



## Factors affecting *Agrobacterium*-mediated genetic transformation in fruit and nut crops – An overview

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### Abstract

In most fruit and nut crops, the frequency of genetic transformation via *Agrobacterium* is quite low, therefore hindering the routine production of transgenics in these crops. It is therefore important to understand the various critical factors involved in *Agrobacterium*-mediated genetic transformation, as each and every factor affects transformation frequency and needs to be optimised for routine production of transgenics in fruit and nut crops. This review highlights and explores the important factors contributing to the success of *Agrobacterium*-mediated transformation in fruit and nut crops. Some of these critical factors affecting success of *Agrobacterium*-mediated gene transfer in fruit crops include: genotype, explant, method of transformation, *Agrobacterium* strain, gene construct, infection/co-cultivation conditions and antibiotics both to kill *Agrobacterium* after infection and for selection of putative transgenic plants. In addition, this review also provides a current 'snapshot' of transgenic fruit and nut crops that have been produced via *Agrobacterium*-mediated transformation, with improved horticultural traits including disease resistance, insect/pest resistance, herbicide tolerance, cold tolerance, salt tolerance and improved plant and fruit characteristics and shelf life. *Agrobacterium*-mediated gene transfer in fruit crops should be considered as an important complementary tool to existing technologies and conventional fruit breeding, not a substitute.

**Key words:** Genotype, explant, method of transformation, infection/co-cultivation conditions, antibiotics, disease resistance, insect/pest resistance, herbicide tolerance, cold tolerance, salt tolerance, shelf life.

### Introduction

Fruit and nut crops, being naturally nutritious, constitute one of the most important components of a healthy human diet. However, to meet the demands of an ever-increasing world population, particularly of developing countries, and to overcome the abiotic and biotic stresses faced by these crops, there is an urgent need for incremental improvement in the existing varieties and/or to develop new varieties. Improvement by conventional breeding has certain limitations. For instance most of these fruit crops are heterozygous, have a long juvenile phase and exhibit nucellar embryony, thereby leading to delay and difficulty in the selection of new genotypes with improved characteristics. The recently understood recombinant DNA technology has potential, via genetic engineering, to incorporate a specific gene which controls a particular trait, without co-transfer of undesirable genes from donor species as occurs in conventional breeding. The commonly used vector (*Agrobacterium*) and vectorless (biolistics) methods of gene transfer have led to production of transgenics in a range of fruit crops including tropical, sub-tropical and temperate species<sup>1-6</sup>. As compared to biolistics, *Agrobacterium*-mediated transformation has several advantages, such as it is economical, reliable and has single gene insertions in plant genomes, thereby lowering the chances of gene silencing at later stages. Thus it is economical and worthwhile to produce transgenics through *Agrobacterium*-mediated transformation. However, to produce transgenics, there are certain pre-requisites such as availability of efficient plant regeneration, gene transfer and selection systems,

which allow the production of transgenics with stable inheritance of traits in further generations. Plant regeneration through somatic embryogenesis is the most efficient method of plant regeneration and has been achieved in several fruit species<sup>7-13</sup>. Somatic embryogenesis has recently been exploited to produce transgenics in several fruit crops, such as citrus<sup>14</sup>, grapes<sup>15-17</sup>, papaya<sup>18</sup> and *Musa* spp.<sup>19</sup>. However, due to low transformation frequencies obtained in most transformation experiments, routine production of transgenic plants with improved horticultural traits is yet to be achieved. Thus keeping in view the advantages of *Agrobacterium*-mediated transformation over biolistics, and the low transformation frequencies in transformation experiments, the present review focuses on highlighting and understanding the critical factors affecting success of *Agrobacterium*-mediated transformation in fruit and nut crops.

### *Agrobacterium*-mediated Transformation

*Agrobacterium tumefaciens* is a naturally occurring soil bacterium, which infects a wide range of dicot plants and produces a tumourous growth on roots by transferring tumour or root inducing plasmids into the host plant genome. The segment of DNA, which is transferred to the host plant, is known as T-DNA. The events involved in T-DNA transfer from *Agrobacterium* to plant cell have been reviewed extensively in the past<sup>20-25</sup>. The stable incorporation of plasmid DNA into higher plant cells was first demonstrated in the late 1970s in tobacco<sup>26</sup>. The natural ability of *Agrobacterium* to transfer a fragment of DNA (T-DNA) to plants has been exploited as a mechanism for introducing foreign DNA

Abbreviations: 2,4-D – 2,4-Dichlorophenoxyacetic acid; IAA – indole-3-acetic acid; IPA – Indole-3-propionic acid; IBA – indole-3-butyric acid; NAA – 1-naphthylacetic acid; TDZ – N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (Thiadiazuron); GUS –  $\beta$  glucuronidase; MS – Murashige and Skoog.

into various plant species. The simple replacement of a wild type T-DNA coding region with any DNA sequence of interest has made *Agrobacterium* the most useful transformation tool<sup>27</sup>. This leads to the transfer of a specific gene(s) within the plant species or from other organisms such as bacteria, fungi or viruses through a vector, an *Agrobacterium*, which now commonly is called vector/*Agrobacterium*-mediated gene transfer.

An *Agrobacterium*-mediated gene transfer system as compared to a vectorless/direct gene transfer system is relatively inexpensive and has the potential to generate transgenic cells at a relatively high frequency, without significant reduction in plant regeneration rates. Moreover, the DNA transferred to the plant genome is defined, it does not normally undergo any major re-arrangements, and it integrates into the genome as a single copy<sup>28</sup>. Gene transfers have been accomplished in a wide variety of dicotyledonous plants via *Agrobacterium tumefaciens* and *A. rhizogenes*<sup>29-32</sup>. *Agrobacterium* has been successfully used in the production of transgenic fruit plants, such as apples<sup>14</sup>, citrus<sup>33</sup>, grapes<sup>34</sup>, papayas<sup>35</sup>, pecan<sup>36</sup>, plums<sup>37</sup>, strawberries<sup>38,39</sup> and Japanese persimmon<sup>40,41</sup>. This technique has also been recently extended to monocots such as bananas<sup>19,42-44</sup> and pineapples<sup>45</sup>. However in most cases, low efficiencies of transformation hinder routine transgenic production in fruit plants<sup>4</sup>. It is a prerequisite to underpin various factors in determining successful *Agrobacterium*-mediated genetic transformation in fruit and nut crops.

#### Factors Affecting *Agrobacterium*-mediated Transformation

**Plant genotype:** Plant genotype plays a significant role in successful *Agrobacterium*-mediated transformations. The different cultivars of a particular fruit plant respond differently to transient as well as stable GUS expression. This holds true for tropical, sub-tropical and temperate fruit plants. Genotype-dependent efficiency of *Agrobacterium*-mediated transformation has been reported in almonds<sup>46</sup>, apple<sup>47</sup>, bananas<sup>48</sup>, scion cultivars of grapes<sup>49-51</sup> and grape rootstocks<sup>15, 52-54</sup>, pears<sup>15, 52-56</sup>, pecan<sup>36</sup> and strawberries<sup>47,57</sup>. It is evident from the past research that the plant genotypes and even the cultivars within the genotype respond differently to plant regeneration and hence the transformation of a particular fruit crop. Thus, the fruit species, which are recalcitrant to regeneration, are difficult to transform.

**Explant:** The explant, its source, pre-treatment prior to inoculation/infection and placement on media with various *Agrobacterium* strains, also plays an important role in the consequent success of transformations. Different explants have been used in various transformation studies in fruit plants. Epicotyl or internodal stem segments have been an explant of choice for transformation studies in citrus species<sup>5, 27, 32, 33, 58-70</sup>. The epicotyls were found to be suitable for culture *in vitro* which resulted in a high shoot formation frequency (86%) and histochemical GUS-assays showed that 70% of the resistant plants identified were GUS positive<sup>71</sup>. Hence, epicotyls are preferred over other explants in citrus. The other explants, such as lateral buds in 'Shatian' pummelo; cell suspension cultures in *C. sinensis* and *C. reticulata*<sup>72</sup>; protoplasts from an embryogenic nucellar derived suspension culture in *C. sinensis* sweet orange cv. Itaborai<sup>73</sup>; callus from cambium obtained from 4 month old seedlings of sour orange *C. aurantium*<sup>74</sup>; hypocotyls segments in *C. aurantifolia*<sup>75</sup>; cotyledons; roots; and crowns have also been used for

transformation studies in citrus<sup>76</sup> with varying responses to the transformation success rate. In grapes, the somatic embryos particularly those obtained from anthers, are the explant of choice for transformation studies<sup>15, 17, 51, 77-79</sup>. Varied responses to transformation in grapes have also been obtained using other explants as well, such as leaf segments<sup>30,31,50,80-82</sup> immature zygotic ovules<sup>54</sup>, hypocotyl, petiole<sup>83</sup> and shoot apex<sup>49</sup>. It has been observed that a direct shoot regeneration system, as from leaves, could produce chimerically transformed plants, but is unsuitable for routine production of uniformly transformed plants<sup>50</sup>. Likewise, on leaves the transformation efficiency of strains LBA 4404 and GV 3101 was much lower than with stem segments as explants.

In *Musa* spp. different explants such as apical meristem, corn slices<sup>42</sup>, friable callus<sup>43</sup>, embryonic cell suspension<sup>19</sup> and protoplasts<sup>44</sup> have been used for transformation experiments with varying responses to transformation outcomes. In Papayas, explants such as leaf discs, stem segments<sup>84</sup>, petioles<sup>84, 85</sup>, cell suspensions<sup>86</sup> and embryonic tissue from immature zygotic embryos<sup>18, 35, 87</sup> have been reported for transformation studies. The transformation frequency was more than double (97%) in leaf explants as compared to cotyledons as explants, in papaya<sup>84</sup>, while in peaches, immature embryos<sup>88</sup> influence the transformation rate. In the pear species, leaf segments are the explants of choice in the cultivars Passe Crassane, Conference, Diyenne du Comice, and Beurre Bose<sup>55, 89-91</sup>. Additionally, cotyledon<sup>92</sup> seedlings directly agro-inoculated on stems<sup>93</sup> have also showed varied response to transformation frequency. Using leaves as explants, up to 42% of transformed buds or bud clusters were obtained in the pear cv. Conference using *Agrobacterium*-mediated gene transfer<sup>89</sup>. Leaf explants on the other hand are the leading explant used for transformation experiments<sup>14, 47, 94-109</sup>.

Likewise in strawberries (*Fragaria* spp.), leaf segments have been used in various *Agrobacterium*-mediated transformation experiments<sup>38, 39, 47, 57, 110-113</sup>. Runners in *Fragaria vesca*<sup>114</sup> and petioles in *F. ananassa* have also been used as explants<sup>39</sup>, with varying responses to transformation frequencies. In *Rubus* spp. leaf explants such as leaf discs<sup>115</sup>, internode stem segments<sup>116, 117</sup> and petioles<sup>118</sup> have been reported in transformation studies. In kiwifruit, hypocotyls, stems<sup>114</sup>, leaf discs callus<sup>29</sup> and young leaves<sup>119</sup> have also affected transformation rates. Whilst in Japanese persimmon, leaf discs are the preferred source of explant material<sup>120, 121</sup>.

