not sufficient to overcome Fe stress because of the buffering effects of bicarbonate (HCO\(_3\)) (Marschner, 1986; Romheld, 1987).

Chaney \textit{et al.} (1972) demonstrated that compounds capable of reducing Fe\(^{3+}\) were excreted from roots. Moreover, high levels of phenolics (such as caffeic acid) and organic acids (citric acid and malic acid) were found in roots of Fe-deficient plants (Brown and Amber, 1973; Olsen and Brown, 1980; Landsberg, 1981; Olsen \textit{et al.}, 1981; Romheld and Marschner, 1981, 1983). Excreted phenolics and organic acids can help mobilize Fe(III) through acidification and chelation.

In addition to the biochemical changes, there are morphological modifications induced by Fe stress. For instance, there is an increase in number and length of root hairs, swelling of the apical root zones, and appearance of transfer cells in the rhizodermis of the apical root zones (Kramer \textit{et al.}, 1980; Marschner, 1986; Romheld and Marchner, 1986; Bienfait, 1988\textit{b}).

**Lime-induced chlorosis**

Lime-induced chlorosis is the most frequent nutritional disorder occurring in plants grown in calcareous soils, which contain high levels of calcium bicarbonate (Marschner, 1986). These soils have a very high pH, usually between 7.5 and 8.5, and contain 20\% to 95\% free calcium carbonate (CaCO\(_3\)). The iron concentrations in the
solutions of these soils is less than $10^{-10}$ M, whereas the Fe concentration required for optimal plant growth is $10^{-6}$ to $10^{-5}$ M (Lindsay, 1974).

In addition to its effect on Fe solubility, high bicarbonate concentrations seem to influence the uptake and transport of Fe in plants. Under buffered, high pH conditions, both $H^+$ efflux and Fe reduction are greatly inhibited (Romheld and Marschner, 1986). Recent evidence indicates that the initial effect of bicarbonate or other high-pH buffers resides in the inhibition of the apoplastic pH shift (Thibaud et al., 1994). As a consequence, reductase activity decreases, since the pH is too high for optimal enzyme activity. Moreover, Fe(III) chelates are less available at the plasma membrane due to electrostatic repulsion between the chelates and the membrane or cell wall, thereby decreasing Fe reduction (Thibaud et al., 1994). The presence of bicarbonate also reduces the release of phenolics, impairs root elongation and inhibits xylem flux from root to shoot, thereby decreasing nutrient transport to the leaves (Dofing et al., 1989).

Plant species vary in their sensitivity to low Fe. Crops that are susceptible to lime-induced chlorosis include soybean, sorghum, dryland rice, peanut, grapevine, citrus, peach, plum, cherry, pear and apple (Chen and Barak, 1982; Vose, 1982). Pronounced differences in lime-induced chlorosis have also been observed between related species and even genotypes belonging to the same species (Olsen and Brown, 1980; Hamze and Nimah, 1982). For instance, of pear species and rootstocks, *Pyrus amygdaliformis* is quite tolerant to low Fe availability, and is followed by *P. communis*, *P. betulaefolia* and *P. calleryana*. *Cydonia oblonga* is more sensitive than any of the *Pyrus* species.
However, even within *C. oblonga*, variation has been observed in this trait (Viti and Cinelli, 1989), making selection a possible avenue to alleviate lime-induced chlorosis.

To control lime-induced chlorosis, Fe chelates are applied, either as drip irrigation or foliar spray (Salisbury and Ross, 1992). Some of the most frequently applied chelates are FeEDDHA (Fe ethylenediamine di(o-hydroxyphenyl) acetic acid; trade name sequestrene) and FeEDTA (Fe ethylenediaminetetraacetic acid; trade name versenate) (Salisbury and Ross, 1992). However, application of chelates is expensive and offers only a temporary solution (Hamze and Nimah, 1982; Salisbury and Ross, 1992). A possible alternative is application of soil- or root-associated bacteria that excrete siderophores (Bar-Ness *et al*., 1992). However, the most lasting solution is genetic improvement of rootstocks for more efficient Fe utilization.

**Regeneration of fruit trees**

The ability to regenerate plants from cultured tissues is a prerequisite for unconventional methods of fruit tree improvement such as selection of somaclonal variation, somatic hybridization, and genetic transformation. A number of reports have been published on regeneration systems of fruit trees such as pear, quince, apple, peach, plum and apricot. Adventitious bud regeneration in *Pyrus* species was first report by Mehra and Jaidka (1979). Shoots were regenerated from pear roots (Yehia, 1985) and shoot internodes (Viseur, 1986); however, the regeneration frequencies were quite low. Higher regeneration rates were obtained with leaf discs as explant materials (Chevreau *et al*., 1989; Predieri *et al*., 1989b; Leblay *et al*., 1991). Both Chevreau *et al*. (1989) and Predieri *et al* (1989b) reported regeneration of about 30 to 40% of the leaf discs.
The best results were obtained on half-strength MS mineral (Murashige and Skoog, 1962) with a \( \text{NH}_4^+:\text{NO}_3^- \) ratio of 1:3 and the growth regulators thidiazuron (TDZ) and \( \alpha \)-naphthaleneacetic acid (Leblay et al., 1991). An initial culture phase of 30 days in the dark enhanced regeneration significantly, with regeneration ranging from 62% to 97% for four cultivars (Leblay et al., 1991). Shoot regeneration from leaf mesophyll protoplasts has also been reported, but only from wild pear material, *Pyrus communis* var. Pyraster L. (Ochatt and Caso, 1986).

Shoot regeneration from leaf discs of quince has been reported by Dolcet-Sanjuan (1991). Leaves were obtained from three-week-old micropropagated shoots of *C. oblonga*. Leaves were dissected into transverse sections, leaving the sections together at one margin and then placed, with abaxial side down, in petri dishes containing MS-N6 medium with 32 \( \mu \)M TDZ and 0.3 \( \mu \)M NAA. All cultures were kept at 25°C in the dark for the first three weeks, and at a 16 h photoperiod (40 umol m\(^{-2}\)s\(^{-1}\)) for the following three weeks. The frequency of regeneration was 78% with 3.2 shoots per regenerating leaf.

There are numerous reports of regeneration of apple, both by somatic embryogenesis and adventitious organogenesis. *In vitro* regeneration of somatic embryos was reported by Eicholtz et al. (1979) and Liu et al. (1983). Kouider et al. (1984) obtained adventitious shoots from apple cotyledons. The most prolific regeneration was achieved with leaves and leaf segments (James et al., 1988; Welander, 1988; Fasolo et al., 1989; Predieri and Fasolo, 1989a; Belazi et al., 1991). Predieri and Fasolo (1989a) reported high frequency shoot regeneration from leaves of the apple rootstock M26.
(Malus pumila Mill.). James et al. (1988) obtained 90% shoot regeneration from leaf strips of cv. Greensleeves on medium containing 2 mg/l BAP and 0.5 mg/l NAA. Dark treatment of leaf explants enhanced regeneration (Welander, 1988; Dufour, 1990) with optimum levels observed following one week (Korban et al., 1992) or two weeks (Welander, 1988; Fasolo et al., 1989) in darkness prior to transfer to lighted condition. Successful shoot regeneration has also been achieved from internode segments (Belazi et al., 1991). In addition, plant regeneration methods for leaf protoplasts cultures of apple were established by Patat-Ochatt et al. (1988) and Perales and Schieder (1993).

Shoots were regenerated from embryo-derived callus of Redhaven peach (Prunus persica L.) that was initiated on MS medium. The percentage of embryo callusing was 49% (Smigocki and Hammerschlag, 1991). Regeneration of peach rootstock cultivars from cotyledons of mature stored seed has been reported by Pooler and Scorza (1995). Shoot regeneration from mature stored seed of three peach rootstock cultivars were highest when cotyledons were cultured for 3 weeks in darkness on MS medium with 2.5% sucrose and a combination of IBA (1.25 or 2.5 μM) and TDZ (6.25 or 12.5 μM). Regeneration rates for 'Flordaguard', 'Nemared' and 'Medaguard' were 60%, 33%, and 6% respectively. In vitro regeneration of two plum genotypes was achieved from leaf explants (Yancheva, 1994) with 46.25% of the explants giving shoots for cv. Stanley and 31.4% for cv. Kjustendilska sliva. Plum can also be regenerated from cotyledon halves of mature seeds (Korte and Casper, 1994). A regeneration system for apricots (Prunus armeniaca cv. Kecskemeter) from cotyledons of immature embryos was established by Machado et al. (1992a,b). The regeneration rate was highest when the cotyledons were
taken between day 68 and 89 after full bloom, both in terms of the number of cotyledons forming shoots, and the number of shoots regenerated per cotyledon.

**Somaclonal variation**

**Overview**

Tissue and cell cultures are a rich source of genetic variation. Although it was initially assumed that plants regenerated from the same tissues would be genetically identical, as a consequence of the genetic variation in cultured tissues, plants derived from such tissues can be different from each other and the parent plant (Larkin and Scowcroft, 1981; Skirvin *et al.*, 1993). The variation among plants derived from cell cultures has been termed somaclonal variation (Larkin and Scowcroft, 1981). Somaclonal variation can be a source of valuable traits; however, its occurrence can be a disadvantage when clonal propagation or transformation is desired.

The first detailed studies on variation in cell cultures and plants derived from them were conducted with sugarcane and included cytological examinations as well as evaluations of phenotypical characteristics (Heinz and Mee, 1969; 1971). Variants were found with heavier tillering, slower growth rates, and increased erectness (Heinz and Mee, 1971). Since that time somaclonal variation has been reported for a number of crops and has been exploited for crop improvement (Poehlman, 1987; Van den Bulk, 1991; Skirvin *et al.*, 1993; Bouharmont, 1994; Donovan *et al.*, 1994a). Traits range from morphological changes such as flower shape, leaf shape, plant height (Skirvin *et al.*, 1993) also difference in yield and protein contents (Donovan, 1994a). These traits
include resistance to disease (Van den bulk, 1991; Donovan, 1994a), resistance to abiotic stress such as salt, aluminium, herbicide, drought and cold resistance (Skirvin et al., 1993; Bouharmont, 1994; Donovan, 1994a).

