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Transgene transmission frequencies between cultivated strawberry (*Fragaria x ananassa* Dutch) and other *Fragaria* species

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Abstract. The transmission of transgenes (*uid-A* and *nptII*) was studied in crosses between transgenic plants, with *Fragaria* x *ananassa* cultivar Teodora as the donor plant and various *Fragaria* species as recipient plants. GUS expression and kanamycin resistance were evaluated in embryos and in seedlings after aseptical germination, both in intra- and inter-specific crosses as demonstrated by different ploidy level. This expression in most of the T_1 seedlings shows that one or more functional transgenes were transferred from T_0 plants to T_1 seedlings. These results, obtained in a greenhouse, confirm the possibility of genetic exchange both at the intra- and inter-specific level, though the inter-specific hybrids seem to have reduced germination ability. These observations need to be further investigated.

Keywords: *Fragaria x ananassa* Dutch, *Fragaria vesca* L., transgene expression, GUS activity, crop-wild hybrid*s*

1. Introduction

Strawberry, of the genus Fragaria within the family Rosaceae, is one of the most economically important fruit crops worldwide due to its adaptability to various environmental growing conditions [1]. The development of *in vitro* regeneration and genetic transformation systems for the cultivated strawberry (*Fragaria x ananassa*) has opened up the opportunity for strawberry improvement through genetic engineering, in addition to traditional plant breeding techniques [2, 3]. Genetic engineering and biotechnology applications are increasingly being used to improve strawberry qualities and expand the breeding base and germplasm utilization. Genetic engineering offers the potential to create novel varieties by selectively targeting a gene or a few heterologous traits for introduction into the strawberry genome, facilitating and speeding the process of obtaining desirable agronomical traits such as increased resistance to pests, herbicides, diseases, environmental stresses as well as enhancement of fruit qualities. Marker genes have been introduced into the cultivated strawberry and in other *Fragaria spp*. by *Agrobacterium*-mediated transformation and by electroporation of protoplasts [4, 5].

Transformation in plant crops could present problems in developing transgenic cultivars [6, 7], such as differences in expression level of the integrated transgene among transformation events. Cultivated strawberry is octoploid ($2n = 8\times = 56$) which may pose problems for the analysis of transgene expression. Studies focused on transmission of transgenes are reported in different species such as *Arabidopsis thaliana* [8], tobacco [9] and rice [10]. In strawberry, trasmission of transgenes in *F. vesca* cv. Alpine [11] and in *Fragaria x ananassa* [12] showed the sexual transmission

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of transgenes to R1 generation progeny but the mechanisms of transmission are unknown. Understanding the transmission of transgenes is important for the development of transgenic cultivars. These cultivars are dependent upon the transformation event which results in the expression of the integrated transgene at a high and stable level over generations and environments.

In order to better understand the transmission of transgenes, we tested the horizontal transfer of transgenes from strawberry to related species including *F. vesca*. We focused our attention on this last species because it is present in different habitats in Italy and is related to cultivated strawberry. We presented some hypotheses with respect to the possibility of genetic exchange both at the intra- and inter-specific level and we also characterized the transformed plants of *Fragaria* x *ananassa* cv Teodora obtained, as reported in [13].

2. Materials and methods

2.1. Characterisation of transgenic donor plants

The donor plant Clone 10 was previously obtained from *Fragaria* x *ananassa* cv Teodora, transformed by *Agrobacterium tumefaciens* strain C5851 harboring the vector pGV3850 with *uid-A* and *nptII* genes [13, 14].

In order to characterize transgenic plants obtained according to [13], genomic DNA was isolated from 1 g of leaves of transgenic clones of *Fragaria x ananassa* cv Teodora: 10, 10a, 228, 230, 247, 252, 253, 257, 264, 430 and 437 by using the "5 Prime" DNA extraction kit (Eppendorf).

Southern analysis was performed with 7 μg of *Eco*RI-*Pst*I (Invitrogen) digested samples; spermidine (final concentration of 0.6 mM) was added to the digestion mix to ensure effective cutting of the DNA by restriction enzymes. Restricted DNA was separated on 1% (w/v) agarose gel, blotted onto positively charged nylon membranes (Roche Applied Science) and fixed by UV irradiation. Filters were probed with a digoxigenine-labelled fragment (Roche Applied Science) of the *uid-A* and *nptII* genes. Both of these were isolated from the plasmid vector pGV3850 and prepared by PCR following the supplier's instructions, using the pairs primer reported in [15].