For transformation success of a particular fruit species, explants play a key role. The part of a plant/explant, which exhibits mass scale propagation ability of true to type plants, such as regeneration via somatic embryogenesis, should be preferred for transformation studies. The year round availability of the explant used for transformation experiments is one of the important criteria to be considered. Cell suspension cultures ensure a year round supply of somatic embryos in the actively growing phase.

**Source:** The explant source also affects transformation efficiency. In the Washington Navel orange, a co-culture of 21-day-old epicotyl stem segments gave greater transformation efficiency than a co-culture of 56-day-old stem segments<sup>63</sup>. Likewise it has been reported that transformation frequency in Citrus was highest when epicotyls were obtained from 2-day-old seedlings<sup>65</sup>. Both age and source of sour orange plant material affected the transformation frequency<sup>74</sup>. *C. paradisi* transgenic plants have

been produced from the explants/epicotyl segments obtained from etiolated seedlings<sup>122</sup>. In papayas, the transformation frequency increased with an increase in the age of the papaya plant (with the maximum in 1 year old plants) among 1yr, 70 d, and 40 d old plants, from which explants were obtained<sup>84</sup>. Higher transformation efficiency was obtained when etiolated internodal explants were used in the apple cv. Royal Gala<sup>123</sup>. The first internodes from etiolated shoots exhibited four-fold more GUS expressing zones compared to leaf explants from non-etiolated shoots<sup>124</sup> using the *Agrobacterium* strain EHA 105. In the blueberry cultivars Bluecrop, Duke and Georgiagem, it was found that GUS expression was significantly higher when explants were derived from shoots cultured on 15 mM sucrose, as compared to shoots cultured on 58 mM sucrose<sup>125</sup>.

The condition of the explants, such as the age or condition of the culture, prior to selecting explants, influences gene delivery. The explants from *in vitro* seedlings or actively growing plant parts/meristem show a better response to *in vitro* manipulation with/without growth hormones and thus are amenable to modification by recombinant technology for producing transgenics on a commercial scale.

**Pre-treatment:** The explant pre-treatment influences transformation rates in different fruit plants. In the grape cv. 110 Richter, 2 weeks before co-cultivation, the somatic embryo callus, when transferred to a hormone-free medium to stimulate formation and development of somatic embryos, also improved transformation frequency<sup>126</sup>. Likewise in peaches, a pre-culture of shoots on a MS medium (before excising stem segments) for 1 week increased transformation efficiency<sup>109</sup>. In apples, a pre-culture of leaf explants prior to co-cultivation slightly increased the number of GUS expressing zones in *M. domestica* cv. Jonagold<sup>97</sup>. Conversely in *Actinidia deliciosa* cv. Hayward, a pre-culture inhibited gene transfer at the cut edge<sup>119</sup>. Explants (leaf segments) taken from conditioned leaves (4 days on shaker) on MS modified liquid media, had 3 times more GUS foci, compared to control experiments in the apple cvs. Delicious and Pink Lady<sup>104</sup>. In contrast, in 'Ducan' grapefruit, it was found that a 1 or 2 day pre-culture of explants on a co-cultivation media reduced the percentage of GUS positive shoots by a factor of 8.4 (0.7% in pre-cultured explants vs. 5.4% without pre-culture)<sup>127</sup>. Pre-treatment allows the conditioning of the explant prior to *Agrobacterium* infection and has been found to increase transformation events in only in some fruit species, such as apples, grapes, kiwifruit and peaches. The conditioning of leaves particularly improves the porosity of cell walls and thus enhances the transformation rate.

**Orientation/placement of explants on media:** The horizontal placement of internodal stem segments on a medium in sour oranges increased regeneration to transformation frequency<sup>62</sup>. Likewise, it has been reported<sup>58</sup> that explant orientation affected GUS activity, i.e. the GUS positive shoot production increased with basal ends upright in the medium in 'Key' lime. In apples, the apical and basal leaf portions of rooted shoots grown *in vitro* were more susceptible to *Agrobacterium* than petiolar regions<sup>95</sup>. In kiwifruit transformation, it has been reported that leaf explants with the adaxia down on media, showed a significantly higher gene transfer frequency than those abaxial surface down<sup>128</sup>. The orientation of the explant cut has been shown to strongly influence transformation efficiency in sweet orange and citrange<sup>129</sup>. Epicotyl

explants prepared by longitudinal cutting increased regeneration frequency from 3.6 to 37.1% in citrange, and transformation frequency from 0.7 to 4.3% in sweet orange, and from 5 to 40% in citrange.

**Method of transformation:** The use of *Agrobacterium*-mediated transformation alone or used in conjunction with other methods, such as biolistics, sonication, vacuum infiltration, whisker transformation or vortexing, have variable responses to transformation in different fruit plants. Biolistics, in conjunction with *Agrobacterium*, have been reported in bananas<sup>42</sup>, citrus<sup>62</sup>, grapes<sup>130-132</sup> and strawberries<sup>112</sup>. In citrus, grapes and bananas, the explants were first bombarded either with gold/tungsten particles and then inoculated/infected with an *Agrobacterium* strain. The wounding by particle bombardment prior to *Agrobacterium* infection did improve transformation efficiency in grapes and bananas, while it was not useful for either Citrus spp. *C. aurantium* or *C. aurantifolia*<sup>62</sup>. In the strawberry cv. Chandler, the gold particles were first coated with *Agrobacterium* cells and then bombarded<sup>112</sup>, which improved transformation frequency. Likewise, sonication in conjunction with *Agrobacterium* transformation has been reported in apples<sup>133</sup>, citrus and passion fruit<sup>67</sup>. In *Citrus depressa*, using epicotyl segments, and in *Passiflora edulis*, using leaf explants, the transient GUS expression improved significantly compared to control treatments<sup>67</sup>. Meanwhile in the semi-dwarf apple rootstock M26 the sonication did not enhance the transformation efficiency<sup>133</sup>. The use of sonication in conjunction with *Agrobacterium* needs to be further investigated for other fruit crops. In another approach to improve transient and stable GUS expression in *Agrobacterium*-mediated transformation, vacuum infiltration has been used in Citrus (Troyer Citrange, *C. sinensis* x *P. trifoliata*)<sup>76</sup> and apples 'Royal Gala'. It has been reported<sup>107</sup> that vacuum infiltration with an acidified medium (pH 3.0), when used in combination with antibiotics improved transformation efficiency indirectly by eliminating *Agrobacterium* from explants and/or reduced contamination with *Agrobacterium tumefaciens* using strain EHA101. The use of silicon carbide whiskers in conjunction with *Agrobacterium tumefaciens* has been reported in grape rootstock 110 Richter (*V. berlandieri* x *V. rupestris*)<sup>131</sup>, however, no transgenic cells were obtained using this method. In the apple cv. Falstaff, when using leaf discs the use of silicon carbide whiskers along with vortexing improved transformation efficiency<sup>102</sup>. In papayas, vortexing explants with tungsten M-15 particles and *Agrobacterium* improved transformation frequency<sup>86</sup>. While in another experiment in *C. papaya*, *Agrobacterium tumefaciens* has been used in conjunction with carborundum in a liquid phase<sup>87</sup> to improve transformation efficiency. In yet another approach to improve transformation frequency in apple rootstock M26, explants were pre-treated with enzymes (cellulose/pectinase) and then infected with an *Agrobacterium* strain. However, enzyme pre-treatment did not enhance transformation efficiency in apples<sup>133</sup>.

The *Agrobacterium*-mediated transformation alone or assisted with other methods has been used in various fruit crop transformation experiments. Most methods including sonication, biolistics, vortexing, whisker transformation and vacuum infiltration, improved transformation, but have been used on a limited scale either in one or a few fruit species. It would be

worthwhile to further explore these methods in other commercial fruit species, particularly those which are recalcitrant. The use of sonication prior to *Agrobacterium* infection, in our studies<sup>2</sup> on passion fruit and citrus, significantly enhanced the GUS expression in epicotyl segments, respectively.

**Agrobacterium strain:** The strain of *Agrobacterium* used for transformation experiments plays a significant role in frequencies of transformation achieved in different fruit plants. *Agrobacterium tumefaciens* has routinely been used in several fruit plants. So far 34 different strains (Table 1) have been used in transformation experiments with tropical, sub-tropical and temperate fruit plants. *A. rhizogenes* has also been used in a few fruit plants such as in Citrus<sup>32</sup>, grapes cv. Grenache<sup>30</sup> and cv. Koshusanjaku<sup>31</sup>, apple rootstock<sup>133, 134</sup>, kiwi fruit<sup>29</sup> and strawberries<sup>114</sup>.

The strain A281 has exclusively been used in temperate fruit plants such as apples, strawberries, pears, and kiwifruit. The strains C58 and EHA 101 have been used only to transform sub-tropical (mango, citrus, and grape) and temperate fruit plants (apple, plum, strawberry, berries and kiwifruit), while the strains EHA 105 and LBA 4404 have been used to transform all major groups of fruit plants such as tropical (banana, papaya), sub-tropical (citrus, grape) and a range of temperate fruit plants. The transformation efficiency/frequency varied amongst different strains. In the Citrus cv. Washington Navel orange, among three strains tested, the strain C58 C1 showed maximum transformation efficiency (45%) followed by EHA 101 (29%) and LBA 4404 (0%)<sup>63</sup>, while LBA 4404 proved effective in Trifoliolate orange<sup>59</sup> and Troyer Citrange (*C. sinensis* x *P. trifoliolate*)<sup>66</sup>. This indicates that a particular strain behaves differently in different genotypes. In a similar study on grapes, cv. Chardonnay and Glevurztraminer<sup>135</sup>, the strain GV 3101 was best and LBA 4404 proved to be an inefficient strain. On the other hand, in the grape rootstock *V. rupestris*<sup>136</sup> and 110 Richter<sup>52, 126</sup>, Rusalka<sup>137</sup> and in *V. vinifera*, cvs. Seyval Blanc<sup>80</sup>, Muller-Thurfan, Riesling cv. Dornfelder<sup>16, 138</sup>, the *Agrobacterium* strain LBA 4404 proved to be effective for grape transformation. To transform the Tunisian autochthonous grapevine<sup>132</sup>, C58 strain was found to be more effective than LBA 4404. In apples, among three strains, LBA 4404, C58C1 and EHA 101, in the *M. domestica* transformation study<sup>97</sup>, the strain EHA 101 yielded at least twice as much GUS expressing leaf zones as strain LBA 4404, whereas strain C58C1 did not show any GUS activity in leaves. Studies in apple cv. Pinova showed the superiority of the *Agrobacterium* strain EHA 105 as compared to the LBA 4404 and KYRT1 strains<sup>139</sup>. Yet, in another study on *M. domestica*, strain C58C1 was quite efficient<sup>140, 141</sup>. In pear transformation, the predominately used strains are the hypervirulent EHA 101 and EHA 105 strains<sup>56, 89, 90, 142</sup>. The *Agrobacterium* strain A281 has been shown to be superior in transformation of kiwifruit as compared to LBA 4404<sup>128</sup>.