Somaclonal variation can be due to pre-existing differences in the plant tissues or to changes induced during the culture period (Evans and Sharp, 1986). Many factors influence the amount of somaclonal variation, including the species or genotype, the explant source, the duration of the culture period, and the culture medium and environmental conditions (Evans and Sharp, 1986; Karp and Bright, 1985; Meins, 1983; Poehlman, 1987). The possible causes of somaclonal variation have been investigated in a number of studies. Changes in chromosome number or structure are frequently occurring events (Lee and Phillips, 1988). Breakage of chromosomes often causes a loss of chromatin, resulting in deletions of portions of chromosome arms or translocations (Lee and Phillips, 1987; McCoy et al., 1982). Point mutations (D'Amato, 1985; Brown and Lorz, 1986), gene amplification (Lee and Phillips, 1988) and DNA methylation (Brown, 1989) are some of the other causes of somaclonal variation. The problem of somaclonal variation is the frequent instability of the acquired traits, indicating that part of the variation may be due to epigenetic changes (Skirvin et al., 1993).

Somaclonal variation of fruit trees

Several somaclonal variants have been reported for fruit trees, including peach, apple, and quince. Hammerschlag and Ognjanov (1990) evaluated peach plants derived from embryo callus for resistance to Xanthomonas campestris pv. pruni. Only one of the two cultivars used gave rise to somaclonal variants with increased resistance to the
pathogen, indicating that the frequency of somaclonal variants in peach is genetically
determined (Hammerschlag and Ognjanov, 1990).

Among 40 clones derived from apple leaf discs, one consistently exhibited
increased resistance to *Phytophthora cactorum* (Rosati et al., 1990). Donovan et al.
(1994a) described the occurrence of somaclonal variants of apple with increased
resistance to the fire blight pathogen, *Erwinia amylovora*. Of the 270 clones derived
from leaf explants, 33% showed some increase in resistance in the greenhouse, and 16
somaclones with relatively high levels of resistance were selected. In the same
population, variation in rooting ability and shoot proliferation was observed (Donovan
et al., 1994b). Differences in isozyme patterns were also observed among apple
regenerants (Martelli et al., 1993).

Somaclonal variants of quince with increased tolerance to Fe-deficient conditions
were obtained (Dolcet-Sanjuan, 1991; Dolcet-Sanjuan et al., 1990, 1992). In the initial
screen, six out of 2,000 shoots regenerated from quince leaves showed increased
tolerance to low Fe availability (on medium containing FeSO₄ and bicarbonate) and two
of these displayed stable tolerance upon repeated testing. The trait was expressed in
shoot cultures, roots of plantlets, root cultures, and cell suspension cultures. The
somaclonal variants had not only higher chlorophyll concentrations in the leaves, but also
increased ability to reduce Fe(III) and acidify the medium when compared to the original
quince clone (Dolcet-Sanjuan et al., 1992). Thus, the typical responses of Strategy I
plants were manifested by these variants.
Plant transformation

Plant transformation is defined as the introduction of genetic material into plant cells, resulting in chromosomal insertion, which is stable through meiosis (Gasser and Fraley, 1989; Walden, 1989; Potrykus, 1991; Glick and Thompson, 1993). The ability to transform plants with foreign DNA encoding specific genetic information is a valuable tool for molecular genetics and crop improvement (Goodman et al., 1987; Poehlman, 1987; Gasser et al., 1989; Marx, 1989; Chen and Sharp, 1990; Kays, 1991). Transgenic plants have been used to analyze and define DNA sequences such as promoters, controlling organ- and tissue-specific expression (Bressan and Handa, 1992). Transformation techniques can lead to transfer of genes across sexual barriers and not only introduce genes from other plant species but even unrelated organisms such as bacteria and fungi (Schaff, 1991). Two approaches can be used to accomplish gene transfer: transformation with Agrobacterium containing altered Ti plasmids and direct transformation with small plasmids (Fraley et al., 1986; Potrykus, 1988, 1990, 1991; Chen and Sharp, 1990). Regeneration techniques, either organogenesis or somatic embryogenesis, are generally required. Therefore, progress in transformation has closely followed the developments in cell culture and regeneration methods of individual plant species (Walden and Schell, 1991; Glick and Thompson, 1993).

Agrobacterium-mediated transformation

Agrobacterium tumefaciens is a soil bacterium and is sometimes referred to as "nature's first genetic engineer." It is a gram-negative rod and belongs to the family Rhizobiaceae (Ream, 1989). All members of this family have large plasmids (larger than
100kb). A. tumefaciens is a plant pathogen causing crown gall disease in a wide range of dicotyledons (Ream, 1989; Hooykaas and Schilperoort, 1992). A. tumefaciens is able to transfer a small piece of DNA (transfer DNA or T-DNA) containing tumorigenic loci from its tumor-inducing (Ti) plasmid into the genome of a large number of plants. The development of the tumor is caused by the expression of these oncogenes. A related species, A. rhizogenes, induces the hairy root disease, an uncontrolled proliferation of branched roots with many root hairs, also by transfer of genes contained on the plasmid (Hooykaas, 1989).

The circular Ti plasmid contains about 100 genes. The T-DNA consists of approximately 20 kb of DNA bordered by 25 bp repeats (Baker et al., 1983; Ream, 1989; Walden, 1989; Rossi et al., 1993). The T-DNA carries genes encoding opine-synthesizing enzymes and enzymes involved in cytokinin and auxin biosynthesis (Ream, 1989; Walden, 1989; Raven et al., 1992). To generate vectors that can incorporate desirable DNA in the plant genome, the plasmids are disarmed by removing the opine- and hormone-related genes (Fraley et al., 1986; Ream, 1989; Walden, 1989). Selectable and screenable markers are inserted to allow selection and identification of transformed plants.

The infection process is initiated by wound-induced release of phenolics by the plant (Fraley et al., 1986; Ream, 1989; Walden, 1989). These phenolics (e.g., 4-acetyl-2,6-dimethoxyphenol; acetosyringone) are the chemical trigger for the cascade of events in the bacteria mediated by the virulence (vir) region of the Ti plasmid (Fraley et al., 1986; Ream, 1989; Walden, 1989). The vir region contains at least six loci, vir A, vir
While the function of the vir region begins with induction of vir A by phenolics, it ends with endonuclease encoded by the vir G gene. This enzyme is involved in the excision of the T-DNA and its random insertion in the plant genome (Fraley et al., 1986; Ream, 1989; Walden, 1989). Two types of Agrobacterium-based vectors are used for plant transformation: co-integrative or cis vectors and binary or trans vectors (Fraley et al., 1986; Walden, 1989). In co-integrative vectors, the modified T-DNA and the vir region occur on the same plasmid, while in binary vectors, they occur separately, on different plasmids. The disadvantage of co-integrative vectors is the low frequency of co-integrate formation, which ranges between $10^{-5}$ to $10^{-10}$ for the pMON200 system (Fraley et al., 1986).

Agrobacterium is generally used in combination with wound tissue, including leaf explants or other excised plant tissues, and callus or protoplast cultures (Fraley et al., 1986). After cocultivation for 2 or 3 days, Agrobacterium is eliminated by incorporation of antibiotics in the medium, while the transformed cells are selected by addition of selective agent. Although most Agrobacterium-mediated transformation involves the use of a regeneration system (Fraley et al., 1986; Horsch et al., 1988; James and Dandekar, 1991), it should be noted that there are some interesting exceptions, in which whole plants are used. For transformation of Arabidopsis thaliana, either seeds can be incubated with the modified bacterium and then germinated (Feldmann and Marks, 1987), or seedlings are infiltrated with the bacterium under vacuum (Bechtold et al., 1993). For
such transformation systems, selection of transformants needs to be delayed until the next generation.

Direct transformation

_Agrobacterium_ has a limited host range and does not infect monocotyledonous plants such as cereals (Paszkowski _et al._, 1988; Cocking, 1990; Raven _et al._, 1992; Fisk and Dandekar, 1993). This has contributed to the development of alternative transformation methods, avoiding the use of _Agrobacterium_-based vectors. Moreover, some of these direct transformation methods have been useful for transient assays, to determine gene expression and identify gene products. Small plasmids, maintained in _E. coli_, are used for direct gene transfer. The plasmids usually contain the same genes as used for vector-mediated transformation are used, including selectable markers and reporter genes.

Direct DNA transfer has been accomplished in several ways. For instance, protoplasts have been used to facilitate DNA uptake, by chemicals, such as PEG and Ca ions (Shillito _et al._, 1985), by electroporation (Shillito _et al._, 1985; D' Halluin _et al._, 1992), and microinjection (Neuhaus _et al._, 1987). The limitation of these types of methods with regard to production of stably transformed plants is the prerequisite of plant regeneration plants from protoplasts. A very widely used method is microprojectile bombardment, using a gene gun, with tungsten or gold particles as microcarriers (Klein _et al._, 1987). Various types of plant materials and tissues have been used for microbombardment, including callus cells, leaf tissues, embryos and meristems (Fisk and Dandekar, 1993). Other, less frequently used methods include fusion of protoplasts with
liposomes (Riggs and Bates, 1986) microinjection into tillers (Fisk and Dandekar, 1993), incubation of pollen (Hess, 1977; De Wet et al., 1986; Ohta, 1986; Potrykus, 1991), DNA transfer during pollen tube growth (Luo and Wu, 1988), electrophoresis (Ahokas, 1989), and laser-beam mediated transfer (Weber et al., 1988). Thus, for some of the direct DNA transfer methods, a tissue culture phase may be avoided. However, it should be noted that the frequencies of transformation achieved by some of these methods are very low (Potrykus, 1990; Fisk and Dandekar, 1993).

