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1 **Title**

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

3

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16

17 **Abstract**

18 Genetic transformation of apple and pear is mainly performed *via Agrobacterium tumefaciens*. The
19 average efficiency of transformation remains low in most laboratories and the success of the
20 transformation experiments is very variable. Improving the efficiency and the reproducibility of
21 apple and pear transformation is thus highly desirable. Adventitious regeneration ability of the
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*
24 inoculation. We report here the results of 36 independent transformation experiments on 'Gala'
25 apple, performed with 10 different binary plasmids, comparing three methods of *A. tumefaciens*
26 inoculation. Agroinfiltration (*Agrobacterium*-mediated vacuum infiltration) of the explants in a

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

36

37 **Keywords**

38 Apple ;pear ; transformation ; agroinfiltration ; surfactant

39

40 **1. Introduction**

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

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

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

74 *Agrobacterium*-mediated vacuum infiltration, also called agroinfiltration, is a standard
75 method for transient expression of foreign genes in plant tissues. It has been used as a simple and
76 rapid method for assaying gene function in a variety of plant species such as tobacco, grapevine,
77 potato, rose, rice and poplar (Palanichelvam et al. 2000, Santos-Rosa et al. 2008, Bashkar et al.
78 2009, Yasmin and Debener 2010, Andrieu et al. 2012, Takata and Erikson 2012). In this case, non-

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

87

88 **2. Material and Methods**

89 **2.1 Biological material**

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

103

104 **2.2 Transformation methods**

105 For apple transformation, the bacteria were re-suspended in liquid apple regeneration medium
106 consisting of MS medium containing 22.7 μ M thidiazuron (TDZ), 2.15 μ M IBA and 100 μ M
107 acetosyringone. Two methods of inoculation were tested: “Crushing” consisted in crushing the leaf
108 blade with non-traumatic forceps prior to immersion into the inoculum at 10^8 bacteria/ml (Fig. 1b);
109 “Agroinfiltration” consisted in vacuum-infiltrating the leaves in an inoculum at 10^8 bacteria/ml
110 containing or not a wetting agent (Silwet L-77® (Lehle Seeds, USA) at 0.002 % v/v) under -0.9 bar
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 co-
113 cultivation medium was apple regeneration medium containing 100 μ M acetosyringone.

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

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

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

139

140 **2.3 DNA extraction and PCR**

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

157

158 **2.4 Statistical analysis**

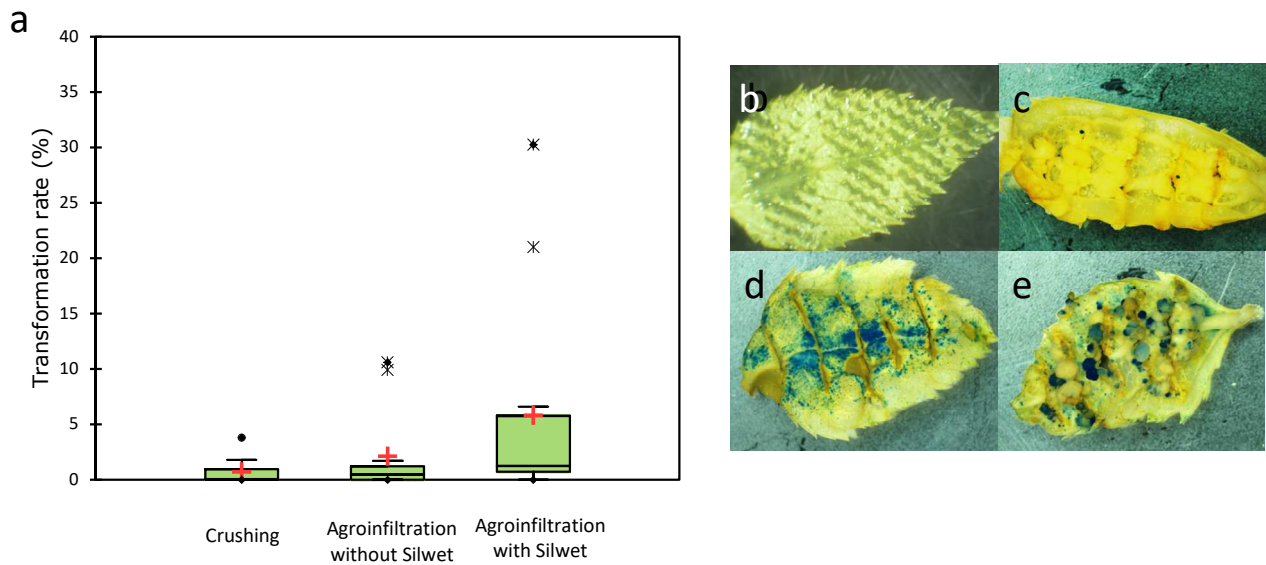
159 Statistical analyses were performed with XLSTAT by using the nonparametric Wilcoxon test
160 ($p < 0.05$) for apple data and a χ^2 independence test ($\alpha = 0.05$) for pear data.