As evident from the literature, several strains have been used for gene transfer studies in fruit crops. The extensively used strains are EHA 101, EHA 105, LBA 4404 and C58. Amongst all, EHA 105 is the most efficient strain, having achieved a transformation frequency of 87.5%, the highest reported in fruit species<sup>143</sup>. EHA 105 has also been successfully used in fruit crops such as apples, pears, bananas, citrus, grapes, plums, strawberries and raspberries.

**Gene construct:** The vector plasmid used to transform *Agrobacterium*, which is then used for the transformation of fruit plants, also plays an important role in transformation efficiency

and the production of transgenics in fruit plants. The gene and the promoter/terminator used are considered to play a role in transformation, as evident from several studies in grapes, apples, citrus, strawberries and kiwifruit.

**Gene/plasmid used:** In grapes, the transformation efficiency was highly increased by using a modified T-6b gene (a gene from pTiTm4), which interferes with normal growth and allows regeneration of plants<sup>135</sup>. Likewise, in the grape cv. Thompson seedless, it was found that the plasmid containing Tom RSV-CP was better than one having the *Shiva* I gene<sup>130</sup>. In apples, the A281 T1 plasmid pTiBo542 was most virulent compared to A208, while the presence of the plasmid PCGN7314 greatly reduced transformation efficiency in A281 from 66 to 22%<sup>95</sup>. Likewise in *M. domestica*, A281 was more effective than C58 and A348 wild type strains<sup>96</sup>. However, in apple cultivar Granny Smith leaf segments, the *Agrobacterium* strain C58 together with the plasmid PGV 2449 performed better than with the plasmid PGV2492 for transformation<sup>98</sup>. The transformation efficiency is influenced by type of plasmid used<sup>144</sup>. The use of co-integrate plasmids PGV3850 :1103 neo and PGV 3850 :1103 *gus*, gave higher transformation frequency than the binary vector PGV 3111 x PKIWI. It has been further reported in apples<sup>141</sup> that both vector and the bacterial background influence the transformation frequency as well. The vector PB121 showed the lowest transformation frequency compared with PCMB-B:GUS. Moreover, in apples the transformation rate varied amongst different plasmid constructs<sup>145</sup>. Using plasmid construct Pla2AMVSP, Att transgenic lines had a low expression of the *att E* (attacin E) gene, compared to other *att E* transgenic lines.

In strawberries, the transformation efficiency was much higher (0.95-6%) when PSS1 was used, regardless of the explant (leaves/petioles), compared to when T1 binary vector PBIN6 was used in the experiment<sup>39</sup>. Likewise, in pecan nut transformation studies<sup>36</sup> the *Agrobacterium* strain EHA 101 containing the plasmid PCGN7314, was better than that containing the plasmid PCGN 7001. In kiwifruit cv. Hayward, of the different plasmids in the *Agrobacterium* strains A281, LBA 4404, C58 and EHA 101, such as pKIWI 105, pKIWI 109 and pLAN 421, the plasmid pLAN 421 was the best<sup>119</sup>. In citrus the vector plasmid pGA482GG had higher transformation frequency than the plasmid pMONG973<sup>62</sup>.

The incorporation of supplementary copies of virulence genes (*virA*, B and G) has been found to greatly stimulate the virulence of *Agrobacterium tumefaciens* in apples<sup>95</sup>. Likewise in a citrus study, conducted to investigate the role of supplementary copies of *virG* from pTiBO542 towards transformation using *Agrobacterium* strain EHA 105, it was found that with *C. limon* the additional copies of *virG* which substantially increased transformation from 1 to 82% was induced by C58/PMP90, while the transformation efficiency was also increased from 0-20% by C58/PMP 90. When PCH 30 was introduced into EHA 105, the transformation frequency increased from 34 to 54% and the transformation efficiency increased from 3 to 7%<sup>143</sup>. The transformation frequency also enhanced (6-36%) and (0-35%) with plasmid PCH 30 and C58/PMP 90 respectively.

Transgenic plants of 'Hayward' kiwifruit expressing *rolA*, B and C genes showed an increased rooting ability as compared to non-transgenic plants<sup>29</sup>. Transgenic plants of M26 apple rootstock expressing Ri T-DNA exhibited higher rooting ability<sup>146</sup> as well as

a well-developed root system. Likewise, transgenic grape plants constitutively expressing RI-T-DNA, including *rol* genes, produced an abundant root system<sup>31</sup>. The dwarfing, as well as improved rooting, was obtained by incorporating *rol* genes, such as *rolA* and B genes in apples<sup>141, 147-149</sup> and *rolC* genes in pears<sup>91</sup>. The plants transformed with specific or a combination of *rol* genes showed altered morphology. Thus *rol* genes have a direct influence on the transformation efficiency of fruit plants and can be properly utilized for the production of dwarf or semi dwarf scion cultivars as well as rootstocks. To have success in transformation, the gene/plasmid used should not interfere with the regeneration of plants. Normal regeneration is the pre-requisite for production of transgenic plants.

**Promoter used:** The promoter used during the genes construct has also been reported to affect transformation efficiency in fruit plants. The plant viral or bacterial origin promoters are tagged to the gene of interest and used for transformation. Some promoters confer constitutive expression while others may be selected to permit time specific expression. The cauliflower mosaic virus (CaMV) 35S promoter is often used because it directs high levels

of gene expression in most tissues<sup>28</sup>. In general, the cauliflower mosaic virus (CaMV 35S) promoter has been used and has been found to be superior to other promoters. In trifoliolate orange, the GUS activities with 35S promoters were higher than those with a *rolC* promoter<sup>59</sup>. In ‘Rio Red’ grapefruit, the use of the CaMV 35S promoter increased transformation efficiency from 5 to 7 fold compared to the figwort mosaic virus 34S promoter, used to drive the beta-glucuronidase (*UidA*) gene<sup>68</sup>. Likewise in apples, using *Agrobacterium* strain A281, the CaMV 35S promoter was better than the nopaline synthase (*nos*) promoter driving the neomycin phosphotransferase (*nptII*) gene<sup>96</sup>. Similarly, in *Malus pumila*, CaMV 35S proved to be better than the Rubisco small sub-unit (SSV) gene promoter<sup>150</sup>. In transformation studies with apple rootstock M26, the CaMV 35S promoter also proved to be effective<sup>151</sup> for transformation experiments. In apple cv. Greensleeves, results showed that the heterologous promoters *rolCP* and *CoYMVP*, expressed the *gusA* gene at a lower level than the CaMV 35S promoter<sup>152</sup>. Other promoters, such as a wound inducible promoter (osmotin) from tobacco, have been used in apples to control the *Cecropin*-MB 39 gene responsible for increasing resistance to *Erwinia amylovora*<sup>123, 153</sup>.

**Table 1.** Different *Agrobacterium tumefaciens* strains used in genetic transformation of various fruit plants.

<i>Agrobacterium</i> Strain	Fruit / Nut crop	Reference(s)
A6	Rubus spp.	Hassan et al., 1993
A136	Peach	Hammerschlag et al., 1989; Smigochi and Hammerschlag, 1991
A208	Apple	Dandekar et al., 1990
	Strawberry	Uratsu et al., 1991
	Mango	Mathews et al., 1992, 1993
A 281	Apple	Dandekar et al., 1990; Maheshwaran et al., 1992
	Strawberry	Uematsu et al., 1991
	Kiwifruit	Janssen and Gardner 1993; Li et al., 2003; Li et al., 2003 <sup>128</sup>
	Pear	Merkulov et al., 1998
A 348	Apple	Maheshwaran et al., 1992
A 415	Citrus	Hikada et al., 1990
A 518	Citrus	Gutierrez et al., 1997
A 722	Strawberry	Uematsu et al., 1991
A Ch 5	Strawberry	Uematsu et al., 1991
AG 162	Grape	Baribault et al., 1990
AGLO	Apple	Schaart et al., 1995
AKE 10	Pear	Kaneyoshi et al., 2001
516	Rubus spp.	Hassan et al., 1993
527	Rubus spp.	Hassan et al., 1993
1065	Citrus	Gill et al., 2000
	Passion fruit	Cancino et al., 1999
5805	Grape	Baribault et al., 1990
15955	Strawberry	Uematsu et al., 1991
B653 x 200	Strawberry	Uematsu et al., 1991
C58	Citrus	Ghorbel et al., 2001
	Grape	Bouamama et al., 2000
	Apple	Maheshwaran et al., 1992; Trifonova et al., 1994
	Strawberry	Uematsu et al., 1991
	Plum	Scorza et al., 1994
	Berries	Hassan et al., 1993
	Kiwifruit	Janssen and Gardner, 1993
C BE21	Apple	Dolgov et al., 1999
	Strawberry	Dolgov et al., 1999
	Pear	Lebedev et al., 2002
C58C1	Apple	Welander et al., 1998; James, 1993; De Bondt et al., 1994; Sedira et al., 2001
	Mango	Mathews et al., 1993
	Wild Pear	Caboni and Lauri, 2002