For species other than those belonging to the graminaceae, where there is usually a choice between Agrobacterium-mediated and direct transformation, the advantages and disadvantages of each method need to be considered. The disadvantage of direct DNA transfer is the generally lower rate of transformation, while its advantage is the absence of bacterial infection and thus avoidance of treatment with antibiotics necessary to eliminate Agrobacterium (Fraley et al., 1986; Miki and Iyer, 1990; Potrykus, 1990).

Selectable and screenable markers

Selectable markers allow selection of transformed cells and tissues, by their ability to grow in the presence of the selective agent. A number of selectable markers have been used, most often the nptII gene, conferring kanamycin resistance, since it is effective in many systems and was the first such gene available (Topfer et al., 1980; De Block et al., 1984; Velten et al., 1984; Jefferson, 1987; Jefferson et al., 1986; 1987; Becker et al., 1992). Other examples of selectable markers include those conferring resistance to methotrexate (Eichholtz et al., 1987; Kemper et al., 1992), bialaphos (De Block et al., 1987; White et al., 1990), hygromycin (Van Den Elzen et al., 1985; Becker
et al., 1992), and glyphosate (Fillatti et al., 1987). The proper choice of gene may depend on the plant tissue; for instance, hygromycin has been reported more effective (Puonti-Kaerlas et al., 1990) and less effective (D’Halluin et al., 1990) than kanamycin. In addition to the selectable marker, a reporter gene such as CAT (encoding chloramphenicol acetyltransferase), GUS (encoding a bacterial β-glucuronidase), or luc (luciferase) is often used, which can aid further characterization of putative transformants (Reynaerts, 1988). The GUS gene has been extremely useful for monitoring transformation during the period of gene transfer from Agrobacterium tumefaciens as well as examining gene expression in transformed plants (Jefferson, 1987; Jefferson et al., 1986; 1987; Janssen and Gardner, 1989). CAT has served an earlier screenable marker in animal and plant cell systems (De Block et al., 1984) and has been used to analyse the activity and regulation of plant promoters (Herrera-Estrella et al., 1985). The luciferase gene from firefly (Photinus pyralis) or the marine bacterium (Vibrio harveyi) is expressed in transgenic plants by light production (Ow et al., 1986).

Demonstration of stable integration of transferred DNA

The recovery of shoots after stringent selection procedures may appear promising; however, the occurrence of escapes dictates that rigorous proof that the foreign DNA is present in the plant genome needs to be provided. Thus, it is imperative that stringent selection criteria are applied to demonstrate integrative transformation. Shoots are usually rooted in medium containing the selective agent, which provides a better test than the initial regeneration on selective medium, because of the intimate contact between roots and medium. If a reporter gene was present on the construct, reporter gene activity
is used as a second indication for transformation. It should be noted that some of the earlier reporter genes such as GUS could be expressed in the bacteria; however, the newer forms of this gene contain either an intron (Vancanneyt et al., 1990) or an eukaryotic binding site (Janssen and Gardner, 1989). Moreover, the gene products of the selectable marker can be determined; for instance, there is an enzymic assay and an immunological test for nptII activity (Jefferson, 1987a; McDonnell et al., 1987; Peng et al., 1993). More definitive proof of transformation is provided by Southern hybridization (Southern, 1975). For digestion of plant DNA, restriction enzymes that do not cut the T-DNA are recommended, since they produce positive fragments of different sizes for the various transformants. Although PCR is often used to demonstrate the presence of transgenes (Triglia et al., 1988), this cannot substitute for Southern analyses (Potrykus, 1991). Particularly when Agrobacterium is used for transformation, there is the risk that some bacteria are still present in the tissues, which could potentially result in false positives. As pointed out by Potrykus (1991), it is not sufficient to demonstrate positive results in some tests, but there should be a tight correlation between the results. Transformed plants should prove positive in all of these tests, whereas the control plants should always be negative. The most definitive proof stems usually from analyses of progeny (Potrykus, 1991), which should show segregation into transformed and nontransformed plants (again, with strict correlations between the results of the various tests). However, this may not be possible for non-flowering plants or trees with a long juvenile phase.
Transformation methods for fruit trees

Various *Agrobacterium*-based vectors in combination with some of the regeneration systems described above have been used for transformation of fruit trees. It should be noted that regeneration from leaf discs or stem segments as reported for apple, pear and quince offers a distinct advantage over regeneration from embryos or seedlings as reported for some of the *Prunus* species, since the genotype of the parental cultivar is maintained. Thus, transformation of apple, pear and quince can directly lead to improved cultivars. There are several examples of successful transformation of apple, using the leaf disc regeneration system (James *et al.*, 1989; Dandekar, 1992). A number of factors influencing gene transfer in apple have been identified (James *et al.*, 1993a; De Bondt *et al.* 1994). The disarmed *A. tumefaciens* strain, EHA101 (pEHA101) was found to be the most effective for apple transformation (De Bondt, *et al.*, 1994). Maheswaran *et al.* (1992) suggested that the wild-type strain A281 was more effective than C58 and A348 for the apple rootstocks M26. De Bondt *et al.* (1994) suggested that cocultivation of leaf explants with *A. tumefaciens* on a medium with a high cytokinin level was more conducive to gene transfer than cocultivation on media with high auxin concentrations. In addition, precultivation of leaf explants, before cocultivation, increased GUS expression when measured immediately after cocultivation, but it decreased the number of transformed calli on the explants 6 weeks after infection. Compounds that increase *Agrobacterium*-based transformation in apple are acetosyringone and betaine phosphate (James *et al.*, 1993b). Other factors that affect transformation of apple are the *Agrobacterium* cell density during infection, the bacterial growth phase, the
nature of the carbon source, explant age, explant genotype and strain modification of
*Agrobacterium* (Schuerman and Dandekar, 1993; De Bondt *et al*., 1994). One more
factor that may affect selection of transformant after transformation with *Agrobacterium*
is methylation. To enhance recovery of transformants, inhibition of methylation
of T-DNA in the transgenic plants was required (Mandal *et al*., 1993). The cytosine
methylation inhibitor, 5-azacytidine, gave increased gene expression in transgenic plants
(Hooykaas and Schilperoort, 1992; Mandal *et al*., 1993; Palmgren *et al*., 1993).

Other fruit tree materials that have been used successfully for *Agrobacterium-
mediated transformation are apricot embryos (Machado *et al*., 1992a, b, 1993), peach
embryos (Smigocki and Hammerschlag, 1991), plum hypocotyls (Mante *et al*., 1991),
and cotyledon halves of mature plum seeds (Korte and Casper, 1994). Machado *et al*.
(1992a,b) reported transformation and regeneration of apricot (*Prunus armeniaca*)
cultivars with *A. tumefaciens* strain LBA 4404 containing various binary plasmids, and
carrying the coat protein gene of plum pox virus (PPV). Reports of *Agrobacterium-
mediated transformation of mature and immature peach tissues have been published
(Hammerschlag *et al*., 1989; Martin *et al*., 1990; Scorza *et al*., 1990). In an attempt to
obtain transgenic peach plants, Hammerschlag *et al*. (1989) infected peach stems with
*Agrobacterium* (tms328::Tn5); however, shoots could not be regenerated from the
transformed cells. Smigocki and Hammerschlag (1991) infected immature peach embryo
with the shooty mutant strain of *Agrobacterium* strain tms328::Tn5, which carries an
octopine-type Ti plasmid with a functional cytokinin gene and a mutated auxin gene.
Shoots were regenerated from embryo-derived callus that was initiated on MS medium
without phytohormone. Shoots showed increased frequency of branching but were difficult to root (Smigocki and Hammerschlag, 1991). Korte and Casper (1994) developed transformed plums with resistance against plum pox virus. They used cotyledons and co-cultivated with *A. tumefaciens* strain LBA 4404 or EHA 101 containing the binary plasmid pLX 222 carrying the GUS gene with intron and *nptII* as the selectable marker.

Transformation of pear is still very difficult (Chevreau and Skirvin, 1992). Only one report described the effect of *in vitro* inoculation of pear shoots by a wild strain of *A. tumefaciens* (pTiT37), but DNA analysis of the regenerated plants showed no evidence of transformation, suggesting that tumor tissues were chimeric (Chevreau and Skirvin, 1992). However, since pear and quince are both susceptible to infection by *Agrobacterium*, and can be regenerated from leaf discs, they should be good candidates for *Agrobacterium*-mediated transformation.
References


CHAPTER II

RESPONSES OF TWO SOMACLONAL VARIANTS OF QUINCE (Cydonia oblonga Mill.) TO Fe-DEFICIENCY STRESS IN THE GREENHOUSE

Abstract

Two somaclonal variants of quince (Cydonia oblonga Mill.), IE-1 and IE-2, were obtained previously (Dolcet-Sanjuan, 1991; Dolcet-Sanjuan et al., 1992). These variants displayed higher tolerance to Fe-deficient conditions in vitro than the original clone, Quince A, as indicated by higher chlorophyll concentrations in the new leaves, higher Fe(III) reduction in roots, and increased acidification of the medium. The variants have now been compared to Quince A under greenhouse conditions, in normal potting soil (pH 5) and in soil obtained from an Fe-deficient orchard, with and without addition of lime and KHCO₃ solution to further increase the pH (up to pH 8.5). Studies conducted in 1993 and 1994 detected significantly higher chlorophyll concentrations in the new leaves of the variants than Quince A when grown in the high-pH (7-8.5) soils. The Fe²⁺ concentrations in the leaves, measured at the end of the 1994 experiment, were also higher in the variants. These results indicate that IE-1 and IE-2, when grown as young plants in the greenhouse, have similar tolerance to Fe-deficiency stress as in vitro and are stable variants.
Introduction

Fe is an important micronutrient and is required for a number of essential processes in the plant, including photosynthesis and respiration (Marschner, 1986; Romheld and Marschner, 1986; Barton and Hemming, 1993). Fe insufficiency is manifested in chlorosis of new leaves. Many soil types, particularly those with high pH, are deficient in Fe. There is large variability among crops in the sensitivity to low Fe; some of the fruit crops, most notably pear and peach, are very sensitive (Lombard and Westwood, 1987; Viti and Cinelli, 1989, 1993). Quince is a widely used rootstock for pear and is particularly important in intensive orchards because of its dwarfing ability. However, a disadvantage of quince is its inefficiency in utilizing Fe present in the soil (Lombard and Westwood, 1987; Duron et al., 1989; Viti and Cinelli, 1989, 1993). In order to improve Fe uptake by this rootstock, attempts have been made to select genotypes with increased Fe utilization (Viti and Cinelli, 1989; Dolcet-Sanjuan, 1991; Dolcet-Sanjuan et al., 1990, 1992). Although further improvement by breeding may be attainable, it is a long-term process and requires evaluation in combination with different pear scions. An alternative strategy is the application of biotechnology, such as selection of somaclonal variants with improved iron utilization or transformation with genes involved in Fe efficiency. Such strategies should allow improvement of established clones, with proven performance, and avoid the lengthy selection process.

