

Agroinfiltration is a key factor to improve the efficiency of apple and pear transformation

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1 Title

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17 Abstract

18 Genetic transformation of apple and pear is mainly performed via Agrobacterium tumefaciens. The average efficiency of transformation remains low in most laboratories and the success of the 19 20 transformation experiments is very variable. Improving the efficiency and the reproducibility of apple and pear transformation is thus highly desirable. Adventitious regeneration ability of the 21 22 explants is not a limiting factor for apple or pear transformation. The present study focuses on 23 improving the frequency of stably transformed cells in the explants following A. tumefaciens inoculation. We report here the results of 36 independent transformation experiments on 'Gala' 24 apple, performed with 10 different binary plasmids, comparing three methods of A. tumefaciens 25 inoculation. Agroinfiltration (Agrobacterium-mediated vacuum infiltration) of the explants in a 26

bacterial suspension containing a surfactant (Silwet L-77) at a low concentration (0.002% v/v) 27 significantly increased the average transformation efficiency (mean rate of 5.8 % and maximum rate 28 of 30%) and reduced the number of failed experiments (16%) compared to the method of crushing 29 30 the explants with non-traumatic forceps prior to immersion into the inoculum. The success of the agroinfiltration method was associated with a very high level of GUS expression one month after 31 inoculation. In addition, agroinfiltration dramatically increased the transformation efficiency of pear, 32 reaching rates of transformation between 50 and 80%, compared to inoculation with a scalpel 33 34 dipped into the inoculum. Altogether, our results demonstrate that the production of large number of transgenic apple or pear lines in a short period of time is feasible using the agroinfiltration method. 35

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37 Keywords

38 Apple ;pear ; transformation ; agroinfiltration ; surfactant

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40 **1. Introduction**

41 Apple (Malus domestica Borkh) and European pear (Pyrus communis L.) are two closely related species belonging to the subtribe Malinae (Potter et al., 2007). Recent analysis of the 42 European pear genome (Chagné et al., 2014) confirmed its similarity to apple. Because of its high 43 commercial importance, apple is one of the first woody plant species which was successfully 44 transformed via Agrobacterium in 1989 (James et al. 1989). Despite this early success and the high 45 number of apple genotypes amenable to genetic transformation, the rate of transformation of apple 46 has remained low. Most published data indicate a rate of transformation below 2% on a per explant 47 basis and transformation rates above 10% are exceptional (Malnoy et al. 2008a). Furthermore, the 48 repeatability of apple transformation experiments is low and it is still difficult for most laboratories 49 to produce large number of transgenic lines in a short period of time. With the recent production of 50 a high-quality de novo assembly of the apple genome (Daccord et al 2017), analysis of gene 51 function has become a major research topic in apple. Thus, improving the efficiency of apple 52

transformation rates is a key issue. The first successful gene transfer in European pear was in 1996 (Mourgues et al. 1996). The transformation rates of pear are generally low (1 - 4%) with a few exceptions reaching 40-50% (Malnoy et al 2008b). Currently, the number of clonal genotypes amenable to genetic transformation is much lower in *Pyrus* (< 20) than in *Malus* (> 50). However this field of research is very active in pear and the need for high transformation rates is important.

Most of the biotechnology protocols that have been first developed on apple were later 58 adapted to pear with success. For both species, the efficiency of Agrobacterium-mediated 59 60 transformation is strongly linked to the regeneration ability, which is highly genotype dependent. In apple as well as in pear, adventitious regeneration from young leaf explants is very efficient and 61 62 several genotypes reach rates of regeneration of 100% with a high number of buds per regenerating leaves in absence of transformation (Yepes and Aldwinckle 1994, Chevreau et al. 1989). The 63 second key parameter for the recovery of transgenic plants is the frequency of stably transformed 64 cells in the explant following Agrobacterium tumefaciens inoculation. Despite a high natural 65 susceptibility of apple and pear to wild A. tumefaciens strains in orchards, stable T-DNA insertion 66 following A. tumefaciens inoculation is a rate-limiting factor for the production of apple and pear 67 transgenic plants. The first inoculation method used for apple transformation was immersion of pre-68 wounded leaves in the inoculum for a few minutes (Malnoy et al 2008a). Crushing the leaf blade 69 with non-traumatic forceps prior to immersion into the inoculum increased the efficiency of apple 70 transformation (Norelli et al. 1996), probably by increasing the number of infection sites for 71 Agrobacterium in the leaves. For pear transformation, the main inoculation procedure is leaf 72 73 wounding with a scalpel dipped into the inoculum (Malnoy et al. 2008b).