Table 1. continued

	Pear	Negri et al., 2002
	Starwberry	Monticelli et al., 2002
C58 GV3101	Grape	Mezzetti et al., 2002
C58Z707	Grape	Xue et al., 1999; Krastanova et al., 2000
CV Ag 11	Juneberry	Hajela et al., 1993
EHA 101	Citrus	Moore et al., 1992; Gutierrez et al., 1997; Costa et al., 2002; Yu et al., 2002; Boscariol et al., 2003
	Grape	Baribault et al., 1989; Colby et al., 1991; Scorza et al., 1996; Mozsar et al., 1998; Hoshino et al., 2000
	Apple	De Bondt et al., 1994; Hammerschlag et al., 2000; Sedira et al., 2001; Gittins et al., 2003; Viss et al., 2003
	Plum	Mante et al., 1991; Scorza et al., 1994
	Pecan	McGranaham et al., 1993
	Pear	Mourgues and Chevreau, 1997; Chevreau et al., 1999, 2000; Reynoird et al., 1999; Bell et al., 1999; Maloney et al., 2003
	Kiwi	Uematsu et al., 1991; Janssen and Gardner, 1993
	Jap. Persimmon	Gao et al., 2000, 2001
	Passion Fruit	Quoirin et al., 2004
EHA 105	Apple	Liu et al., 1998; Hanke et al., 1999; Bolar et al., 1999; Liu et al., 2000; Ko et al., 2002; Hanke et al., 2002; Szankowski et al., 2003
	Strawberry	Zhang et al., 2001; Monticelli et al., 2002
	Red raspberry	Mathews et al., 1995
	Blueberry	Cao et al., 2003
	Citrus	Pena et al., 1995, 1997, 1998; Cervera et al., 1998; Dominguez et al., 2000; Piestun et al., 2000; Malfa et al., 2000; Ghorbel et al., 2001; Costa et al., 2002; Li et al., 2002; Almeida et al., 2003; Molinari et al., 2004
	Pear	Caboni and Lauri, 2002; Maloney et al., 2003
	Plum	Padilla, et al., 2003
	Grape	Scorza et al., 1996; Colova-Tsolova et al., 2003
	Banana	Ganapathi et al., 2001
GV 3010	Citrus	Hidaka et al., 1990
GV 3101	Grape	Baribault et al., 1990; Berres et al., 1992; Chen et al., 2001
	Rubus spp.	Hassan et al., 1993
GV 3111	Papaya	Pang and Sanford, 1988
	Passiflora	Manders et al., 1994
K12	Strawberry	Uematsu et al., 1991; Dandekar and Martin, 1988.
K12 x 562 E	Strawberry	Uematsu et al., 1991; Dandekar and Martin, 1988.
K12 x 167	Strawberry	Uematsu et al., 1991; Dandekar and Martin, 1988.
K61	Walnut	McGranahan et al., 1988
KYRT1	Apple	Hanke et al., 2002
LBA 4404	Citrus	Kaneyoshi et al., 1994; Gentile et al., 1999
	Grapes	Baribault et al., 1989; Mullins et al., 1990; Berres et al., 1992; Martinelli and Mandolino, 1994; Gall et al., 1994; Krastanova et al., 1995; Golles et al., 1997; Lannini et al., 1997; Harst et al., 2000; Bornhoff et al., 2000; Das et al., 2002; Gribaudo et al., 2003
	Banana	May et al., 1995; Khayat et al., 1998; Hoshino et al., 2000; Krastanova et al., 2000; Spielmann et al., 2000
	Papaya	Yang et al., 1996; Cheng et al., 1996; Ying et al., 2000; Chen et al., 2001; Bau et al., 2003
	Mango	Cruz-Hernandez et al., 1997
	Apple	James et al., 1989; Norelli et al., 1994; Sriskandarajah et al., 1994; De Bondt et al., 1994; Yao et al., 1995; Welander et al., 1998; Zhu et al., 1998; Cheng et al., 1998; Song et al., 2000; Hanke et al., 2002
	Strawberry	Mansouri et al., 1996; Owens et al., 2002; Monticelli et al., 2002; Gabriel Ricardo et al., 2003
	Apricot	Machado et al., 1992; Machado et al., 1999
	Plum	Machado et al., 1994; Machado et al., 1999; Padilla, et al., 2003
	Almond	Archilleti et al., 1995
	Passionfruit	Cancino et al., 1999
	Pear	Zhu et al., 1998
	Blueberry	Graham et al., 1996
	Redraspberry / Blackberry	Graham and McNical, 1990
	Rubus spp	Graham et al., 1990
	Kiwifruit	Janssen and Gardner, 1993; Li et al., 2003; Li et al., 2003 <sup>128</sup>
MP 90	Strawberry	Nehra et al., 1990

In pears, both CaMV 35S<sup>55, 90, 93</sup> and *nos* promoters<sup>154</sup> have been used for gene transfer studies. However in cherries, the use of CaMV 35S and Calmodulin promoters have been reported<sup>155</sup> to affect the transformation efficiency of the sweet cherry (*Prunus avium*) cv. Summit.

Thus the gene construct used for transformation plays a significant role in transformation frequency and efficiency of production of transgenics.

**Agro-infection conditions:** The infection of explants with an *Agrobacterium* strain is affected by several factors, such as bacterial culture initiation, bacterial density, infection time and microenvironment.

**Bacterial culture initiation:** Overnight bacterial culture (broth) is raised to infect the explant for *Agrobacterium*-mediated transformation. In general, overnight culture is raised from 2-3 day old single colonies grown from  $-80^{\circ}\text{C}$  freezer cultures on LB media fortified with required antibiotics for selection. The bacterial culture initiation is done in liquid medium containing suitable antibiotics for selection, on shaker, in dark for 12-16 h. The use of overnight culture without antibiotic selection has also been reported in Washington Navel orange<sup>63</sup>. The overnight culture can be supplemented with flavonoids such as acetosyringone to enhance/activate virulence of the bacteria. The use of acetosyringone (20  $\mu\text{M}$ ) has been reported to improve transformation frequency in peach<sup>88, 109</sup>, apricot<sup>156</sup> and *Rubus* spp.<sup>117</sup>, whilst in mango, a concentration of 30  $\mu\text{M}$  has been reported to be beneficial<sup>157-159</sup>. Likewise in kiwifruit, it was found that acetosyringone (20  $\mu\text{M}$ ) in *Agrobacterium* growth medium increased transient GUS expression 2-fold as compared to growth medium without acetosyringone. In apple, overnight cultures have been raised containing 100  $\mu\text{M}$  acetosyringone<sup>99</sup>. However in pecan, *Agrobacterium* growth medium supplemented with either acetosyringone (100  $\mu\text{M}$ ) alone or in combination with betaine phosphate (1 mM) had little effect on transformation frequency<sup>36</sup>.

Similarly in strawberry<sup>39</sup>, the use of 20 or 200  $\mu\text{M}$  acetosyringone in bacterial culture prior to overnight growth had no effect at any concentration in regards to transformation efficiency.

Phenolic compounds such as acetosyringone have been extensively used to enhance transformation efficiency in field crops. However, in fruit crops, it has been used to a limited extent. The potential of acetosyringone and other phenolic compounds such as naringenin should be further explored to improve the efficiency of *Agro*-infection in commercial fruit crops.

**Bacterial density/dilution and inoculation time:** The density of bacterium used for infection is adjusted either by monitoring the time of overnight cultures during incubation or by diluting the overnight cultures. The bacterial density and inoculation time is directly co-related. The optimum bacterial culture densities varied in different fruit crops (Table 2). In trifoliolate orange, bacterial density of  $5 \times 10^8$  cells  $\text{ml}^{-1}$  was used to infect epicotyl segments for 15 mins<sup>59</sup>. While in cv. Washington Navel orange 10 min. inoculation time was sufficient<sup>63</sup> to infect epicotyl segments with the same bacterial density. In citrus cv. Mexican lime,  $1 \times 10^8$  cells  $\text{ml}^{-1}$  bacterial density was found suitable to infect internodal stem segments for 45 min<sup>32</sup>. However  $10^7$  cells  $\text{ml}^{-1}$  were found optimum<sup>61</sup> when infected for only 15-30 min. in some citrus cultivars. In sweet orange,  $4 \times 10^7$  cells  $\text{ml}^{-1}$  of overnight culture was placed as a drop on the cut end of stem segments<sup>60</sup>, and this dilution was found to be better than  $4 \times 10^8$  cells  $\text{ml}^{-1}$  in Carrizo citrange. In mango, different bacterial concentrations were tested for optimising co-culture, with  $10^8$  cells  $\text{ml}^{-1}$  the best with 20% transformation<sup>159</sup>. While at  $10^6$  and  $10^7$  cells  $\text{ml}^{-1}$ , transformation frequency decreased to 10% and at higher concentration, the plant tissue darkened immediately and no white tissue was recovered. Likewise in grapes, variable bacterial density of overnight culture was used to infect different explants. To infect hypocotyl and petioles of cv. Rupestris St. George, an overnight culture of  $10^8$  cells  $\text{ml}^{-1}$  of was used and only 30 sec. soaking was required<sup>52, 83</sup>.

**Table 2.** Optimum bacterial density used in *Agrobacterium*-mediated gene transformation in different fruit plants.

Fruit species	Common name/ cultivar	Bacterial density (cell: $\text{ml}^{-1}$ )	Reference(s)
<i>Carya illinoensis</i> (Wangenh) K. Koch.	Pecan nut/ Elliot 6, Schley 5/3, Wichita 9.	$2.5 \times 10^8$	Mc Granahan et al., 1993.
<i>Citrus aurantifolia</i> Swing.	Citrus/ Mexican lime	$10^7$	Pena et al., 1997
<i>Citrus aurantifolia</i> Swing.	Citrus/ Mexican lime	$1 \times 10^8$	Perez and Ochoa, 1998.
<i>Citrus paradise</i> (Macf.)	Grapefruit / Duncan	$5 \times 10^8$	Costa et al., 2002
<i>Citrus sinensis</i> L.Osb.	Citrus/ Washington navel orange	$5 \times 10^8$	Bond and Roose, 1998.
<i>Citrus sinensis</i> L. Osb.	Citrus/ Pineapple orange	$4 \times 10^7$	Pena et al., 1995
<i>Juglans hindsii</i> (Jeps). Jeps.	Walnut/ California black walnut	$5 \times 10^8$	Dandekar and Martin, 1988.
<i>Juglans regia</i> L.	Walnut/ Scharch	$5 \times 10^8$	Mc Granahan et al., 1988.
<i>Malus x domestica</i> Borkh.	Apple/ M. 26	$5 \times 10^8$	Maheshwaran et al., 1992.
<i>Malus pumila</i> Mill.	Apple/ Greensleeves	$5 \times 10^8$	Dandekar et al., 1990.
<i>Malus x domestica</i> Borkh.	Apple/ M. 26	$2 \times 10^9$	Norelli et al., 1994
<i>Malus x domestica</i> Borkh.	Apple/ Jonagold	$2.5 \times 10^9$	De Bondt et al., 1994.
<i>Mangifera indica</i> L.	Mango/ Hindi	$10^8$	Cruz and Litz, 1997.
<i>Prunus domestica</i> L.	Plum/ selection B 70146	$10^5 - 10^6$	Mante et al., 1991
<i>Prunus persica</i> L. (Batsch)	Peach/ Redhaven	$5 \times 10^8$	Smigocki and Hammerschlag, 1991.
<i>Vitis berlanderi</i> Planch x v. <i>riparia</i> S.	Grape/ 110 richter, Rupestris St. George	$10^8$	Mullins et al., 1990; Krastanova et al., 1995.
<i>Vitis berlanderi</i> Planch x v. <i>riparia</i> S.	Grape/ Chardonnay, Gewvr, Kober, 5BB.	$10^9$	Berres et al., 1992.
<i>Vitis vinifera</i> L.	Grape/ Cabernet Sauvignon	$10^6$	Baribault et al., 1990.