Previously, two somaclonal variants, IE-1 and IE-2, have been selected from a population of about 2,000 quince shoots derived from cultured leaf discs (Dolcet-Sanjuan, 1991; Dolcet-Sanjuan et al., 1992). These variants had significantly higher chlorophyll
concentrations in the leaves than the unselected Quince A controls when grown under Fe-deficient in vitro conditions. Moreover, they displayed a higher ability to reduce Fe(III) and acidify the culture medium. These traits were expressed in unrooted shoots as well as plantlets. To determine whether this higher efficiency in Fe utilization is a stable trait, the performance of variants and controls was further evaluated in the greenhouse. In this chapter the results of this evaluation, comparing responses under Fe-sufficient and Fe-deficient conditions, are reported.

Materials and Methods

Plant Materials

The materials used for the experiments were Cydonia oblonga Mill. Quince A (East Malling) and two somaclonal variants of Quince A, IE-1 and IE-2 (Dolcet-Sanjuan, 1991; Dolcet-Sanjuan et al., 1992). Plants were obtained from shoot cultures which had been maintained by monthly transfers on medium containing MS mineral nutrients (Murashige and Skoog, 1962), 30 g/l sucrose, 100 mg/l myo-inositol, 1 mg/l thiamine, 1 mg/l nicotinic acid, 1 mg/l pyridoxine, and 5 \( \mu \text{M} \) 6-benzyladenine (BAP). The medium for rooting of shoots contained the same nutrients as for multiplication, with the exception of the \( \text{NH}_4\text{NO}_3 \) and \( \text{KNO}_3 \) concentrations (which were halved) and the replacement of BAP by 5\( \mu \text{M} \) \( \alpha \)-naphthaleneacetic acid (NAA). After 7 days on the rooting medium, shoots were cultured on rooting medium without growth regulators for 28 days (Dolcet-Sanjuan et al., 1990). All cultures were grown at 25°C and 16 hours of coolwhite light (40 \( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \)).
Plantlets were transferred to flats in the greenhouse (soil-perlite mix) and placed on a misting bed. After four weeks, the plants were transplanted to pots and placed on the greenhouse bench.

Design of Experiments

Two experiments were performed, one in 1993, the other in 1994. In 1993, plants were moved to the greenhouse in July and the experiment, with four soil treatments (A-D; see below), was started on August 4. The pots were arranged into four blocks, each consisting of one soil type. A block contained eight plants each of Quince A, IE-1, and IE-2, randomly arranged. Leaf samples for chlorophyll determinations were taken from each plant after six weeks (September 15). The upper three leaves from two plants were pooled for chlorophyll extraction.

In 1994, plants were moved to the greenhouse in March and the experiment, with five soil treatments (A-E), was started on April 15. A nested design was used, with four blocks of five rows each. Each row represented one soil type and contained six plants, two each of Quince A, IE-1 and IE-2, randomly arranged. Leaf samples for chlorophyll determinations were taken on June 15, July 15, and August 15. The upper three leaves of a branch of each plant were taken for chlorophyll extraction. Fe$^{2+}$ concentrations were determined on August 15.

For treatment A a potting soil mixture containing peat and perlite and nutricote slow-release fertilizer was used. It was watered twice weekly with tap water. Treatment B used a 1:1 mixture of perlite and soil retrieved from an orchard near Medford where trees showed symptoms of Fe-deficiency chlorosis. It was watered once weekly with tap
water and once weekly with a 1/4 Hoagland solution (without Fe). For treatments C, D and E the same soil was used as for treatment B but lime was added (500 mg hydrate lime and 500 mg dolomite lime per 50 g soil). C was watered as B; for D and E the 1/4 Hoagland solution contained 1 mM and 10 mM KHCO₃, respectively. Saucers were placed under the pots to prevent loss of water or nutrients.

**Chlorophyll Measurements**

Leaves were cut into pieces, weighed, and incubated in N,N-dimethylformamide (DMF) at 4°C in the dark (Moran, 1982). The absorbance of the extracts was determined at 647 and 664.5 nm. The amount of chlorophyll was determined by the equation \(17.90A_{647} + 8.08A_{664.5}\) (Inskeep and Bloom, 1985). The chlorophyll content of the leaves was expressed as \(\mu g/mg\) fresh weight of leaves.

**Determination of Fe²⁺ Concentration**

The Fe²⁺ concentration of the upper leaves was determined by extraction with 2,2'-bipyridyl (Abadia et al., 1984). Tissues were incubated on an orbital shaker in the dark for 24h. After filtration through Whatman No. 1 filters, the absorption at 522 nm was determined. A standard curve was obtained with a number of concentrations of Fe²⁺SO₄·7H₂O, reduced by treatment with hydroxylamine-HCl. The Fe²⁺ concentration was expressed as \(\mu g\) per g fresh weight.

**Data Analyses**

For the 1993 experiment (chlorophyll concentrations), comparisons were made between the genotypes within each soil type. The StatGraphics program was used for
data analyses. The 1994 experiment was set up as a split-plot design with soil treatments as the main plot and clones as the sub-plot. Data were analyzed with the SIPS program (Oregon State University).

Results

The experiment conducted in 1993 showed clear differences between the original Quince A clone and the two variants derived from it. The new leaves of Quince A were chlorotic after four weeks in high-pH soil (treatments B-D), whereas those of the variants remained green. The chlorophyll contents of the leaves reflected this difference (Fig. II-1). Although the design of the experiment did not allow comparisons between the soils, there were significant clonal differences (p<0.001) within each of the three treatments (B, C, and D) using high-pH soil. The differences were due to the lower chlorophyll concentrations in the original clone; the two variants did not differ from each other. Also, there was no difference (p = 0.44) between the clones on normal soil (A).

The experiment conducted in 1994 was more comprehensive, with complete blocks and different times of chlorophyll analysis (June 15, July 15, and August 15). This allowed determination of the interactions as well as the main effects. Again, there was a clear contrast between the performance of the variants and Quince A (Figs. II-2 and II-3). Under all high-pH treatments, the variants remained much greener than Quince A. There were highly significant interactions between all factors (Table II-1). This seems to be caused by the rapid decrease in chlorophyll concentration of Quince A early in the experiment, while the decrease in the variants occurred mainly between July 15 and August 15 (as illustrated for soil E in Fig. II-4). Again, the severity of chlorosis
Fig. II-1. Chlorophyll concentrations of the upper leaves of Quince A and two somaclonal variants, IE-1 and IE-2, after 6 weeks in normal potting soil (A) and Fe-deficient soil (B-D) (1993 experiment). See Materials and Methods for details on soil conditions.
Fig. II-2. Plant of IE-2 (left) and Quince A (right) after three months in soil D
Fig. II-3. Chlorophyll concentrations of the upper leaves of Quince A and two somaclonal variants, IE-1 and IE-2, in normal potting soil (A) and Fe-deficient soil (B-D) on June 15 (a), July 15 (b), and August 15 (c) (1994 experiment). See Materials and Methods for details on soil conditions. The LSD (0.05) is 0.138.
Table II-1. Analysis of variance of chlorophyll concentration in leaves as influenced by soil, genotype, and date (1994 experiment)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block</td>
<td>3</td>
<td>0.0339</td>
<td>1.0</td>
<td>0.43</td>
</tr>
<tr>
<td>Soil</td>
<td>4</td>
<td>7.9782</td>
<td>233</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Error (a)</td>
<td>12</td>
<td>0.0342</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clone</td>
<td>2</td>
<td>13.9370</td>
<td>298</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Soil x Clone</td>
<td>8</td>
<td>0.9421</td>
<td>52.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Error (b)</td>
<td>30</td>
<td>0.0180</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date</td>
<td>2</td>
<td>1.9721</td>
<td>141</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Date x Soil</td>
<td>8</td>
<td>0.0737</td>
<td>5.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Date x Clone</td>
<td>4</td>
<td>0.2229</td>
<td>16.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Date x S x C</td>
<td>16</td>
<td>0.0395</td>
<td>2.8</td>
<td>0.001</td>
</tr>
<tr>
<td>Error (c)</td>
<td>90</td>
<td>0.0140</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling Error</td>
<td>180</td>
<td>0.0228</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table II-2. Average soil pH at the start of the experiment (April 15), and at the three dates of chlorophyll analyses (June 15, July 15, August 15)

<table>
<thead>
<tr>
<th></th>
<th>Soil A</th>
<th>Soil B</th>
<th>Soil C</th>
<th>Soil D</th>
<th>Soil E</th>
</tr>
</thead>
<tbody>
<tr>
<td>April 15</td>
<td>5.0</td>
<td>7.3</td>
<td>8.3</td>
<td>8.3</td>
<td>8.3</td>
</tr>
<tr>
<td>June 15</td>
<td>4.8</td>
<td>7.2</td>
<td>8.1</td>
<td>8.1</td>
<td>8.1</td>
</tr>
<tr>
<td>July 15</td>
<td>4.6</td>
<td>7.0</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>August 15</td>
<td>4.6</td>
<td>7.0</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>
Fig II-4. Chlorophyll concentrations of the upper leaves of Quince A and two somaclonal variants, IE-1 and IE-2, in soil E over the duration of the 1994 experiment.
chlorophyll concentration were related to the presence of lime and pH of the soil. It should be noted that the pH of the soils remained stable during the experiment (Table II-2). Even though the interactions were highly significant (Table II-1), the mean square for clones was much higher than those for the interactions. This indicates that the differences between the clones are of particular relevance.

