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Abstract

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 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 explants is not a limiting factor for apple or pear transformation. The present study focuses on 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' apple, performed with 10 different binary plasmids, comparing three methods of *A. tumefaciens* inoculation. Agroinfiltration (*Agrobacterium*-mediated vacuum infiltration) of the explants in a

bacterial suspension containing a surfactant (Silwet L-77) at a low concentration (0.002% v/v) significantly increased the average transformation efficiency (mean rate of 5.8 % and maximum rate of 30%) and reduced the number of failed experiments (16%) compared to the method of crushing 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 inoculation.In addition, agroinfiltration dramatically increased the transformation efficiency of pear, reaching rates of transformation between 50 and 80%, compared to inoculation with a scalpel 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.

Keywords

Apple ;pear ; transformation ; agroinfiltration ; surfactant

1. Introduction

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 European pear genome (Chagné *et al*., 2014) confirmed its similarity to apple. Because of its high commercial importance, apple is one of the first woody plant species which was successfully transformed via *Agrobacterium* in 1989 (James et al. 1989). Despite this early success and the high number of apple genotypes amenable to genetic transformation, the rate of transformation of apple has remained low. Most published data indicate a rate of transformation below 2% on a per explant basis and transformation rates above 10% are exceptional (Malnoy et al. 2008a). Furthermore, the repeatability of apple transformation experiments is low and it is still difficult for most laboratories to produce large number of transgenic lines in a short period of time. With the recent production of a high-quality de novo assembly of the apple genome (Daccord et al 2017), analysis of gene 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-

integrated copies of T-DNA present in the nucleus of plant cells permit to reach a level of expression 1000 fold higher than in stable transformants (Jansen and Gardner 1989). By applying a selection pressure after the period of co-culture, agroinfiltration has also be used for the production 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 of the capacity of agroinfiltration to enhance gene delivery to increase both the efficiency and the regularity of transformation of apple and pear and we propose an optimized protocol based on agroinfiltration of leaf explants.

2. Material and Methods

2.1 Biological material

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

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 µM thidiazuron (TDZ), 2.15 µM IBA and 100 µM
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 µM acetosyringone.
114 For pear transformation, the bacteria were re-suspended in liquid pear micropropagation
115 medium (described above) with 100 µM 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 µMl TDZ, 2.68 µM naphthalene acetic acid (NAA) and 100 µM 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

were micropropagated on the same medium as their mother plants, with the addition of 300 mg/l cefotaxime, 150 mg/l timentin and 100 mg/l kanamycin. Because all the regenerated buds appeared on the wounds, it was possible to isolate very clearly independent regeneration events occurring on 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 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 estimated as the number of PCR positive lines per 100 leaves.

2.3 DNA extraction and PCR

Presence of transgenes and absence of contaminating agrobacteria were monitored by PCR. Genomic DNA of apple and pear leaves was extracted as described in Fulton et al. (1995). Here are the primers used for the detection of (i) *A. tumefaciens* presence (GTAAGAAGCGAACGCAGGGAACT and GACAATGACTGTTCTACGCGTAA on 23S ribosomal RNA coding gene CP014260.1 gene locus_tag="AWN88_17620" 1310643..1313449), (ii) *nptII* gene (ATCGGGAGCGGCGATACCGTA and GAGGCTATTCGGCTATGACTG on *nptII* gene of plasmid pK7WG2D from Karimi et al. (2002)) and (iii) elongation factor 1α (*EF1α*) coding gene as a marker of plant DNA suitability for PCR (CTCTTGGTGTCAGGCAAATG and TCAAGGTTGGTGGACCTCTC on AJ223969). Amplifications were performed using GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA) according to the manufacturer's recommendations. The PCR reaction conditions were identical for the three genes except the hybridization step which was at 55°C and not 58°C for *A. tumefaciens* detection primers: 95°C for 5 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 72°C for 5 min. The PCR products were separated on a 1.5 % agarose gel. A typical result of this detection method applied during transformation experiment 249 (Supplementary Table 2) is given 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 Khi^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).