On the other hand inoculation time of 1 h was found optimum in cv. Cabernet Sauvignon, Sultana<sup>49</sup> and in other *V. vinifera* cvs. and its hybrids<sup>135</sup> using bacterial density of  $10^6$ - $10^9$  cells ml<sup>-1</sup>. A subsequent study reported while using leaves and embryonic callus as explants, the bacterial density of overnight culture was adjusted to  $1 \times 10^8$  cells ml<sup>-1</sup> and used for infection for 10 min.<sup>31</sup> In peach (*Prunus persica*), bacterial density of overnight culture was adjusted to  $1 \times 10^8$  cells ml<sup>-1</sup> to infect stem explants<sup>109</sup>, while to infect embryos, the final density of overnight culture was adjusted to  $5 \times 10^8$  cells ml<sup>-1</sup> by MS medium<sup>88</sup>. In banana, overnight cell culture was diluted to 1:10 with MS medium<sup>160</sup> and used for infecting apical meristem and corn slices for 30 min.<sup>42</sup> In apple, the density of overnight bacterial culture was adjusted to  $5 \times 10^8$  cells ml<sup>-1</sup> to infect leaf explants<sup>95,96</sup>. In another apple cultivar,  $2 \times 10^9$  cells ml<sup>-1</sup> was found to be optimum<sup>99</sup>, while in other reports on apple, the optical density of overnight culture was adjusted, which have direct co-relation to bacterial density. In *Malus domestica* cv. Jonagold, inoculation density  $2.5 \times 10^9$  cells ml<sup>-1</sup> showed higher transformation efficiency compared to  $0.1 \times 10^7$  cells ml<sup>-1</sup><sup>97</sup>. Likewise in *M. domestica*, cv. Delicious, overnight culture OD was adjusted to 0.042 at 650 nm and was used to inoculate leaf explants on shaker for 20 min.<sup>144</sup>. On the other hand, in *M. domestica*, explants were inoculated in overnight culture having a final OD adjusted to 0.5 at 450 nm for 5 min.<sup>95</sup> and for 5 h<sup>140</sup>. In apple cultivar Royal Gala, overnight culture OD<sub>600nm</sub> was adjusted to 0.8<sup>14</sup> and in cv. Golden Delicious OD<sub>600nm</sub> was adjusted to 0.3-0.5, to infect explants for 4-6 min.<sup>100</sup>. While in apple rootstock M26 overnight culture of OD<sub>420nm</sub> was adjusted to 1.5-2.0 for infecting explants for 5 min.<sup>141</sup>.

In strawberry, overnight culture OD<sub>600nm</sub> at 0.8 was found optimum<sup>39</sup> to infect explants for 20-30 min., while overnight culture was diluted to 1:10 with MS and gently shaken for 20 min. to infect leaf disks<sup>161</sup>. In apricot, immature embryos were infected for 5-10 sec. with overnight culture having OD<sub>600nm</sub> 0.6 and diluted to 1:50 in MS medium<sup>156</sup>. In plum, the overnight culture was diluted with MS to have final OD  $10^5$ - $10^6$  cells ml<sup>-1</sup> and was found to be optimum<sup>162</sup> for transformation. In almond, OD of 0.6 and a 30 min. inoculation time was found to be the best<sup>46</sup>. In walnut, overnight culture density of  $5 \times 10^8$  cells ml<sup>-1</sup>  $\cong$  0.5 OD at 550 nm was found optimum<sup>163,164</sup>, while in pecan,  $2.5 \times 10^8$  cells ml<sup>-1</sup> overnight culture and inoculation time of 10-15 min. was found optimum<sup>36</sup>. In passion fruit, on the other hand overnight culture was diluted 1:10 (v:v) with APM medium and explants were inoculated for 30 min.<sup>165</sup>, while in juneberry, overnight culture diluted to 1:1 was used to infect explants overnight<sup>166</sup>. In kiwifruit, a 10-fold dilution of overnight culture having optical density of 0.05-0.075 at 550 nm was found optimum for transformation<sup>119</sup>.

In general, MS basal liquid medium is used to dilute overnight cultures to achieve the appropriate OD for infecting explants. However, apart from MS media, APM medium in passionfruit<sup>165</sup>; LB medium in peach<sup>109</sup>; 3% sucrose solution in *V. vinifera*<sup>137</sup> and 523 medium in *V. vinifera*<sup>50</sup> have also been used to dilute overnight cultures, while in kiwifruit transformation studies, overnight culture was diluted with distilled water<sup>114</sup>. In most of the cases, direct dilution of overnight bacterial culture/broth was done with various media as described earlier. However, in some cases, the overnight culture was first pelleted down by centrifugation and then re-suspended in liquid media. This practice has been reported in transformation experiments with a range of fruit plants such as grapes<sup>50,52,130</sup>, peach<sup>109</sup>, apple<sup>94,95,97,140,141</sup>, strawberry<sup>38,39,167</sup>,

plum<sup>37,162</sup>, pecan<sup>36</sup> and Passiflora<sup>165</sup>.

The bacterial density or the number of cell ml<sup>-1</sup> of the bacterial culture plays a significant role in transient GUS expression. However, the bacterial cells to be used for infection of explants should be in an actively growing (log phase) condition as this ensures high transformation rates.

**Infection microenvironment:** The infection is done in varying conditions/microenvironment such as inoculating explants either by hand dipping or using shakers and under varying temperature or light conditions. In grape spp. *V. vinifera*, embryonic callus was infected with overnight culture by keeping explants on rotary shaker (100 cycles mm<sup>-3</sup>) for 10 min.<sup>31</sup>, at room temperature. Likewise somatic embryos of *V. vinifera* were inoculated for 5-10 min. at room temperature<sup>137</sup>. However in 'Delicious' apple, the leaf explants were inoculated with overnight culture on shaker at 75 rpm for 20 min. at varying temperatures, such as 23, 30, 35, 40 and 45°C. The inoculation temperature infected transformation efficiency and the maximum transformation efficiency was obtained at 35°C<sup>144</sup>. While in *M. domestica* cv. Jonagold, leaves were shaken gently in bacterial suspension culture for 1 min at room temperature<sup>97</sup>. In contrast, the virulence induction was done at 25°C for 5 h with agitation at 200 rpm and then explants were inoculated<sup>140</sup>. In strawberry cv. Red Coat, leaf discs were shaken gently in the bacterial suspension for 20 min.<sup>167</sup>, while in apricot, immature embryos were immersed only for 2-10 sec. in overnight raised *Agrobacterium* suspension<sup>156</sup>. Likewise in plum, hypocotyl segments were inoculated by immersing in *Agrobacterium* suspension, but for 10-20 minutes, without shaking<sup>37</sup>. In pear cv. Conference, Doyenne-du-Comice (DC) and Passe-Crassane (PC), just dipping the scalpel wounded leaf explants was sufficient for transformation events to occur<sup>89</sup>. In black currant, explants were co-incubated with overnight culture for 24 h at 24°C, under light conditions<sup>116</sup>. In kiwifruit, the explants were inoculated with diluted overnight culture and were shaken vigorously for 20 min.<sup>114</sup>. This was in contrast to several other reports where gentle shaking was done during infection.

The infection microenvironment should be such that there is maximum virulence induction of *Agrobacterium*, which consequently helps to enhance the transient and stable transformation events. However, the infection time varies with different fruit crops and needs to be standardized with different explants.

**Co-cultivation conditions:** The co-cultivation conditions include co-cultivation time (days), co-cultivation media, co-cultivation temperature, light/dark co-incubation and pre-culture period. The range of co-cultivation conditions is reported for fruit plants.

**Co-cultivation time:** The co-cultivation of explants with *Agrobacterium* is needed to allow the bacterial cells to infect explant cells. The explant after infection with *Agrobacterium* is blotted dry on sterile filter papers and then co-cultivated on shoot regeneration medium for few days. However, the optimum co-cultivation time varied among different fruit crops (Table 3). In Citrus, explants after agro- inoculation are co-cultivated for 3 days<sup>27,32,65</sup>, *C. sinensis* and *C. reticulata* (3 days)<sup>72</sup>, Mexican lime (3 days)<sup>61</sup> and sweet orange, (2 days)<sup>33,60</sup>. In *C. sinensis* L. Osbeck, it was found that it was not possible to co-cultivate for 3 days due to overgrowth problems, therefore a 1 day co-cultivation period

**Table 3.** Optimum co-cultivation time *Agrobacterium* and explant in different fruit species.

Fruit species	Common name	Co-cultivation period, days	Reference(s)
<i>Vitis vinifera</i> L.	Grape	1	Baribault et al., 1990
<i>Actinidia deliciosa</i> A. Chev.	Kiwifruit	1	Uematsu et al., 1991
<i>Malus x domestica</i> Borkh	Apple	1	Trifonova et al., 1994
<i>C. sinensis</i> L. Osb	Orange	1	Almeida et al., 2003
<i>Prunus persica</i> L. (Batsch)	Peach	2	Hammerschlag., 1989
<i>Carica papaya</i> L.	Papaya	2	Pang and Sanford, 1988; Yang et al., 1996; Cheng et al, 1996.
<i>Malus x domestica</i> Borkh	Apple	2	Dandekar et al., 1990; Norelli et al., 1994; Schaart et al., 1995.
<i>Prunus armeniaca</i> L.	Apricot	2	Machado et al., 1992; Scorza et al., 1994.
<i>Prunus amygdalus</i> (Batsch)	Almond	2	Archilletti et al., 1995
<i>Juglans regia</i> L.	Walnut	2	Mc Granahan et al., 1988, 1990.
<i>Actinidia deliciosa</i> A. Chev.	Kiwifruit	2	Janssen and Gardner, 1993
<i>Fragaria ananassa</i>	Strawberry	2	Nehra et al., 1990
<i>Vitis vinifera</i> L.	Grape	2	Colby et al., 1991; Scorza et al., 1996; Harst et al., 2000.
<i>Vitis rupestris</i> S.	Grape	2	Gall et al., 1994; Krastanova et al., 1995; Soloki et al., 1998.
<i>Vitis vinifera</i> L, <i>Vitis rupestris</i> S	Grape	2	Golles et al., 1997
<i>Vitis vinifera</i> L.	Grape	3	Baribault et al., 1990; Nakano et al. 1994, Das et al., 2002
<i>Vitis rupestris</i> S	Grape	3	Martinelli and Mandolino, 1994.
<i>C. sinensis</i> L. Osb x <i>Poncirus trifoliata</i> L. Raf.	Citrange	3	Cervera et al., 1998
<i>C. aurantifolia</i> Swing.	Mexican lime	3	Perez and Ochoa, 1998
<i>C. sinensis</i> L. Osb, <i>C. limon</i> , <i>Poncirus trifoliata</i> L. Raf.	Sweet orange, lemon, trifoliolate orange.	3	Han et al., 1999
<i>C. sinensis</i> L.Osb, <i>C reticulata</i> Blanco.	Sweet orange, Mandarin.	3	Hidaka et al., 1990
<i>Citrus paradise</i> (Macf.)	Grapefruit	3	Costa et al., 2002
<i>Musa</i> spp.	Banana	3	May et al., 1995
<i>Carica papaya</i> L	Papaya	3	Ying et al., 2000
<i>Malus pumila</i> Mill.	Apple	3	James et al. 1989; El Mansouri et al. 1996
<i>Passiflora edulis</i> fv. Flavicarpa.	Passionfruit	3	Manders et al., 1994
<i>Actinidia deliciosa</i> A. Chev.	Kiwifruit	3	Rugini et al., 1991
<i>Actinidia deliciosa</i> , <i>Actinidiachinensis</i>	Kiwifruit	3	Li et al., 2003 <sup>128</sup>
<i>Fragaria ananassa</i> Duch.	Strawberry	3	James et al., 1990
<i>Fragaria vesca</i> L.	Strawberry	3	Mansouri et al., 1996
<i>Diospyros kaki</i> Thunb.	Jap. Persimmon	3	Gao et al., 2000, 2001
<i>Rubus</i> spp.		4	Hassan et al., 1993
<i>Malus x domestica</i> Borkh.	Apple	4	De Bondt et al., 1994
<i>Pyrus communis</i>	Pear	4	Mourgues and Chevreau, 1997

was used<sup>168</sup>. In grapes, co-cultivation period of 1-5 days has been reported. In *V. vinifera*, co-cultivation periods of 1 day<sup>49</sup>, 2 days<sup>16, 50, 52, 126, 130, 131, 137</sup>, 3 days<sup>31, 49, 82, 136</sup> and 5 days<sup>81</sup> have been found optimum.