Agrobacterium-mediated vacuum infiltration, also called agroinfiltration, is a standard method for transient expression of foreign genes in plant tissues. It has been used as a simple and rapid method for assaying gene function in a variety of plant species such as tobacco, grapevine, potato, rose, rice and poplar (Palanichelvam et al. 2000, Santos-Rosa et al. 2008, Bashkar et al. 2009, Yasmin and Debener 2010, Andrieu et al. 2012, Takata and Erikson 2012). In this case, non-

integrated copies of T-DNA present in the nucleus of plant cells permit to reach a level of 79 expression 1000 fold higher than in stable transformants (Jansen and Gardner 1989). By applying a 80 selection pressure after the period of co-culture, agroinfiltration has also be used for the production 81 82 of stable transgenic lines of recalcitrant plants species such as cotton, grapevine or common bean (Haq 2004, Zottini et al. 2008, Mukeshimana et al. 2013). In the present report, we took advantage 83 of the capacity of agroinfiltration to enhance gene delivery to increase both the efficiency and the 84 85 regularity of transformation of apple and pear and we propose an optimized protocol based on agroinfiltration of leaf explants. 86

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88 2. Material and Methods

89 **2.1 Biological material**

The experiments were performed on two genotypes: the apple 'Gala' and the pear 90 'Conference'. Both genotypes have a very high adventitious regeneration potential. In vitro 91 proliferating shoot cultures of the apple 'Gala' were micropropagated on Murashige and Skoog 92 (MS) (1962) medium supplemented with 2.22 µM 6-benzyladenine (BA) and 0.5µM 3-93 indolebutyric acid (IBA). Cultures of the pear 'Conference' were micropropagated as described by 94 Leblay et al. (1991) on a derivative of Lepoivre's medium supplemented with 2.22 µM 6-BA and 95 0.5 µM IBA. All cultures were grown at 22–24° C with a 16:8 h light:dark photoperiod (cool white 96 fluorescent tubes, 40–60 μ mol m⁻² s⁻¹) and transferred to fresh medium every 4 weeks. The 97 youngest leaves of 4-week-old in vitro shoots were used for A. tumefaciens inoculation. The 98 bacterial strain used in all transformation experiments was the A. tumefaciens EHA105 containing 99 the ternary plasmid pBBR1MCS-5 with a constitutive virG gene (van der Fits et al. 2000). The 100 101 binary plasmids used in the different transformation experiments are described in Supplementary Table 1. 102

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104 2.2 Transformation methods

For apple transformation, the bacteria were re-suspended in liquid apple regeneration medium 105 consisting of MS medium containing 22.7 µM thidiazuron (TDZ), 2.15 µM IBA and 100 µM 106 acetosyringone. Two methods of inoculation were tested: "Crushing" consisted in crushing the leaf 107 blade with non-traumatic forceps prior to immersion into the inoculum at 10⁸ bacteria/ml (Fig. 1b); 108 "Agroinfiltration" consisted in vacuum-infiltrating the leaves in an inoculum at 10⁸ bacteria/ml 109 containing or not a wetting agent (Silwet L-77® (Lehle Seeds, USA) at 0.002 % v/v) under -0.9 bar 110 111 during one minute. After inoculation, the leaves were wounded transversely with a scalpel and 112 plated adaxial side down on co-cultivation medium for two days in the dark at 22-24 °C. The cocultivation medium was apple regeneration medium containing 100 µM acetosyringone. 113

114 For pear transformation, the bacteria were re-suspended in liquid pear micropropagation medium (described above) with 100 µM acetosyringone. Two methods of inoculation were tested: 115 "Scalpel" consisted in wounding with a scalpel dipped into an inoculum at 10^8 bacteria/ml : 116 "Agroinfiltration" consisted in vacuum-infiltrating the leaves in an inoculum at 10⁸ bacteria/ml 117 without wetting agent. After inoculation, the leaves were wounded transversely with a scalpel in 118 case of "Agroinfiltration" and plated abaxial side down on co-cultivation medium for two days in 119 the dark at 22-24 °C. The co-cultivation medium was pear regeneration medium (Mourgues et al. 120 1996) containing 9 µMl TDZ, 2.68 µM naphthalene acetic acid (NAA) and 100 µM acetosyringone. 121