Comparisons of the Fe$^{2+}$ concentrations in the leaves showed that, as expected, the Fe$^{2+}$ decreased with increasing lime (and pH) of the soil (Fig. II-4). There were

![Bar chart showing Fe$^{2+}$ concentrations in leaves, with data for Quince A, IE-1, and IE-2 in different conditions.

Fig. II-5. Fe$^{2+}$ concentrations of the upper leaves of Quince A and two somaclonal variants, IE-1 and IE-2, in normal potting soil (A) and Fe-deficient soil (B-E) on August 15 (1994 experiment). See Materials and methods for details on soil conditions. The LSD (0.05) is 2.35.
highly significant interactions between soil and clone, as was the case with chlorophyll concentrations. However, the patterns of Fe$^{2+}$ were somewhat different from the chlorophyll contents. The variants differed from the Quince A control under all conditions and had particularly high Fe$^{2+}$ in normal potting soil, indicating that they may have higher Fe-reducing ability and Fe uptake even under conditions where Fe is readily available to the plant. Moreover, there were differences between the two variants under treatments A, C, and D, with higher Fe$^{3+}$ concentrations in IE-2 than IE-1 (Fig. II-4). However, these differences were not reflected in chlorophyll content differences (Fig. II-3c).

Since it is important that the performance as rootstock is retained, the variants were examined for any signs of changes in morphology and growth. No visible differences were noticed between the variants and Quince A at any time, with the exception of the color of the leaves.
Discussion

The results of the chlorophyll analyses show that there are pronounced differences between Quince A and the somaclonal variants under greenhouse conditions and that such differences are magnified with progressively more severe Fe-deficiency stress. The two somaclones remained green and maintained normal chlorophyll concentrations under most stress conditions. Only after a prolonged period of extreme stress did the chlorophyll concentration decrease. This indicates that the higher Fe efficiency of the variants previously observed under in vitro conditions (Dolcet-Sanjuan et al., 1992) was also maintained after transfer to the greenhouse, suggesting stability over time, environmental conditions, and growth phases.

The same trends as observed for chlorophyll content were also found for Fe$^{2+}$ content. For Quince A, the Fe$^{3+}$ concentrations in the leaves decreased with increasing lime and pH of the soil. Although Fe$^{2+}$ also decreased in the variants, the Fe$^{2+}$ levels in the variants were always higher than those in Quince A. These data are consistent with the concept that chlorosis is related to the levels of Fe$^{2+}$ in the leaves. Above a certain threshold of Fe$^{2+}$, the physiology of the plant and chlorophyll synthesis are normal, but below that threshold, chlorophyll synthesis is limited, which is manifested by the appearance of chlorotic leaves.

The variants were selected from about 2,000 somaclones, which means that 0.1% of the clones were more Fe efficient. Although the systems used for generating and selecting variants are often not comparable and differences in frequencies should be expected, the frequency of quince variants seems within the frequency range of variants
obtained in different studies (Lorz and Scowcroft, 1983; Irvine, 1984; Evans et al., 1984; Hammerschlag, 1990; Skirvin et al., 1993). Of particular interest are the variants with resistance to *Erwinia amylovora* selected from ‘Greensleeves’ apple regenerants (Donovan et al., 1994). A comparison between quince and apple seems relevant, not only because they are temperate fruit tree species belonging to the Rosaceae, but also because the regeneration system of apple is similar to that of quince. The comparison may also indicate some further promise for the quince variants since the ‘Greensleeves’ variants have been shown to be stable in the field (David James, personal communication).

The stability of the variant phenotypes over time (more than five years) indicates that the changes in tolerance to Fe-deficiency stress are more likely genetic than epigenetic. However, the exact nature of the changes is still unknown. As a host of events, including aberrations in chromosome numbers and structure, point mutations, and methylation, can lead to somaclonal variation (D’Amato, 1985; Brown and Lorz, 1986; Brown, 1989; Lee and Phillips, 1988), it is difficult to pinpoint the exact basis of the changes, especially in woody species. Nevertheless, in agronomic terms, the most important consideration is the stability of the improved trait, and the somaclonal variants will be of value in pear production even if the origin of the changes remains obscure.

The objective of improvement by somaclonal variation is to obtain clones with desired changes in a single characteristic leaving other desirable traits unaltered. It is conceivable that also other changes may have occurred during the tissue culture phase but are not readily detectable. Although the performance of the variants as rootstocks
for pear scions has not been determined yet, it is encouraging that their growth habit and all apparent morphological traits are indistinguishable from the original Quince A clone. Final assessment of these somaclonal variants must be derived from field studies, after grafting the variants with various pear scions.
References


CHAPTER III

REGENERATION AND TRANSFORMATION OF QUINCE AND PEAR

Abstract

As prerequisites for generating stably transformed quince and pear, efforts were made to improve the efficiency of regeneration systems for Quince A and RV.113 (a dwarfing pear rootstock), to select the best antibiotic for elimination of Agrobacterium tumefaciens while maintaining high levels of shoot regeneration, and to establish protocols for transformation. Shoot regeneration from quince leaves was elevated from 70% to 90-100% by substitution of agar with gelrite (1.6 g/l). Other modifications of the medium, including replacement of sucrose with equimolar amounts of glucose, addition of 100 mg/l ribose, xylose and mannose, and addition of 10 mg/l AgNO₃ did not enhance regeneration. Regeneration of pear was promoted by storing micropropagating source material at 4°C for 2 weeks, followed by exposure to 25°C for 1 week. Timentin was better than carbenicillin and cefotaxime both in repressing growth of the A. tumefaciens strain EHA105 and allowing regeneration of quince shoots. There was a negative linear relationship between antibiotic concentration and regeneration percentage as well as number of shoots per regenerating leaf. Co-cultivation of quince leaves with EHA105 containing the vector pGiPTV-BAR (containing the bar gene for bialaphos resistance and the intron-containing uidA gene for β-glucuronidase (GUS) activity), led to numerous GUS-positive sites on the leaves if damage to leaves was minimized by cutting them immersed in liquid medium. Bialaphos-resistant shoots have been regenerated from
cocultivated quince leaves. The same procedures applied to pear resulted in only few GUS-positive sites.

**Introduction**

Genetic transformation allows the incorporation of desirable genes, either from plant sources or unrelated organisms, for the improvement of economically important crops. The potential benefits of transformation are particularly relevant for fruit tree species, since conventional breeding is complicated by high heterozygosity and a long generation time. Examples of successful transformation of fruit trees include the transfer of genes encoding endotoxin protein of *Bacillus thuringiensis* and cowpea trypsin inhibitor to apple (James *et al.*, 1993a). The methodologies were based on use of disarmed *Agrobacterium* as a vector and a regeneration system from leaf discs (James *et al.*, 1989; James and Dandekar, 1991). Similar approaches could lead to genetic improvement of pear and quince, a dwarfing rootstock for pear. For instance, pear may benefit from incorporation of genes involved in disease resistance, pest resistance, and delay of ripening. Quince, which is inefficient in iron uptake in calcareous soils (Lombard and Westwood, 1987; Duron *et al.*, 1989; Viti and Cinelli, 1989, 1993), may be modified to become more Fe efficient by insertion of genes enhancing iron utilization. Although transformation protocols have not been established for pear or quince, effective procedures for regeneration of quince are available (Dolcet-Sanjuan, 1991; Dolcet-Sanjuan *et al.*, 1991). Regeneration procedures for pear have also been reported (Mehra and Jaidka, 1979; Viseur, 1986; Cheveau *et al.*, 1989; Predieri *et al.*, 1989; Leblay *et al.*...
al., 1991; Dolcet-Sanjuan, 1991) but the regeneration efficiencies vary widely with genotype.

The objectives of this research are to further improve regeneration of quince and pear from leaf discs and to establish methods for Agrobacterium-based transformation.

Materials and Methods

Regeneration of quince and pear

The initial regeneration protocols for quince, *Cydonia oblonga* Mill. East Malling Quince A, were those described by Dolcet-Sanjuan *et al.* (1990). Leaves from 3-week-old micropropagated shoots were cut transversely at six places, leaving the sections together at one margin. Five leaves were placed with the abaxial side down in each petri dish containing 25 ml medium. The medium contained a mixture of the MS (Murashige and Skoog, 1962) and N6 (Chu, 1978) minerals (with N,P,K and S from N6), sucrose (30 g/l), *myo*-inositol (100 mg/l), thiamine-HCl (1 mg/l), nicotinic acid (1 mg/l), pyridoxine-HCl (1 mg/l) and Difco Bacto agar (6 g/l). The pH was adjust to 5.7 before autoclaving at 120°C. Thidiazuron (TDZ) and α-naphthaleneacetic acid (NAA) were dissolved in dimethylsulfoxide (DMSO) and added after autoclaving (25μl DMSO per 25 ml medium). Unless specified, the TDZ and NAA concentrations in the medium were 32 and 0.3μM, respectively. The cultures were placed in the dark at 25°C. After 21 days, the leaves were transferred to fresh medium and placed in a lighted culture room with a photoperiod of 16h (40 μmol.m².s⁻¹) for 3 or 4 weeks.
Medium modifications designed to improve regeneration of quince included replacement of 3% sucrose with an equimolar amount of glucose; addition of 100 mg/l xylose, ribose and mannose; addition of 10 mg/l AgNO₃; and replacement of agar with gelrite (1.6 g/l). Other modifications included: various lengths of the dark culture period (5, 10, 20, and 30 days); and source of explants, including leaves with and without petioles, petioles only, and the middle third of the leaf blade (around the midrib) with the petiole.