Likewise in other fruit plants, co-cultivation period also ranged from 1-5 days in various transformation experiments. The co-culture period of 1 day was found optimum for transformation in kiwifruit<sup>114, 119</sup> and apple<sup>98</sup>, 2 days in peach<sup>109</sup>, papaya<sup>35, 84, 85</sup> apple<sup>95, 99, 100</sup> strawberry<sup>38, 167</sup>, apricot<sup>37, 156</sup>, plum<sup>169</sup>, almond<sup>46</sup> and walnut<sup>164, 170</sup>. The culture period of 3 days has been found optimum for transformation in banana<sup>42</sup>, papaya<sup>86</sup>, apple<sup>39, 113, 161, 171</sup>, passion fruit<sup>165</sup> and kiwifruit<sup>29</sup>. In *Rubus* spp., 4 days<sup>117</sup> was found optimum for co-cultivation, however in apple, the co-cultivation periods of 2, 3, 4 or 5 days had no effect on transformation efficiency<sup>14</sup>, thus 2 day co-cultivation period was used. It has also been reported in *M. domestica*, that with increase in co-cultivation time (max. up to 4 days) GUS expressing leaves increased<sup>97</sup>.

In mango, co-cultivation was done in liquid medium contrary to other cases where generally co-cultivation is done in solid medium. In this case, 3 g of pro-embryonic masses of somatic embryos were co-cultured in 50 ml liquid medium containing 0.05 ml of log phase culture of *Acetosyringone* activated (30  $\mu$ M) *Agrobacterium*. The culture was incubated on shaker at 120 rpm in dark. The pro-embryos were transferred to fresh maintenance medium every 24 h for 3 days. No additional bacterium was added at this stage<sup>157-159</sup>.

After infection with actively growing *Agrobacterium* cells, the explants are co-cultivated for 1-5 days to allow the interaction of bacterial cells with the explant cells. The optimum co-cultivation duration however varies from one fruit crop to another. However, in the majority of fruit crops such as apple, apricot, almond, banana, citrus, grape, kiwifruit, papaya, passion fruit and strawberry, 2-3 days co-cultivation time is considered to be optimum.

**Co-cultivation media:** The co-cultivation media and the condition of the explants, such as whether they are kept wet or dry during co-cultivation, has a significant role in transformation efficiency. Likewise, the use of a feeder layer during co-cultivation has been found useful in some fruit species.

The use of plant growth regulator-free co-culture medium has been reported in Trifoliolate orange<sup>59</sup>, grape cv. 110 Richter<sup>126</sup> and papaya cvs. Sunrise Solo and Kapoho Solo<sup>84</sup>. While in several other fruit plants, plant growth regulators have been found beneficial for transformation. The co-cultivation of explants on the medium rich in auxins (IAA, IPA, 2,4-D) has been reported in Citrus cv. Citrange<sup>27</sup>. Likewise in apple cultivar Royal Gala, explants grown on medium supplemented with auxins (IBA, NAA), yielded more than twice as many GUS expressing zones and calluses compared with those grown on medium without auxins<sup>172</sup>. In contrast to this observation in *M. domestica*, the co-cultivation medium with high cytokinins was more conducive to gene transfer compared to media with high auxins<sup>97,98</sup>. The co-cultivation media supplemented with both auxins and cytokinins have also been reported in several fruit plants such as grape<sup>83</sup>, papaya<sup>85</sup>, and *Rubus* spp.<sup>117</sup>.

Phenolics are known to activate *Agrobacterium* virulence genes<sup>173</sup>. Acetosyringone has been routinely used in transformation experiments with fruit plants. In Tamarillo, addition of acetosyringone to bacterial culture and co-cultivation medium increased transformation efficiency (35%) significantly<sup>174</sup>. Likewise, transformation frequency of embryonic calli evaluated by GUS histochemical assay was increased by the addition of acetosyringone to co-culture medium in *V. vinifera* cv. Koshusanjaku<sup>81</sup>. The beneficial use of acetosyringone in co-cultivation medium towards transformation has also been reported in walnut<sup>164, 170</sup>, apple<sup>95</sup>, grape<sup>31, 49, 50, 130, 175</sup>, plum<sup>37, 162</sup>, pecan<sup>36</sup>, banana<sup>42</sup> and citrus<sup>27, 59, 65, 76</sup>. The acetosyringone concentration of 12.5 µM was found optimum in liquid suspension cultures<sup>175</sup>. In solid medium the acetosyringone concentration ranged from 20-100 µM, while the latter was routinely used in transformation experiments and was found to be more effective than lower concentration used. Naringenin, another phenolic compound has also been found effective in improving transient GUS expression in *Passiflora*<sup>176</sup>.

The use of feeder layer and filter paper during co-cultivation on the culture media had a significant effect on transformation efficiency. The use of feeder layer (tomato cell suspension) during co-cultivation increased transformation efficiency in Citrus<sup>27, 61</sup>. Likewise, in apple, using a feeder layer of cell suspension from potato or apple callus, improved transformation frequency<sup>94</sup>. Keeping explants dry during co-culture has also been found effective in improving transformation rates of sweet orange cv. Washington Navel<sup>63</sup>. Likewise, in *Passiflora*, explants placed on filter paper covering the surface of media during co-cultivation improved transformation frequency<sup>165</sup>. In *Rubus* spp., the co-cultivation on Whatman 3 filter paper, saturated with liquid MS, without growth regulators<sup>117</sup> was found to be useful. Likewise, some compounds, which promote cell division in tobacco, have been shown to induce virulence genes *Agrobacterium tumefaciens*<sup>177, 178</sup>.

**Co-cultivation microclimate:** During co-cultivation, the cultures are generally kept in the dark at a temperatures ranging from 22-28°C. Recently, it has been demonstrated that light strongly

promotes gene transfer from *Agrobacterium tumefaciens* in plant cells<sup>179</sup>. Similarly, in *Rubus* spp. red raspberry, black berry and black currant, co-cultivation at 24°C under light conditions<sup>115, 116</sup> have been reported to be beneficial for transformation. Co-cultivation of *Agrobacterium* inoculated explants at 22°C have been reported in grape cv. Rupestris St George and *V. vinifera* sp<sup>83</sup>. Incubation of explants during co-cultivation has also been done at 24°C in plum<sup>162</sup>, pecan<sup>36</sup>, red raspberry, black currant and black berry<sup>115</sup>. Likewise, incubation at 25°C in grapes<sup>50</sup>, apple<sup>94, 97, 100, 107</sup>, strawberry<sup>39</sup>, at 26°C in papaya<sup>84</sup>, at 27°C in grapes<sup>31</sup> and at 28°C in grapes<sup>49, 126, 136</sup> have been used and found optimum for gene transfer. Temperature during bacterial culture initiation and co-cultivation plays a significant role in transformation efficiency. The influence of temperature based on the basic finding<sup>180</sup> that the maximum tumour formation occurred between 24-48 h after wounding if during that time the plants were held at 25°C temperature. The loss of tumour initiating ability in *Agrobacterium tumefaciens* has also been reported<sup>181</sup> by incubation at high temperatures.

The supplementation of co-cultivation media with hormones such as auxins, cytokinins, phenolic compounds such as acetosyringone and naringenin has been found to enhance transient GUS expression. The use of naringenin needs to be explored in other fruit species as well. The use of feeder layer should be encouraged in somatic embryogenesis based transformation experiments and in particular, will be beneficial for recalcitrant fruit species. The use of filter paper allows the better aeration of explants during co-cultivation and thus helps to achieve high success rates in transformation.

**Pre-culture:** The number of days required after the co-cultivation period of 3-5 days, on shoot regeneration/basal medium prior to transfer to selection medium/post-co-cultivation and pre-selection period significantly affects gene transfer in fruit plants. Delay in initiating selection on kanamycin is necessary to allow cells to recover from infection, divide and produce larger amount of NPT II<sup>46</sup>. In almond, pre-culture period of 6 days on medium free of kanamycin (selection) was best for cv. MNS1 and of 3 or 6 days was best for cv. Supernova, while a 10 day pre-culture period was very deleterious to transformation efficiency. In apple, a pre-culture period of 0-5 days did not affect transformation efficiency in cvs. Gala and Golden Delicious<sup>14, 100</sup>. In contrast, for citrus cv. Swingle citrumelo it was found that 1 week delay between co-cultivation and selection for kanamycin resistance was necessary for the formation of putative transgenic buds<sup>70</sup>. In strawberry cv. Red Coat, a pre-culture period of up to 10 days on non-selective medium improved transformation frequency by 7%<sup>38</sup>. In *V. vinifera* and *V. rupestris* cv. 110 Richter, kanamycin was added as a selection agent after 2 months of proliferation without selection presence<sup>137</sup>. In papaya, a pre-culture for 3 weeks on callus induction medium followed by transfer of explants to kanamycin selection medium was found useful<sup>35, 85</sup>. Likewise in plum, a pre-culture in non-selective media for 6-10 days<sup>162</sup> and for 10-14 days<sup>37</sup> have been reported to be beneficial for transformation. Likewise in kiwifruit, only 2 weeks after infection, the segments were transferred to selection medium containing kanamycin<sup>29, 114</sup>.

After co-cultivation for 2-3 days, the immediate transfer of explants to the selection medium containing antibiotics is often found to be deleterious for survival of explants. This pre-culture

period allows re-establishment of explants on culture media. Thus, the gradual shifting of explants to selection media enhance the survival of explants and thereby the regeneration of putative transgenic plants from such explants.