For apple and pear, at the end of the co-culture, the leaves were plated (adaxial side down for 122 apple and abaxial side down for pear) on their respective regeneration medium containing 300 mg/l 123 cefotaxime, 150 mg/l timentin and 100 mg/l kanamycin. The explants were kept in the dark and 124 transferred to fresh medium every month for six months. When the inoculated A. tumefaciens strain 125 contained a GUS marker gene, histochemical GUS test was performed one month after inoculation. 126 Samples were incubated overnight at 37°C, in a solution containing 3 mM 5-bromo-4-chloro-3-127 indolyl-b-D-glucuronic acid, 4 mM potassium ferricyanide, 0.05 mM potassium ferrocyanide, 10 128 mM EDTA and phosphate buffer (0.02 M, pH 7.2). Clearing was achieved using 70% (v/v) ethanol. 129 Appearance of adventitious buds was monitored for a period of six months. All regenerated buds 130

were micropropagated on the same medium as their mother plants, with the addition of 300 mg/l 131 cefotaxime, 150 mg/l timentin and 100 mg/l kanamycin. Because all the regenerated buds appeared 132 on the wounds, it was possible to isolate very clearly independent regeneration events occurring on 133 134 separate wounds. If multiple buds arose from the same wound, only one shoot was cloned and counted as a transformation event, to avoid overestimation of the transformation rate. Presence of 135 136 transgenes and absence of contaminating agrobacteria were monitored by PCR. After PCR analysis of the lines which survived on the kanamycin containing medium, the final transformation rate was 137 estimated as the number of PCR positive lines per 100 leaves. 138

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140 **2.3 DNA extraction and PCR**

Presence of transgenes and absence of contaminating agrobacteria were monitored by PCR. 141 Genomic DNA of apple and pear leaves was extracted as described in Fulton et al. (1995). Here are 142 the primers for the detection of Α. tumefaciens 143 used (i) presence (GTAAGAAGCGAACGCAGGGAACT and GACAATGACTGTTCTACGCGTAA on 23S 144 ribosomal RNA coding gene CP014260.1 gene locus_tag="AWN88_17620" 1310643..1313449), 145 (ii) nptII gene (ATCGGGAGCGGCGATACCGTA and GAGGCTATTCGGCTATGACTG on 146 *nptII* gene of plasmid pK7WG2D from Karimi et al. (2002)) and (iii) elongation factor 1α (*EF1* α) 147 coding gene as a marker of plant DNA suitability for PCR (CTCTTGGTGTCAGGCAAATG and 148 TCAAGGTTGGTGGACCTCTC on AJ223969). Amplifications were performed using GoTaq® 149 Flexi DNA Polymerase (Promega, Madison, WI, USA) according to the manufacturer's 150 recommendations. The PCR reaction conditions were identical for the three genes except the 151 hybridization step which was at 55°C and not 58°C for A. tumefaciens detection primers: 95°C for 5 152 min, followed by 35 cycles at 95°C for 30 s, 58°C for 45 s, 72°C for 1 min, with a final extension at 153 72°C for 5 min. The PCR products were separated on a 1.5 % agarose gel. A typical result of this 154 detection method applied during transformation experiment 249 (Supplementary Table 2) is given 155 in Supplementary Fig. 1. 156

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158 **2.4 Statistical analysis**

159 Statistical analyses were performed with XLSTAT by using the nonparametric Wilcoxon test 160 (p < 0.05) for apple data and a Khi² independence test ($\alpha = 0.05$) for pear data.

- 161
- 162 **3. Results**

We report here the synthesis of 36 independent transformation experiments on 'Gala' apple, 163 performed with 10 different binary plasmids on a total of 12,971 explants. Results of each 164 inoculation method were recorded on 12 independent experiments performed with 4 to 5 different 165 166 binary plasmids. The rate of failure (experiments producing none transgenic lines) was 50% for "Crushing", 33.3% for "Agroinfiltration without Silwet" and 16.7% for "Agroinfiltration with 167 Silwet". PCR analyses indicated that 90% of the kanamycin resistant lines contained a complete T-168 DNA and no Agrobacterium (Supplementary Table 2 and Fig. 1). They were counted as transgenic 169 lines. Despite the great variability of transformation rates during these experiments, box-plots in 170 171 Fig. 1a indicate a slight increase of transformation rate (non-significant using Wilcoxon test) with "Agroinfiltration without Silwet" compared to "Crushing". "Agroinfiltration with Silwet" was 172 significantly more efficient than "Crushing" (Wilcoxon test, p = 0.049), with a mean rate of 5.8 % 173 and a maximum rate of 30.2%. No correlation between the rate of transformation and the plasmid 174 used was observed. The success of this method can be linked to a very high level of GUS 175 expression one month after inoculation (Fig. 1 d and e) compared to the "Crushing" method (Fig. 176 1c). 177

The protocol described here for 'Gala' apple was successfully tested in one experiment for the transformation of other apple genotypes: 'Ariane', 'Golden Delicious', 'Greensleeves' and 'M26' with transformation rates between 1 and 9% (Table 1).