For regeneration of pear, *Pyrus communis* L. RV.113 (Brossier series), the same medium and conditions as described above for quince were used, but with agar replaced by gelrite (1.6 g/l). To test whether other concentrations of TDZ or NAA could enhance regeneration, media with all combinations of 1, 3, 10 and 32μM TDZ and 0.1, 0.3, 1, 3, and 10μM NAA were used. To determine the effects of cold treatment of the micropropagated source materials on regeneration, 3-week-old micropropagated cultures were placed at 4°C for 2 weeks, after which they were returned to the 25°C lighted culture room for 1, 3, 5, or 7 days before leaf explants were taken.

For all experiments, ten petri dishes with five leaves per petri dish were used for each treatment. The experiments were repeated once. The regeneration frequency (% of leaves having adventitious shoots) and the number of shoots per regenerating leaf were recorded after a culture period of 6 weeks. Analyses of variance were performed for the regeneration frequency with the StatGraphics program.
Selection of antibiotic for elimination of *Agrobacterium* after co-cultivation

*Agrobacterium tumefaciens* EHA105 (Hood *et al.*, 1993) was obtained from E.
Hood. Bacteria were cultured in liquid YEP medium on a reciprocal shaker at 28°C until
an O.D. of 0.5 (550 nm) was reached. To determine the effects of the antibiotics
timentin, cefotaxime, and carbenicillin on growth of this strain, 50μl of the bacterial
culture was added to culture tubes containing 10 ml YEP medium with the antibiotics at
0, 5, 10, 25, 50, 75 and 100 mg/l, with two tubes per treatment. After a growth period
of 24h at 28°C, the O.D of the cultures was determined. The experiment was repeated
twice.

To determine the effects of the antibiotics on quince regeneration, timentin,
cefotaxime and carbenicillin were added to regeneration medium (after autoclaving) at
concentrations of 0, 50, 100, 150, 200, and 250 mg/l. The standard conditions as
described above for quince regeneration were employed, with agar replaced by gelrite
(1.6 g/l). Ten dishes with five leaves each were used per treatment. The regeneration
frequency and number of shoots per regenerating leaf were recorded after a culture
period of 7 weeks. The experiment was repeated twice. Analyses of variance and
regression analyses were performed for regeneration % and number of shoots per
regenerating leaf with the SIPS program (Oregon State University).

**Choice of selective agent, its concentration, and time of application**

The effective concentrations of kanamycin and bialaphos as selective agents were
determined in the standard regeneration system for quince (with 1.6 g/l gelrite). The
concentrations tested were 0, 10, 20, 30, 40, 50 and 60 mg/l for kanamycin and 0, 1,
5, 10, 25, and 50 mg/l for bialaphos, both added after autoclaving of the medium. Ten dishes with five leaves each were used per treatment. The regeneration frequency and number of shoots per regenerating leaf were determined after a culture period of 7 weeks.

To determine whether application of bialaphos could be delayed after co-cultivation, five treatments were compared: the control without bialaphos, presence of 2 mg/l bialaphos during the entire culture period, and during the last 6, 5 and 4 weeks of the 7-week culture period. Five dishes with five leaves each were used per treatment. The experiment was repeated once. The regeneration frequencies and number of shoots per regenerating leaf were determined. Analyses of variance were performed for the regeneration frequency with the StatGraphics program.

Transformation of quince and pear

The following two vectors were used for transformation:

pGSFR280 (Fig. III-1 A; De Block et al., 1987) and pGiPTV-BAR (Fig. III-1 B; Becker et al., 1992). Both were placed in A. tumefaciens strain EHA 105 by tri-parental mating (Comai et al., 1983). Bacterial cultures were grown from frozen cultures (-80°C) in YEP medium, EHA105/pGSFR280 in the presence of 50 μg/l spectinomycin and streptomycin and EHA105/pGiPTV-BAR in the presence of 50 μg/ml kanamycin. Cultures were kept on a reciprocal shaker at 28°C for about 2 days, until the O.D. at 550 nm was 0.5. After centrifugation of cultures at 2500 rpm, the pellet was dissolved in SIM medium with 100μM acetosyringone. The cultures were placed on a shaker at 28°C
Fig III-1  Vectors used for transformation: A. pGSFR280 (De Block et al., 1987); B. pGiPTV-BAR (Becker et al., 1992). bar: bialaphos resistance; nptII: kanamycin resistance; uidA: B-glucuronidase (GUS)
for 5 h., after which one part was added to nine parts of MS medium (incubation medium 1) or the SIM medium was replaced by MS medium (incubation medium 2).

The transformation procedure can be divided in the following phases: a preculture period of the leaf explants (optional); an incubation period of leaf discs with Agrobacterium for 10 min.; a co-cultivation period of 3 days; and a regeneration phase consisting of 18 days in the dark and 28 days at a 16h photoperiod. Several transformation experiments were performed, with slightly different conditions, as illustrated in Tables III-1 and III-2 for quince and Table III-3 for pear. For the preculture period, the standard medium and conditions for regeneration were used, but the growth regulators in the medium varied. For incubation with Agrobacterium, leaves were left in incubation medium 1 or 2 for 10 min., after which they were carefully blotted dry with sterile paper, and placed on regeneration medium. Cultures were kept in the dark at 25°C for 3 days for cocultivation with Agrobacterium. For regeneration and selection, the leaves were transferred to fresh regeneration medium containing 200 mg/l timentin and bialaphos (see tables for concentrations). After 18 days, leaves were transferred to fresh regeneration medium with 200 mg/l timentin and 5 mg/l bialaphos, with or without 3 mg/l 5-azacytidine (see tables). After 28 days, regenerating shoots were transferred to micropropagation medium (Dolcet-Sanjuan et al., 1990) with 200 mg/l timentin and 5 or 10 mg/l bialaphos and placed in the lighted culture room at 25°C.

GUS assays

GUS activity of leaves cocultivated with EHA105/pGiPTV-BAR was tested 6 days after incubation with Agrobacterium using the histochemical staining procedure described
Table III-1. Conditions and results of the first series of transformation experiments\(^a\) with quince leaves and EHA105/pGiPTV-BAR

<table>
<thead>
<tr>
<th>Expt.</th>
<th>No. of leaves(^b)</th>
<th>Pre-culture</th>
<th>Incubation medium(^d)</th>
<th>Azacytidine during selection</th>
<th>GUS assay</th>
<th>No. of shoots regenerated(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Time (days)</td>
<td>NAA ((\mu)M)</td>
<td></td>
<td>Day(^e)</td>
<td>No. pos. leaves(^b)</td>
</tr>
<tr>
<td>1-2</td>
<td>200</td>
<td>0</td>
<td>-</td>
<td>1</td>
<td>yes</td>
<td>6</td>
</tr>
<tr>
<td>1-3a</td>
<td>100</td>
<td>0</td>
<td>-</td>
<td>1</td>
<td>no</td>
<td>10</td>
</tr>
<tr>
<td>1-4a</td>
<td>100</td>
<td>2</td>
<td>0.3</td>
<td>2</td>
<td>no</td>
<td>6</td>
</tr>
<tr>
<td>1-4b</td>
<td>100</td>
<td>2</td>
<td>0.3</td>
<td>2</td>
<td>yes</td>
<td>6</td>
</tr>
<tr>
<td>1-5a</td>
<td>50</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>no</td>
<td>6</td>
</tr>
<tr>
<td>1-5b</td>
<td>50</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>yes</td>
<td>6</td>
</tr>
<tr>
<td>1-6</td>
<td>50</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>yes</td>
<td>6</td>
</tr>
<tr>
<td>1-7</td>
<td>50</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>no</td>
<td>6</td>
</tr>
<tr>
<td>1-8</td>
<td>50</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>yes</td>
<td>21</td>
</tr>
<tr>
<td>1-9</td>
<td>50</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>no</td>
<td>21</td>
</tr>
</tbody>
</table>

\(^a\)Leaves were cut in petri dishes without any medium. All selection media contained 5 mg/l bialaphos.

\(^b\)In each experiment, ten leaves were used for GUS assays and the rest retained for regeneration of shoots.

\(^c\)All pre-culture media contained 32\(\mu\)M TDZ in addition to NAA.

\(^d\)Medium 1 consisted of a mixture of MS and SIM medium (9:1); medium 2 of MS medium.

\(^e\)Day on selection medium
Table III-2. Conditions and results of the second series of transformation experiments with quince leaves and EHA105/pGiPTV-BAR

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Pre-culture</th>
<th>Incubation medium</th>
<th>Bialaphos conc. (mg/l) during selection</th>
<th>GUS assay</th>
<th>No. of shoots regenerated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (days)</td>
<td>NAA (μM)</td>
<td></td>
<td>No. pos. leaves</td>
<td>Spots/pos. leaf</td>
</tr>
<tr>
<td>2-1</td>
<td>2</td>
<td>0.3</td>
<td>2</td>
<td>5</td>
<td>&gt;40</td>
</tr>
<tr>
<td>2-2</td>
<td>2</td>
<td>0.3</td>
<td>2</td>
<td>5</td>
<td>&gt;40</td>
</tr>
<tr>
<td>2-3</td>
<td>2</td>
<td>0.3</td>
<td>2</td>
<td>5</td>
<td>&gt;40</td>
</tr>
<tr>
<td>2-4</td>
<td>2</td>
<td>0.3</td>
<td>2</td>
<td>3</td>
<td>&gt;40</td>
</tr>
<tr>
<td>2-5</td>
<td>2</td>
<td>0.3</td>
<td>2</td>
<td>3</td>
<td>&gt;40</td>
</tr>
<tr>
<td>2-6</td>
<td>2</td>
<td>0.3</td>
<td>2</td>
<td>5</td>
<td>&gt;40</td>
</tr>
<tr>
<td>2-7</td>
<td>2</td>
<td>0.3</td>
<td>2</td>
<td>5</td>
<td>&gt;40</td>
</tr>
<tr>
<td>2-8</td>
<td>2</td>
<td>0.3</td>
<td>2</td>
<td>3</td>
<td>&gt;40</td>
</tr>
<tr>
<td>2-9</td>
<td>2</td>
<td>0.3</td>
<td>2</td>
<td>3</td>
<td>&gt;40</td>
</tr>
<tr>
<td>2-10</td>
<td>0</td>
<td>-</td>
<td>2</td>
<td>3</td>
<td>&gt;40</td>
</tr>
<tr>
<td>2-11</td>
<td>7</td>
<td>3</td>
<td>1</td>
<td>5</td>
<td>&gt;40</td>
</tr>
<tr>
<td>2-12</td>
<td>10</td>
<td>5μM BAP</td>
<td>1</td>
<td>5</td>
<td>large</td>
</tr>
</tbody>
</table>

*Leaves were cut in petri dishes with liquid MS medium. The selection medium contained azacytidine.