**Antibiotics to kill *Agrobacterium*:** A range of antibiotics are used to kill *Agrobacterium* after co-cultivation with explants. This is essential, as the explants are killed/exhausted by *Agrobacterium*, if no antibiotics are used. After a co-cultivation period of 3-5 days, explants are usually washed with an antibiotic solution, blotted dry on Whatman filter paper and then incubated on selection media, with the antibiotics to kill *Agrobacterium*. The antibiotic, cefotaxime with a concentration ranging from 100-500 mg l<sup>-1</sup> has been reported for transformation experiments. The concentration of 100 mg l<sup>-1</sup> has been used effectively in peach<sup>88, 109</sup>, apple<sup>103</sup>, mango<sup>157, 159</sup>, and strawberry<sup>39</sup>. The use of 200 mg l<sup>-1</sup> to kill *Agrobacterium* has been reported in plum<sup>57</sup>, pear<sup>55, 89</sup> and grape<sup>182</sup>. In pear, it has been used along with another antibiotic ticarcillin (100 mg l<sup>-1</sup>)<sup>89</sup> and in plum along with carbenicillin (500 mg l<sup>-1</sup>), cefotaxime (250 mg l<sup>-1</sup>) along with vancomycin at same concentration have also been reported<sup>27</sup>. The use of cefotaxime alone (300 mg l<sup>-1</sup>) has been reported in Tamarillo<sup>174</sup>, Citrus cv. Mexican lime<sup>32</sup>, apple cv. Jonagold<sup>183</sup> and grape rootstock, *V. rupestris*<sup>136, 137</sup> and along with carbenicillin (200 mg l<sup>-1</sup>) in *V. vinifera*<sup>130</sup>. The use of cefotaxime alone (500 mg l<sup>-1</sup>) has also been reported in grapes<sup>31, 52, 135, 175</sup>, mango<sup>157, 158</sup>, walnut<sup>164, 170</sup>, pecan<sup>36</sup>, kiwifruit<sup>114, 119</sup> and in combination with carbenicillin at same concentration in strawberry<sup>167</sup> and in combination with vancomycin (250 mg l<sup>-1</sup>) in Mexican lime<sup>61</sup>.

The use of other antibiotics such as mefoxin (200 mg l<sup>-1</sup>) in Citrus<sup>62</sup>, carbenicillin (500 mg l<sup>-1</sup>) in grapes<sup>50, 83</sup>, papaya<sup>35, 84, 85</sup>, strawberry<sup>161</sup>, walnut<sup>163, 164</sup>, ticarcillin (150 mg l<sup>-1</sup>) in combination with combactam (50 mg l<sup>-1</sup>) in apple<sup>171</sup> and Timentin<sup>®</sup> in plum<sup>169</sup> (300 mg l<sup>-1</sup>) have been reported to be beneficial to control *Agrobacterium* during transformation experiments. The use of antibiotic augmentin<sup>166</sup> in juneberry and ticarcillin in blue berry<sup>184</sup> at 250 mg l<sup>-1</sup> concentration has been found to effectively control the *Agrobacterium* overgrowth on explants after co-cultivation.

The use of an appropriate antibiotic, which would kill/suppress the growth of bacterium after co-cultivation, is very important. The antibiotics such as cefotaxime, carbenicillin, ticarcillin, vancomycin, augmentin, mefoxin, combactam and Timentin<sup>®</sup> have commercially been used for transformation experiments. The optimum dose varies with fruit species and should be such that it inhibits *Agrobacterium* growth and allows the normal regeneration of putative transgenic tissues.

**Selection medium:** The use of kanamycin to select the presence of selectable marker gene *nptII* has been widely reported in most of the transformation experiments in fruit plants. The kanamycin concentration for selection, range from 25-300 mg l<sup>-1</sup> depending upon the fruit plant species used. The use of 25 mg l<sup>-1</sup> kanamycin for selection has been reported in strawberry cvs. Chandler<sup>111, 161</sup>, Senga Sengana and K1349<sup>57</sup>, while in cv. Tudla, 40 mg l<sup>-1</sup> kanamycin concentration was found optimum for selection<sup>113</sup>. In citrus, for selection of transgenic plants, a range of concentrations of kanamycin such as 25-50 mg l<sup>-1</sup> in Swingle citrumelo<sup>70</sup>, 50 mg l<sup>-1</sup> in *P. trifoliata*<sup>65</sup>, 80 mg l<sup>-1</sup> in Mexican lime<sup>32</sup>, 100 mg l<sup>-1</sup> in citrange<sup>27</sup>, sweet orange<sup>33, 60, 129</sup>, Mexican lime<sup>61</sup>, 100-200 mg l<sup>-1</sup> in Washington

Navel orange<sup>63</sup> and trifoliolate orange<sup>59</sup> and 300 mg l<sup>-1</sup> concentration in Troyer citrange<sup>76</sup> have been reported. In grapes, 50 mg l<sup>-1</sup> of kanamycin was used in hybrid grape cv. Georgikon 28<sup>78</sup> and in *V. vinifera* cv. Neo Muscut<sup>185</sup>. In papaya, kanamycin selection ranged from 100 mg l<sup>-1</sup> in cv. Tai-nong<sup>18</sup> to 150 mg l<sup>-1</sup> in cv. Sunrise. In mango cv. Hindi, a polyembryonic cultivar, step-wise phased selection 200-400 mg l<sup>-1</sup> in solid and 50-100 mg l<sup>-1</sup> in liquid medium, was essential for recovery of pure transformants from the chimeral masses<sup>157, 158</sup>. Likewise, kanamycin selection gradually raised from 100 to 200 mg l<sup>-1</sup> has also been reported in mango<sup>159</sup>. After 4 months on kanamycin (100 mg l<sup>-1</sup>), almost all tissue turned brown due to phenolics, while some regions were white indicating new growth. Likewise in papaya, during initial selection a 300 mg l<sup>-1</sup> concentration was used for 3 weeks, followed by 150 mg l<sup>-1</sup> concentration for the next 6 weeks<sup>86</sup>. In apple, kanamycin selection up to 100 mg l<sup>-1</sup> has been reported in cv. McIntosh Wijcik<sup>107</sup>, while 25 mg l<sup>-1</sup> was used in cv. Jonagold<sup>183</sup> and 20 mg l<sup>-1</sup> used for cv. Bramley Seedling<sup>103</sup>. In plum, kanamycin (75 mg l<sup>-1</sup>) has been reported<sup>37, 162</sup>, while in kiwifruit, kanamycin selection ranged from 50-200 mg l<sup>-1</sup><sup>29, 119</sup>. Use of kanamycin overlay (7 ml of 100 mg l<sup>-1</sup> kanamycin in water) 2 weeks after transfer to selection medium, having 100 mg l<sup>-1</sup> kanamycin, was found to improve the selection for transgenic plants in sour orange<sup>62</sup>. An extended, dark incubation period on selective medium (kanamycin 100 mg l<sup>-1</sup>) containing relatively high concentration of TDZ and NAA was successful in gene transfer in pear<sup>89</sup>. However, the use of *nptII* as a selectable marker and thereby the kanamycin selection, has been found to have deleterious effects on the morphogenesis and development of some fruit crops. *Vitis* sp. have been extremely sensitive to kanamycin<sup>50, 175</sup>. At extremely low levels, kanamycin inhibits growth of grape cultivars and also reduces the recovery of transgenic plants<sup>83</sup>. Likewise, kanamycin at higher concentrations inhibited repetitive somatic embryogenesis in walnut<sup>164</sup> and resulted in the vitrification of passion fruit shoots<sup>165</sup>. The kanamycin selection has also been found unsuitable for *Rubus* species, due to regeneration problems<sup>115, 186</sup>.

As an alternative to *nptII* gene, the *hpt* gene can be used. In red raspberry, *hpt* has been found to be more efficient than *nptII* for recovering transgenic plants. Likewise, the use of *hpt* gene for hygromycin resistance as selective marker instead of *nptII* gene has been found to increase transformation frequency up to 11.5% in pear<sup>187</sup>. Hygromycin selection has also been reported in grape cultivar 110 Richter<sup>126</sup>. Hygromycin selection protocols have also been used successfully to produce transgenic plants in banana<sup>44</sup>, pecan<sup>36</sup>, strawberry<sup>188</sup>, sweet orange<sup>72</sup> and grapefruit<sup>127</sup>. In another experiment, with citrus cv. Citrange, the exposure of explants to darkness for 4 weeks during selection increased transformation efficiency and selection of transgenic plants, while using 100 mg l<sup>-1</sup> kanamycin for selection<sup>27</sup>. In carrizo citrange, it has been reported that the extended time periods of selection improved the recovery of transformed shoots and reduced escapes<sup>33</sup>. In banana cv. Rasthali, due to the incorporation of *als* gene, herbicide Glean was used as selective agent<sup>19</sup>. Paromycin has also been used as selective agent in apple cvs. Pinova, Pirae, Pilot, Pingo, Remo, Elstar, Liberty<sup>105</sup> while in cvs. Marshall and McIntosh, kanamycin proved to be more effective than paromycin as a selective agent<sup>189</sup>. In apple cv. Royal Gala, kanamycin and chlorsulfuron have also been used as selective agents<sup>14</sup>.

The use of an alternate negative selectable agent Basta has

been reported in the transformation of 'Ponkan' mandarin. In this study, the phosphinothricin acetyltransferase gene (*bar*) was used in combination with the herbicide Basta at 50 mg l<sup>-1</sup> which resulted in a 20% transformation efficiency. Whilst the use of 'negative' selection agents such as antibiotics have dominated fruit transformation up to this point in time, a 'positive' selection method has been reported for the first time in *Citrus sinensis* L. cvs. Valencia, Hamlin, Natal and Pera Osbeck, using the phosphomannose-isomerase (PMI) gene as a selectable marker on mannose<sup>190</sup>. Depending on the cultivar, a transformation efficiency ranging between 3 and 23.8% was achieved using mannose (73 mM) as the selectable agent.

With *nptII* as the most commonly used selectable marker gene in transformation experiments, kanamycin has been widely used as a selective antibiotic to kill non-transformed cells/tissues. Kanamycin concentration to select transformed cells varies from

25-300 mg l<sup>-1</sup>. Some fruit crops such as *Vitis* spp., *Rubus* spp., walnut and passion fruit are sensitive to kanamycin or have regeneration problems. Hygromycin selection (*hpt* gene) has been used in such fruit crops and should be further explored in other fruit crops.

#### Transgenics Produced with Horticultural Important Traits

Transgenic fruit plants with horticulturally important traits (Table 4) such as for disease resistance, insect pest resistance, herbicide tolerance, cold tolerance, salt tolerance and with improved plant and fruit characteristics have been produced.

**Table 4.** Transgenic fruit plants with improved horticultural traits developed via *Agrobacterium*-mediated gene transformation.