161

162 **3. Results**

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

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



182

183 **Fig. 1 Main transformation results on apple.** **a** Transformation rate (%) of ‘Gala’ apple according 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
 191

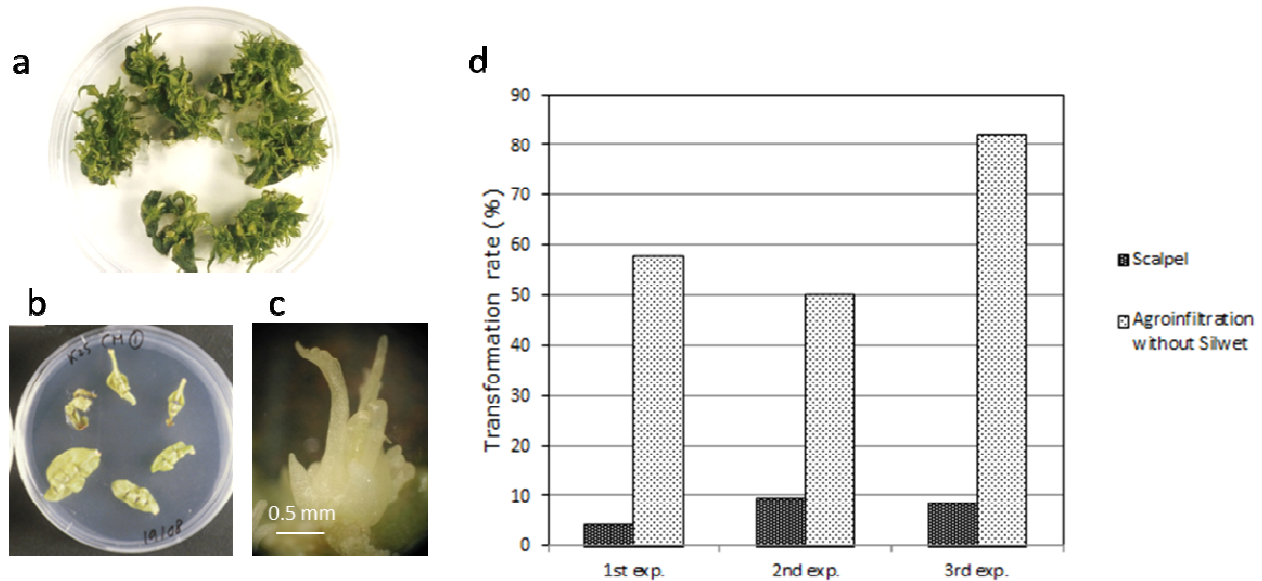
192 **Table 1** Transformation rates of several apple varieties after agroinfiltration with Silwet

Genotype	# leaves inoculated	# regenerated buds	rate of regeneration	# transgenic lines	rate of 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 %

193

194 In our hands, ‘Conference’ pear presents a very high ability for adventitious bud regeneration (Fig.
 195 2a) and susceptibility to kanamycin selection (Fig. 2b). This genotype is amenable to transformation
 196 with rates of success between 2 and 10% (Fig. 2c and d). Here we tested the “Agroinfiltration”
 197 method without Silwet compared to the standard “Scalpel” method, in three independent
 198 experiments performed with the same binary plasmid (392p9N35s-Mr5FB1orf). PCR analyses
 199 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 (Khi^2 test, $p < 0.0001$) with rates of transformation between 50 and 80%.



203

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

211

212

213 4. Discussion

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

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

234

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239

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322

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324 **Author Contribution Statement**

325 E.C. and E.V. were the main investigators in this study. E.C. designed the study, performed part of
326 the experiments, analyzed the data and drafted the manuscript; E.V. performed part of the
327 experiments, analyzed the data and revised the manuscript; N.D, C.J., A.R. and A.C. performed part
328 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.