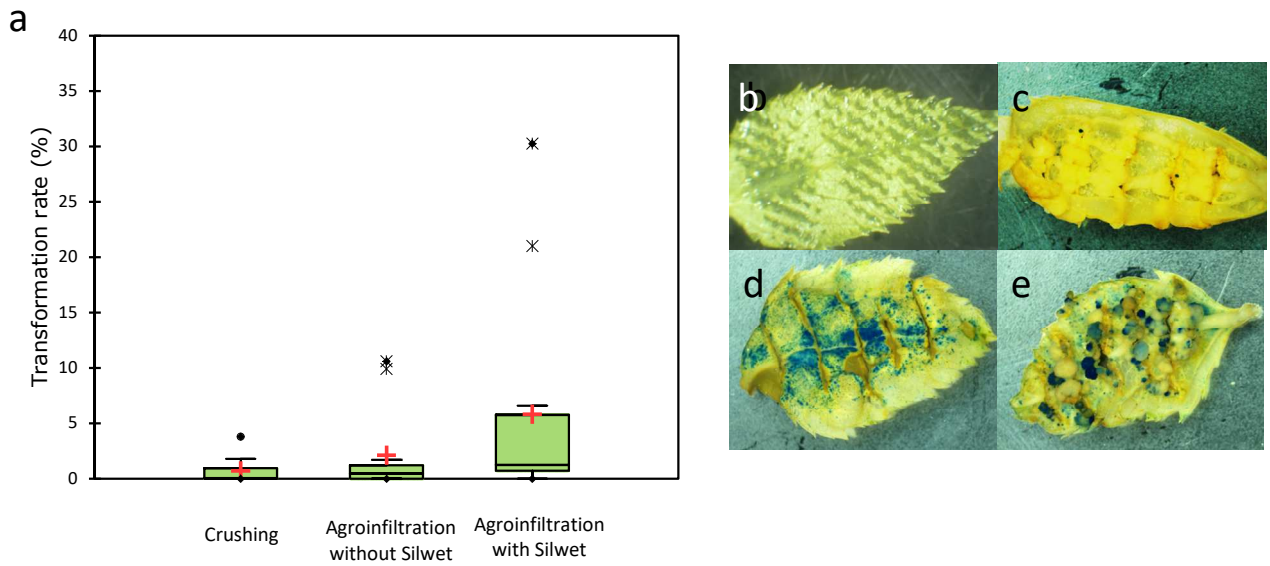

Fig. 1 Main transformation results on apple. a Transformation rate (%) of 'Gala' apple according to the method of *A. tumefaciens* inoculation: "Crushing", "Agroinfiltration without Silwet" and "Agroinfiltration with Silwet". Each box-plot corresponds to 12 independent experiments performed on 150 to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

Table 1Transformation 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 %

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

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

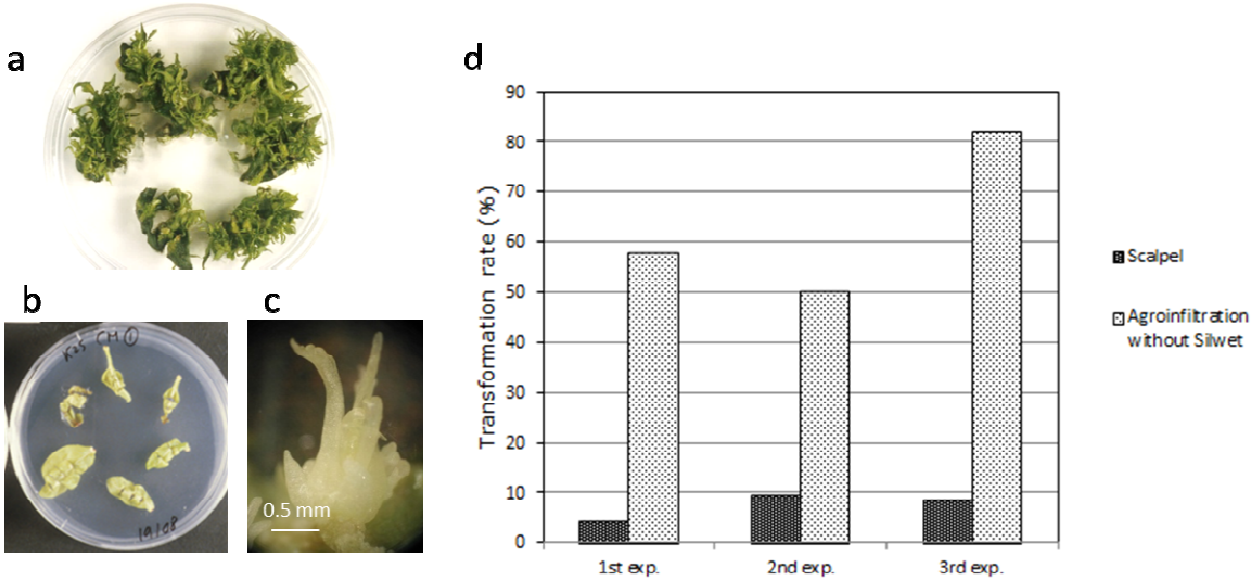

Fig. 2 Main transformation results on pear. a High efficiency of adventitious bud regeneration on 'Conference' pear without *Agrobacterium* inoculation. **b** Absence of bud regeneration on 'Conference' pear without *Agrobacterium* inoculation, under kanamycin selection (100 mg/l). **c** Transgenic 'Conference' 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.

4. Discussion

Agroinfiltration is frequently used as an efficient method for transient transformation assays because the high density of agrobacteria forced into the intercellular spaces increases the efficiency of T-DNA delivery. Here we demonstrated that this method also improved the stable integration of T-DNA into apple and pear cells. Penetration of a bacterial solution by a simple syringe infiltration is not efficient in apple or pear leaves. It is necessary to use a low-pressure vacuum to efficiently 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 efficiency. Previous studies have already shown that the addition of a surfactant in the inoculation

medium increases the transient transformation efficiency in rose (Yasmin and Debener 2010) or poplar (Takata and Erikson 2012). But in some cases, the addition of a surfactant at high concentrations can also be toxic to plant tissues (Wu et al. 2003). In our study, higher concentrations of Silwet (0.005% and 0.01%) caused severe necrosis of apple leaves in a preliminary regeneration experiment without agroinfiltration. Finally, we also showed that agroinfiltration of pear leaves led to very high rates of transformation, even in the absence of surfactant. Positive results were obtained by Habashi et al (2012) using agroinfiltration on the pear 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 '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 after minor optimization of some parameters such as hormonal concentrations.

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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.

Disclosure of potential conflicts of interest

The authors declare that they have no conflict of interest.