Horticultural trait	Gene used	Fruit crop	Reference(s)
<b>Virus resistance</b>			
Arabis mosaic virus (ArMV)	Coat protein (cp) gene	Grape	Golles et al., 2000; Spielmann et al., 2000.
Citrus tristeza virus (CTV)	Coat protein (cp) gene	Citrus	Moore et al., 1993; Han et al., 1999; Dominguez et al., 2000; Ghorbel et al., 2000; Moore et al., 2000; Piestun et al., 2000.
	Coat protein (cp) and Galanthus nivalis agglutinin ( <i>gna</i> ) gene	Citrus	Yang et al., 2000
Grapevine leaf roll virus (GLRV)	GLRa V-3 gene	Grape	Xue et al., 1999
	GLRa V-2 and GLRa V-3 gene	Grape	Krastanova et al., 2000
Grapevine virus A (GVA)	Coat protein (cp) gene	Grape	Golles et al., 2000; Martinelli et al., 2000.
Grapevine virus B (GVB)	Movement protein (mp) gene	Grape	Golles et al., 2000; Martinelli et al., 2000.
Grapevine fan leaf virus (GFLV)	Coat protein (cp) gene	Grape	Krastanova et al., 1995, 2000; Xue et al., 1999; Golles et al., 2000; Gutoranov et al., 2001; Gribaudo et al., 2003; Li et al., 2003
Grapevine chrome mosaic virus	Coat protein (cp) gene	Grape	Gall et al., 1994.
Papaya ring spot virus (PRSV)	Coat protein (cp) gene	Papaya	Cheng et al., 1996; Yeh et al., 1998..
	PRSV replicase (rp) gene	Papaya	Chen et al., 2001.
	PRSV TK (cp) gene	Papaya	Bau et al., 2003
Plum pox virus (PPV)	Coat protein (cp) gene	Apricot	Machado et al., 1992, 1994
	Coat protein (cp) gene	Plum	Machado et al., 1994, 1999; Ravelonandro et al., 1998, 2000
<b>Bacterial resistance</b>			
Bacterial resistance	<i>Shiva-1</i> (Insect cecropin)	Apple	Yao et al., 1995
	Antibacterial AP-D (synthetic antibacterial peptide D) gene	Citrus	Zheng et al., 1999
Crown gall resistance	dsRNA from <i>iaaM</i> and <i>ipt</i> sequences	Apple	Viss et al., 2003
Black heart resistance	PPO (poly phenol oxidase gene)	Pineapple	Graham et al., 2000
Fire blight	<i>Attacin E</i> gene	Apple	Norelli et al., 1994, 1996; Janik et al., 1996; Aldwinckle et al., 1999; Ko et al., 1999; Borejsza et al., 1999; Hanke et al., 2000; Norelli et al., 1999 2000; Ko et al., 2002
	<i>Lysozyme</i> gene	Apple	Hanke et al., 2000
	<i>Attacin E</i> gene	Pear	Reynoird et al., 1999; Chevreau et al., 2000.
	Table 1 contd. SB-37, <i>Shiva-1</i> (cecropin and its analogues)	Pear	Chevreau et al., 1999

**Table 4.** Continued

Horticultural trait	Gene used	Fruit crop	Reference(s)
Fire blight	SB-37 gene	Pear	Reynoird et al., 1999.
	ϕEa1-depolymerase gene	Apple	Hanke et al., 2002
	SB-37 gene	Apple	Aldwinckle et al., 1999; Norelli et al., 1999.
	T4 lysozyme gene	Apple	Hanke et al., 1999; Aldwinckle et al., 1999; Norelli et al., 1999; Ko et al., 2002
	<i>hrpN</i> (harpin) gene	Apple	Abdul-Kader et al., 1999
	Cecropin MB 39 gene (modified SB-37 gene). <i>Shiva-1</i>	Apple	Liu et al., 1999, 2001. Norelli et al., 1999
Fungal resistance			
Apple scab	Chitinase gene	Apple	Mehlenbacher, 1995; Hanke et al., 2000.
	<i>Ech 42</i> (endochitinase) and <i>Nagl</i> (exochitinase) genes	Apple	Norelli et al., 2000
	<i>ThEn-42</i> (endochitinase) gene	Apple	Bolar et al., 1999, 2000.
	AMP (antimicrobial peptide) gene	Apple	Cubber et al., 2000
Fungal/ microbial resistance	PD (plant defensin) gene-cysteine rich antimicrobial peptide	Apple, Pear	Dolgov et al., 1999
Fungal resistance	Stilbene sysnthase ( <i>Vst1</i> ), PGIP genes.	Apple	Szankowski et al., 2003
Powdery mildew and Anthracnose	RCC 2 (rice chitinase gene)	Grape	Yamamoto et al., 2000
Insect-pest resistance			
Insect resistance (BT)	<i>Cry 1A(c)</i> gene	Walnut	Mc Granahan et al., 1988; Dandekar et al., 1989, 1994.
	<i>Cry 1A(c)</i> and ICP gene	Apple	Dandekar et al., 1991
	<i>btk</i> -ICP gene (B. thuringiensis var kurstak gene)	Juneberry	Hajela et al., 1993
Herbicide tolerance			
Triasulfuron and metsulfuron-methyl	<i>Als</i> (acetolactate synthase) gene	Pepino	Atkinson and Gardner, 1993
	<i>Als</i> gene	Tamarillo	Atkinson and Gardner, 1993
	<i>Als</i> gene	Apple	Yao et al., 1995
Glyphosate	CP4.EPSP	Strawberry	Morgan et al., 2002
Basta (glufosinate reistance)	<i>Bar</i> gene	Citrus	Piestun et al., 2000; Li et al., 2002
Stress tolerance			
Cold/ freezing tolerance	Antifreeze gene	Grape	Tsvetkov et al., 2000; Gutoranov et al., 2001
	CBF1 gene	Strawberry	Owens et al., 2002
Salinity tolerance	HAL 2 (halotolerance) gene	Citrus	Cervera et al., 2000
	<i>codA</i> gene & cDNA S6PDH	Persimmon	Gao et al., 2000, 2001
Ethylene control	ACC (1-aminocyclopropane-1-carboxylate) oxidase gene	Apple	Yao et al., 1995
	Antisense gene for polygalacturonase and ACC synthase	Grape	Mehlenbacher, 1995
	ACC oxidase gene	Grape	Ayub et al., 1998
	SA Mase gene (S-adenosyl methionine hydrolase)	Red raspberry	Mathews et al., 1995
	ACS (1-carboxylic acid synthase) gene	Apple	Hrazdina et al., 2000
	Antisense CS-ACSI (chilling inducible ACC synthase) gene	Citrus	Wong et al., 2001
	ACC oxidase gene ( <i>CMe-Aco1</i> )	Passion Fruit	Quoirin et al., 2004
Flavour improvement	Thaumatococin II gene	Apple, Pear	Dolgov et al., 1999; Lebedev et al., 2002
Reduced polyphenol oxidase activity/ browning	Antisense PPO (polyphenol oxidase) gene	Apple	Murata et al., 2001

**Table 4.** Continued

Horticultural trait	Gene used	Fruit crop	Reference(s)
Plant structure			
Dwarfing, improved rooting	<i>rol ABC genes</i>	Kiwifruit	Rugini et al., 1991
	<i>rol genes</i>	Apple	Lambert and Tepfer, 1992
	<i>rol genes</i>	Grape	Nakano et al., 1994
	<i>rol A gene</i>	Apple	Holefors et al., 1998
	<i>rol B gene</i>	Apple	Welander et al., 1998, 1999; Sedira et al., 2001; Zhu et al., 2001.
	<i>rol C gene</i>	Pear	Bell et al., 1999
	<i>rol A, rol ABC, Oat phy A and Arabidopsis phy B gene.</i>	Apple	Holefors et al., 1999
	<i>Arabidopsis phy B gene.</i>	Apple	Holefors et al., 2000
Parthenocarpic fruit	<i>rol A, rol B, rol C gene</i>	Citrus	Gentile et al., 1999
Parthenocarpic fruit	<i>DefH9-iaaM gene</i>	Grape	Mezzetti et al., 2002
Seedlessness	<i>SF4</i>	Grape	Colova-Tsolova et al., 2003
Induce male sterility (Parthenocarpic fruit)	<i>Chimeric ribonuclease gene (barnase)</i>	Citrus	Li et al., 2002

### Conclusions and Future Research

Genetic engineering through recombinant-DNA technology have made it possible to improve an otherwise commercial cultivars lacking one or two characteristics, such as fruit colour, by incorporation of specific gene controlling that character. This is hard to achieve by conventional breeding, which involves random mixing of desirable and undesirable genes in the obtained crosses. *Agrobacterium*-mediated gene transfer and biolistics are two commonly used approaches to obtain transgenic plants. However, the *Agrobacterium*-mediated DNA transfer being simple<sup>191</sup> and less expensive technique is the most common and widely used technique for transformation of dicotyledonous plants<sup>192</sup>. Probably the greatest advantage of the system is that it offers the potential to generate transgenic cells at relatively high frequency, without significant reduction in plant regeneration rates. Moreover, the DNA transferred to the plant genome is defined, it does not normally undergo any major rearrangements and it integrates into the genome as a single copy<sup>28</sup>. With the recent understanding of mechanisms involved in *Agrobacterium*-mediated gene transfer and the importance of virulence gene induction, the monocotyledonous plants have also been transformed successfully.

*Agrobacterium*-mediated transformation has resulted in the production of transgenics with improved horticultural traits in a wide range of fruit and nut crops. Virus resistant plants have been produced by the introduction of coat protein, movement protein or replicase genes in apricot, citrus, grape, papaya and plum. While, the introduction of *shiva-1*, *attacin-E*, lysozyme gene, SB-37 gene, MB-39 gene and *hrpN* gene have lead to production of fireblight resistant plants in apple and pear. The introduction of chitinase (endo/exo) genes, on the other hand, have lead to the production of transgenic in apple, pear and grape resistant against fungal diseases such as apple scab, powdery mildew and anthracnose. The *bt* endotoxin *cry Ia(c)* and insecticidal crystal protein (ICP) genes have lead to production of transgenic against insect pest resistance in apple, junberry and walnut. The introduction of *als* gene in apple, pepino, tamarillo and bar gene in citrus, have lead to herbicide tolerance. Likewise, antifreeze gene for cold tolerance in grape; halotolerance (HAL-2) gene for salinity tolerance in citrus; thaumatin II gene for flavour improvement in apple and pear; ACC synthase, ACC oxidase, SA-Mase genes to delay

ripening in apple, citrus, grape and red raspberry have been introduced. Moreover, to induce dwarfing and to enhance rooting ability *rolA*, *rolB* and *rolC* genes have been introduced in apple, citrus, grape, pear and kiwifruit.

However, in most of the fruit and nut crops, the frequency of transformation is quite low, therefore hindering the routine production of transgenics in such crops. It is therefore important to understand the various factors involved in *Agrobacterium*-mediated genetic transformation which include plant genotype, explant, method of transformation, *Agrobacterium* strain, gene construct, infection and co-cultivation conditions and antibiotics both to kill *Agrobacterium* after infection and for selection of putative transgenic plants. As discussed earlier in the chapter, each and every factor needs to be optimised for routine production of transgenics in fruit and nut crops.

The safety concerns need priority, however, before actual release of transgenics for routine production. The speculations about risk of transgenics are not always true. The transgenic risk is low<sup>193</sup> and can be further reduced if proper bio-safety regulations are followed prior to release of transgenics<sup>194</sup> on commercial scale. Thus, the genetic engineering via *Agrobacterium*-mediated transformation has great potential in upgrading the genetic potential of fruit and nut crops. However, the new techniques of biotechnology must be considered as an important supplement to the existing technologies and in no way a substitute to conventional fruit breeding.

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