*bFor each experiment, 200 leaves were used: ten for GUS assays and the rest for regeneration of shoots.

*cAll pre-culture media containing NAA also contained 32μM TDZ.

*dMedium 1 consisted of a mixture of MS and SIM medium (9:1); medium 2 of MS medium.

*eAll GUS assays were performed after 6 days on selection medium.
Table III-3. Conditions and results of transformation experiments* with pear leaves and EHA105/pGiPTV-BAR

<table>
<thead>
<tr>
<th>Expt.</th>
<th>No. of leaves&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Pre-culture incubation medium&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Bialaphos conc. (mg/l) during selection</th>
<th>GUS assay&lt;sup&gt;e&lt;/sup&gt;</th>
<th>No. of shoots regenerated&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Time (days)</td>
<td>NAA&lt;sup&gt;c&lt;/sup&gt; (μM)</td>
<td></td>
<td>No. pos. leaves&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3-1</td>
<td>200</td>
<td>2</td>
<td>0.3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3-2</td>
<td>200</td>
<td>0</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3-3</td>
<td>200</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3-4</td>
<td>200</td>
<td>0</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3-5</td>
<td>200</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3-6</td>
<td>200</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3-7</td>
<td>200</td>
<td>0</td>
<td>-</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3-8</td>
<td>200</td>
<td>7</td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>3-9</td>
<td>200</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>3-10</td>
<td>200</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3-11</td>
<td>100</td>
<td>10</td>
<td>5μM BAP</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

*Leaves were cut in petri dishes with liquid MS medium. The selection medium contained azacytidine.

<sup>b</sup>In each experiment, ten leaves were used for GUS assays and the rest retained for regeneration of shoots.

<sup>c</sup>All pre-culture media containing NAA also contained 32μM TDZ.

<sup>d</sup>Medium 1 consisted of a mixture of MS and SIM medium (9:1); medium 2 of MS medium.

<sup>e</sup>All GUS assays were performed after 6 days on selection medium.
by Jefferson (1987) with slight modifications. The leaves were stained overnight at 37°C in 0.2 M sodium phosphate buffer (pH 7.0) containing 0.1 M K$_4$Fe(CN)$_6$, 0.1 M K$_4$Fe(CN)$_6$, 0.5 M EDTA and 10% X-Gluc (5-bromo-4chloro-3-indolyl β-D-glucuronide). After the overnight staining, the leaves were cleared with 70% ethanol and then 95% ethanol until the chlorophyll was removed. The same procedure was used to determine GUS expression of regenerated shoots.

**Southern analyses**

Genomic DNA was isolated from regenerated quince shoots using the modified CTAB method of Doyle and Doyle (1988). Approximately 10 μg of DNA was digested with the restriction enzyme EcoRI and Bam overnight. Enzyme-digested DNA was separated on a 0.8% agarose gel and transferred to a nylon filter (Zeta Probe from BioRad). The blots were prehybridized and hybridized following the manufacturer’s protocols (BioRad). Hybridization was performed at 65°C overnight. Three washes were performed in 2x SSC and 0.1% SDS for 30 min. each. Filters were exposed to film for several days at -80°C and developed with a Kodak RR X-OMAT Processor.

**Results**

**Regeneration of quince and pear**

When shoots were regenerated from quince leaf discs on modified MS-N6 medium containing 32 μM TDZ and 0.3 μM NAA according to the procedures described by Dolcet-Sanjuan et al. (1991), the percentage of leaf discs regenerating shoots was around 70%. No further improvements in regeneration were obtained by replacement.
of sucrose with glucose; addition of xylose, ribose, and mannose; or addition of AgNO₃. It was noted that many shoots were formed at the proximal ends of the leaves, but a comparison between leaves with and without petioles indicated that the presence of petioles had no significant effect on regeneration (Fig. III-2). The transversely cut leaves gave better results than other types of leaf explants, such as the lower halves of leaves or the midrib section (Fig. III-2). One factor that enhanced shoot regeneration significantly was substitution of agar with gelrite. With healthy young leaves of three-week-old micropropagated shoots, regeneration percentages of 90-100% were attained consistently (Fig. III-3). Therefore, in further experiments, gelrite was included in the medium instead of agar.

When the optimal conditions for quince regeneration were applied to the pear rootstock RV.113, regeneration did occur, but the regeneration percentage (26%) was much lower than for quince. Other combinations of TDZ and NAA concentrations did not give better results. Shortening the dark period to 10 days and increasing it to 30 days gave regeneration frequencies of 24% and 16%, respectively. The effects of cold treatment of the micropropagated shoots used for leaf explants were tested, since cold treatment is generally followed by a flush of growth, which may stimulate regeneration. Although initially the cold treatment was inhibitory, when the shoots were left at 25°C for 7 days, regeneration increased to 44%, which was significantly higher than the 26% of the controls (Figs. III-4). About two shoots emerged from the regenerating leaves (Figs. III-4 and III-5). Thus, of the treatments tested, cold treatment of source material seems the most promising for enhancing regeneration.
Fig. III-2. Regeneration % and number of shoots per regenerating leaf as influenced by the type of quince explant after a culture period of 7 weeks. Different letters indicate significant differences in regeneration % (p = 0.05).
Fig. III-3. Regenerating shoots on quince leaves cultured on medium containing gelrite for 8 weeks.
Regeneration % and number of shoots per regenerating leaf as influenced by storage of pear (RV. 113) source material at 4°C for 2 weeks, followed by exposure to 25°C for 1-7 days. Treatments: control (standard conditions; A); storage at 4°C, followed by 1 (B), 3 (C), 5 (D) and 7 (E) days at 25°C. Different letters indicate significant differences in regeneration % (p=0.05).
Fig. III-5. Regenerating shoots on pear (RV.113) leaves cultured for 8 weeks.
Selection of antibiotic for elimination of *Agrobacterium* after co-cultivation

Experiments were performed to choose the best antibiotic for elimination of *Agrobacterium* while maintaining high regeneration capacity. Timentin was better than cefotaxime and carbenicillin in suppressing growth of EHA105 (Fig. III-6). Timentin inhibited growth of EHA105 completely when supplied at 5 mg/l, while 10 mg/l cefataxime or 50 mg/l carbenicillin was required to produce the same effect. Timentin was also the least inhibitory to the quince regeneration system (Fig. III-7a and b). Analyses of variance indicated highly significant effects of antibiotics and antibiotic concentrations on regeneration percentage (P < 0.0001), but no interaction between the two. Regression analyses showed a negative, linear relationship between concentration and regeneration percentage for all three antibiotics. The regression line for timentin was significantly different from the common regression line for cefotaxime and carbenicillin (Fig. III-7a). A similar linear relationship was found between the antibiotic concentration and the number of shoots per regenerating leaf disc (Fig. III-7b). Because of its better performance in inhibiting bacterial growth and sustaining shoot regeneration, timentin was chosen as the antibiotic for transformation experiments, at the concentration of 200 mg/l.

Selectable markers

Many vectors are available for transformation, most often containing the *nptII* gene, conferring kanamycin resistance, or the *bar* gene, conferring bialaphos resistance. The concentrations of kanamycin and bialaphos effective for selection of transformed shoots were determined. Kanamycin concentrations of 20 mg/l and higher completely
Fig. III-6. Effects of timentin, cefotaxime, and carbenicillin on growth of *A. tumefaciens* EHA105 as measured by O.D. of the cultures after 24h.
Fig. III-7. Effects of timentin, cefotaxime, and carbenicillin on regeneration percentage (a) and number of shoots per regenerating leaf (b) after a culture period of 8 weeks of quince leaves on regeneration medium.
inhibited regeneration from quince leaves that had not been co-cultivated with *Agrobacterium*, while 10 mg/l caused partial inhibition (with 60% of leaf discs regenerating) (Fig. III-8). Bialaphos reduced regeneration to 32% of the leaf discs at 1 mg/l and to 0% at 5 mg/l or more (Fig. III-8). Even 2 mg/l bialaphos was sufficient for suppression of regeneration (Fig. III-9). However, when selection was delayed til one week or more after explants were cultured, regeneration was partially restored (Fig. III-9). This indicates that selection pressure should be applied immediately after the co-cultivation period as delaying selection would result in many escapes (untransformed regenerating shoots). Overall, bialaphos appeared to be a better selective agent due to the healthier appearance of the leaf explants. Therefore, vectors with the *bar* gene as selectable marker were chosen for transformation experiments.

**Transformation experiments**

In the first series of transformation experiments with quince leaves, the strain-vector combination EHA105/PGSF280 was used. Some shoots were recovered, but Southern analyses indicated that these did not contain the *nptII* insert and thus were not transformants. The lack of a reporter gene made it difficult to evaluate whether transformation events had taken place; therefore, a second vector was obtained, pGiPTV-BAR, which contains the *bar* gene as well as the *uidA* gene conferring GUS activity, making it possible to monitor transformation events. It should be noted that the *uidA* gene has an intron and is not expressed in a prokaryotic system such as *Agrobacterium*.