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Fig. 1 Main transformation results on apple. a Transformation rate (%) of 'Gala' apple according 183 to the method of A. tumefaciens inoculation: "Crushing", "Agroinfiltration without Silwet" and 184 "Agroinfiltration with Silwet". Each box-plot corresponds to 12 independent experiments 185 performed on 150 to 850 explants. +: mean, •: maximum, ×: outlier. b Numerous wounds visible on 186 'Gala' apple leaf just after crushing with non-traumatic forceps. c Low efficiency of GUS 187 histochemical test performed on 'Gala' apple leaf one month after "Crushing" inoculation. d, e 188 High efficiency of GUS histochemical test performed on 'Gala' apple leaf one month after 189 "Agroinfiltration with Silwet" inoculation: adaxial side (d) and abaxial side (e). 190

1	19	1	

	192	Table 1Transformatio	n rates of several	apple varieties af	ter agroinfiltration	with Silwet
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Construns	# leaves	# regenerated	rate of	# transgenic	rate of
Genotype	inoculated	buds	regeneration	lines	transformation
M26	140	23	16.4 %	12	8.6 %
Greensleeves	141	8	5.7 %	3	2.1 %
Golden Delicious	245	18	7.3 %	4	1.6 %
Ariane	329	5	1.5 %	3	0.9 %

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In our hands, 'Conference' pear presents a very high ability for adventitious bud regeneration (Fig. 2a) and susceptibility to kanamycin selection (Fig. 2b). This genotype is amenable to transformation with rates of success between 2 and 10% (Fig. 2c and d). Here we tested the "Agroinfiltration" method without Silwet compared to the standard "Scalpel" method, in three independent experiments performed with the same binary plasmid (392p9N35s-Mr5FB1orf). PCR analyses indicated that 93% of the kanamycin resistant lines analyzed contained a complete T-DNA and no

200 *Agrobacterium* (Supplementary Table 3). They were counted as transgenic lines. Results in Fig. 2d 201 indicate that "Agroinfiltration" was significantly more efficient than "Scalpel" in the three 202 experiments (Khi² test, p < 0.0001) with rates of transformation between 50 and 80%.





Fig. 2 Main transformation results on pear. a High efficiency of adventitious bud regeneration
on 'Conference' pear without *Agrobacterium* inoculation. b Absence of bud regeneration on
'Conference' pear without *Agrobacterium* inoculation, under kanamycin selection (100 mg/l). c
Transgenic 'Conference' adventitious bud regeneration after two months of kanamycin selection. d
Transformation rate (%) of 'Conference' pear according to the method of *A. tumefaciens*inoculation: "Scalpel", "Agroinfiltration without Silwet". Each bar is the result of one experiment
on 105 to 180 explants.

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213 4. Discussion

Agroinfiltration is frequently used as an efficient method for transient transformation assays 214 because the high density of agrobacteria forced into the intercellular spaces increases the efficiency 215 of T-DNA delivery. Here we demonstrated that this method also improved the stable integration of 216 T-DNA into apple and pear cells. Penetration of a bacterial solution by a simple syringe infiltration 217 is not efficient in apple or pear leaves. It is necessary to use a low-pressure vacuum to efficiently 218 219 force the bacterial suspension into the apple or pear leaves. In addition we demonstrated that, for apple, the addition of a surfactant at a low concentration (0.002%) boosts the transformation 220 efficiency. Previous studies have already shown that the addition of a surfactant in the inoculation 221

medium increases the transformation efficiency in rose (Yasmin and Debener 2010) or 222 poplar (Takata and Erikson 2012). But in some cases, the addition of a surfactant at high 223 concentrations can also be toxic to plant tissues (Wu et al. 2003). In our study, higher 224 concentrations of Silwet (0.005% and 0.01%) caused severe necrosis of apple leaves in a 225 preliminary regeneration experiment without agroinfiltration. Finally, we also showed that 226 agroinfiltration of pear leaves led to very high rates of transformation, even in the absence of 227 surfactant. Positive results were obtained by Habashi et al (2012) using agroinfiltration on the pear 228 229 cultivars 'Bartlett' and 'Harrow Delight' with much lower transformation efficiencies (7 to 12%).In conclusion, the protocols of transformation described here are efficient for 'Gala' apple and 230 231 'Conference' pear. They permit to reduce the frequency of failed experiments and to increase the maximum rate of transformation of these genotypes. They should be efficient for other genotypes 232 after minor optimization of some parameters such as hormonal concentrations. 233

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324 Author Contribution Statement

E.C. and E.V. were the main investigators in this study. E.C. designed the study, performed part of the experiments, analyzed the data and drafted the manuscript; E.V. performed part of the experiments, analyzed the data and revised the manuscript; N.D, C.J., A.R. and A.C. performed part of the experiments and revised the manuscript. All authors read and approved the final manuscript.

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330 Disclosure of potential conflicts of interest

331 The authors declare that they have no conflict of interest.