The initial experiments with EHA105/pGiPTV-BAR resulted in very few sites on the leaf discs showing GUS activity (Table III-1). Various conditions were tested, but
Fig. III-8. Effects of bialaphos (a) and kanamycin (b) on regeneration of quince leaves.
Fig. III-9. Effect of 2 mg/l bialaphos, given at the start (0) and after 1, 2, and 3 weeks of culturing, on regeneration percentage and number of shoots per regenerating leaf after a culture period of 8 weeks of quince leaves on regeneration medium. The medium for the control did not contain bialaphos. Different letters indicate significant differences in regeneration % (p=0.05).
none of them resulted in higher GUS activity. Thus, even though shoots were regenerated, it was not expected that they were transformed. Indeed, staining of these shoots for GUS activity gave negative results.

Quince leaves are very thin and it is possible that the cut edges dry out rapidly after cutting; therefore, in the next experiments (Table III-2), leaves were cut under liquid medium. With this modification, many blue spots appeared upon assaying leaves for GUS activity (Figs. III-10 and III-11, and Table III-2), although a portion of the leaves did not have any spots. Small shoots were recovered from each experiment (Table III-2). After transfer of the shoots recovered from the first ten experiments to micropropagation medium with 10 mg/l bialaphos, 23 out of 56 shoots survived. These shoots are potential transformants. Experiment 2-12 resulted in formation of much more callus than the other experiments, because of the pre-incubation period on medium containing 2,4-D. When the callus tissues were stained for GUS activity, many blue spots and entirely blue areas became visible (Fig. III-12), indicative of stable transformation. However, no shoots were generated from these calli.

Transformation experiments with pear (RV.113) resulted in positive GUS assays for 10 to 40% of the leaves, with only a few spots per leaf (Table III-3). At most, two shoots were obtained per 200 leaves.
Fig. III-10. Leaf of quince (experiment 2-1) showing GUS activity 6 days after incubation with *A. tumefaciens* EHA105/pGPTV-BAR
Fig. III-11. Leaf of quince (experiment 2-2) showing GUS activity 6 days after incubation with *A. tumefaciens* EHA105/pGPTV-BAR
Fig. III-12  Callus on quince leaves showing GUS activity after a culture period of 6 weeks following incubation with *A. tumefaciens* EHA105/pGPTV-BAR. For details of the conditions see Table III-2 (experiment 2-12).
Discussion

A prerequisite for transformation by cocultivation of explants with _Agrobacterium_ is an efficient regeneration system. For quince and pear, shoots can be regenerated from leaf discs (Chevreau _et al._, 1989; Predieri _et al._, 1989; Dolcet-Sanjuan _et al._, 1991; Leblay _et al._, 1991); however, in several of these studies the regeneration frequency was still low. The regeneration system reported earlier (Dolcet-Sanjuan, 1991; Dolcet-Sanjuan _et al._, 1991) resulted in recovery of regenerants from about 70% of the shoots. In the present study further improvement was made, leading to regeneration of shoots from 90 to 100% of the leaves, with about 20 to 25 shoots per leaf after a culture period of 7 weeks. The most important change was replacement of agar with gelrite. Limited efforts were made to enhance regeneration of shoots from leaf discs of pear (RV.113). The concentrations of TDZ and NAA that were optimal for quince (32 and 0.3 μM respectively) were also optimal for pear. However, the percentage of leaves giving shoots was much lower than for quince. Regeneration may have been enhanced by stimulating a rapid flush of growth, through cold treatment followed by a short culture period at 25°C. After seven days at 25°C, the regeneration rate was higher in the cold-treated material than in the controls. Further experiments need to be conducted to confirm to identify the optimal length of the cold period as well as the length of the periods at 25°C before and after the cold treatment.

The use of antibiotics to eliminate bacteria after co-cultivation is essential. The most frequently used antibiotics are carbenicillin and cefotaxime (Horsch _et al._, 1988; James _et al._ 1989, 1993a). Timentin is a newer anti-bacterial mixture consisting of the
antibiotic ticarcillin and a β-lactamase inhibitor (30:1). Addition of these antibiotics to the bacterial growth medium resulted in the inhibition of growth of EHA105, as expected, but the efficacies of the antibiotics differed. Timentin was more effective than cefotaxime or carbenicillin. Of the three antibiotics, timentin was also least inhibitory on regeneration of quince, while cefotaxime and carbenicillin reduced regeneration equally. The inhibitory effect of cefotaxime is in contrast to results obtained for wheat (Mathias and Boyd, 1986).

Several different selectable markers have been used for plant transformation, conferring resistance to antibiotics or herbicides. One compound of each group, kanamycin and bialaphos, was tested with regard to effects on quince regeneration. Although both repressed regeneration, bialaphos was judged the better compound because it had the least adverse effect on the appearance of the leaf discs. Since the quality of leaves has been found to be an extremely important factor in regeneration of quince (Dolcet-Sanjuan et al., 1991), vectors containing the bar gene were chosen for the transformation experiments.

Another important consideration is the Agrobacterium strain used for co-cultivation. EHA105 and EHA101 (from which EHA105 was derived by deletion of the bacterial kanamycin resistance gene; Hood et al., 1993) are effective strains for transformation of plants and have been used for transformation of several woody species (DeBondt et al., 1994; Yancheva, 1994; Korte, 1994). EHA101 was the best strain for transformation of apple out of three strains tested (DeBondt et al., 1994). The numerous
blue spots observed after co-cultivation with this strain indicate that quince is also receptive to this strain.

There are many other factors that can influence gene transfer in Agrobacterium-mediated transformation. Vir-gene induction by acetosyringone was first demonstrated by Zambryski et al. (1988) and acetosyringone is included routinely in co-cultivation medium. A pre-incubation period of about 5 hours with acetosyringone enhanced transformation of apple (James and Dandekar, 1991; James et al., 1993b) and was included in the transformation experiments described here.

In the first transformation experiments, only sporadic GUS activity was observed on leaf discs. The most important improvement in methodology concerned the handling of the leaf discs. Cutting leaves in a liquid medium resulted in a significant increase in the number of blue spots. This suggests that the leaves may dry out quickly at the edges. Some leaves did not show any transformation sites, even when cut under liquid medium, indicating that even under this condition damage may still have occurred. GUS expression was measured relatively early in the culture period, when T-DNA may yet to be stably integrated. Although the level of GUS expression generally decreases over time, early GUS activity and stable transformation are closely correlated (Janssen and Gardner, 1989). Thus the many GUS-active sites on quince leaves found in this study signify important progress towards stable transformation.

Thusfar, a number of putative transformants have been obtained, which have yet to be analyzed. Low frequency in transformation is usual for woody plant species (Schuerman and Dandekar, 1993). Often, hundreds or even thousands of explants need
to be included in transformation experiments in order to generate a few transformants (Schuerman and Dandekar, 1993). The reasons for the low success rates are not clear, although a low frequency of coinciding regeneration and transformation sites has been suggested (James et al., 1989). Another problem may be the necrosis of the leaf edges during the regeneration phase, resulting in gradual elimination of the transformation sites. Repression of gene expression may be another possible factor. The inclusion of azacytidine, a methylation inhibitor (Mandal et al., 1993), in the medium may have minimized such incidence.

There are many permutations of factors in tissue culture and transformation protocols that remain to be tested. For example, the duration of the pre-incubation period may be critical. Pre-incubation of leaf discs can sometimes stimulate transformation (McHughen et al., 1989), although the pre-incubation must not be too long since wound repair may then have been completed (Schuerman and Dandekar, 1993). Factors enhancing early cell division after leaf excision, such as auxin concentrations, are likely to be important as well. It is expected that the quest for better transformation protocols will continue by testing many of the conditions not yet examined in the present study.
References


SUMMARY AND CONCLUSIONS

This thesis describes progress towards the application of tissue culture and gene transformation techniques for the improvement of pear and quince, a rootstock for pear. As woody species are generally not very amenable to established methods for herbacious species, a stepwise progression towards the final goal is expected.

The first part of the study involved greenhouse testing of two somaclonal variants tolerant to low Fe discovered previously in the laboratory of M. and D. Mok (Dolcet et al. 1992). Under Fe-limiting conditions, the two variants performed significantly better than the Quince A controls, from which they had been derived. The results indicate that the variant traits selected for in vitro are maintained in young greenhouse-grown plants. The next step is to test the improved rootstocks in Fe-deficient orchards, in combination with pear scions. If the changes are stable over time, these variants will be valuable as rootstock clones for commercial pear orchards, particularly in areas with calcareous soils.

The second part of the study concerned regeneration and transformation of quince and pear. Regeneration of quince was significantly improved by substitution of agar with gelrite as the gelling agent, resulting in regeneration of shoots from 90-100% of the leaf explants. As transformation requires high efficiency of regeneration, this success allowed testing of transformation protocols. Regeneration of pear was also enhanced, using cold treatment of the shoot cultures used for explant materials. The frequency of regeneration was 44%.

The antibiotic timentin was identified as the most appropriate antibiotic for removal of Agrobacterium after the transformation process, since it was most inhibitory
to growth of the bacterium and least inhibitory to regeneration. For the selection of transformed cells, plasmids harboring the bar gene, conferring resistance to the herbicide bialaphos, were preferred over those containing the nptII gene, conferring kanamycin resistance, since the leaves used as explants remained healthier. Substantially higher frequency of transformation, as measured by GUS activity, was obtained when damage to cut surfaces of leaf explants was minimized by cutting the leaves immersed in liquid medium.

Transformed tissues exhibiting GUS activity and resistance to bialaphos have been obtained, but homogeneously transformed shoots have not yet been identified. It is expected that experiments to further optimize the transformation process will be conducted.

The research represents method development for genetic engineering of woody species in general with an emphasis on pear and quince. Successful transformation of quince will allow insertion of genes involved in Fe efficiency. Similar applications in pear will follow when regeneration frequencies in that species can be further enhanced.
BIBLIOGRAPHY