2.2. In vivo pollination

In a test for potential gene flow, potted plants of non-transgenic species of F*ragaria* (Table 1) acclimatized in a glasshouse, were used as recipient plants and fertilized using the pollen of transgenic *Fragaria x ananassa* cv Teodora clone 10. Pollen was collect by emasculation of blossoms, dried under an incandescent lamp and stored at 4°C. Pollen viability was detected by fluorescence emission under UV in a solution of fluorescein diacetate 0.5%. Germinability was assessed by observing the development of the pollen tubes under the microscope, after incubation at 20°C for 6 hrs on a medium containing yeast extract 1 g L^{-1}, sucrose 100 g L^{-1}, boric acid 100 mg L^{-1}, agar 10 g L^{-1}. For each sample and for both viability and germinability, a minimum of 400 pollen grains were numbered. Recipient plants

Table 1
Cross pollination of different *Fragaria spp*

Donor plant	Recipient plants	Ploidy	No. of pollinated flowers	No. of achenes collected
	Clone '10'	8*n*	17	382 (20 + 362)
	cv Capitola	8*n*	15	29 (8 + 21)
	F. virginiana	8*n*	20	102 (16 + 86)
Fragaria x ananassa	*F. chiloensis*	8*n*	8	0
cv 'Teodora' Clone 10	*F. moscata*	6*n*	24	185 (133 + 52)
	F. vesca cv Ilaria	2*n*	21	152 (24 + 128)
	F. vesca cv Snovit	2*n*	22	86 (12 + 74)
	F. vesca cv Zimbaro	2*n*	31	266 (221 + 45)

were emasculated prior to manual pollination. Pollination was carried out in the glasshouse, for a period of 3 weeks (March-April 2006) and each flower was pollinated twice and isolated using a bag. Achenes were collected from the mature fruits.

2.3. Evaluation of transmission of transgenes to the progenies

In order to evaluate the transmission of transgenes, achenes were divided in two sets: the first was directly used to perform GUS histochemical assay (Fig. 3a) performed according to [17] with modifications. Samples were incubated overnight at 37°C in a solution of 3 mM 5-bromo-4-chloro-3indolyl-d-glucuronide-cyclohexy (x-Gluc, Sigma), 0.4 mM potassium ferricyanide, 0.05 mM potassium ferrocyanide, 10 mM EDTA, 10 mM DL dithiotreitol, 0.1% triton x-100 and 20% methanol in 50 mM sodium phosphate buffer (pH 7.0). Chlorophyll was then removed with a solution of methanol-glacial acetic acid (3 : 1 v/v) for three hours and the explants were fixed in 70% ethanol. The second group of achenes was sterilised and placed on germination MS medium [16] content nicotinic acid 1 mg L^{-1}, piridoxine 1 mg L^{-1}, thiamine 1 mg L^{-1}, inositole 100 mg L^{-1}, sucrose 30 g L^{-1}, pH 5.6. Plants obtained were growth on a MS propagation medium containing giberellic acid (GA_3) 0.1 mg L^{-1}, benzyladenine (BA) 0.1 mg L^{-1}, indole-3-butyric acid (IBA) 0.3 mg L^{-1}, sucrose 30 g L^{-1}, 50 mg L^{-1} of kanamycin, pH 5.7, to test kanamycin resistance. Leaves from the same plants were also used to perform both GUS histochemical assays as above reported (Fig. 3b) and PCR analysis for *nptII* and *uidA* genes. DNA extraction for PCR analysis was performed as reported for Southern hybridisation analysis. Amplifications were conducted in a Biometra T thermal cycler, with a preliminary step of 5 min at 95°C, 30

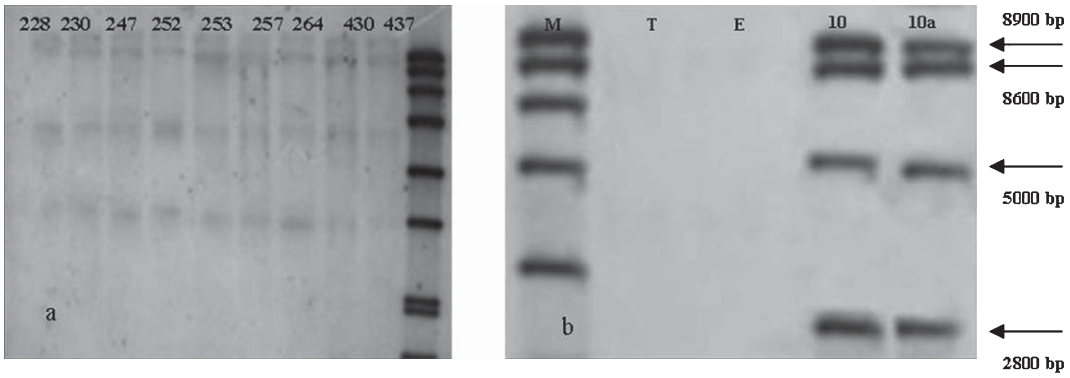

Fig. 1. a) Southern blot analysis of *nptII* gene. (M, DNA Molecular weight marker VII Dig-labelled; b) T, cv Teodora non-transformed; E, cv Egla, non-transformed).

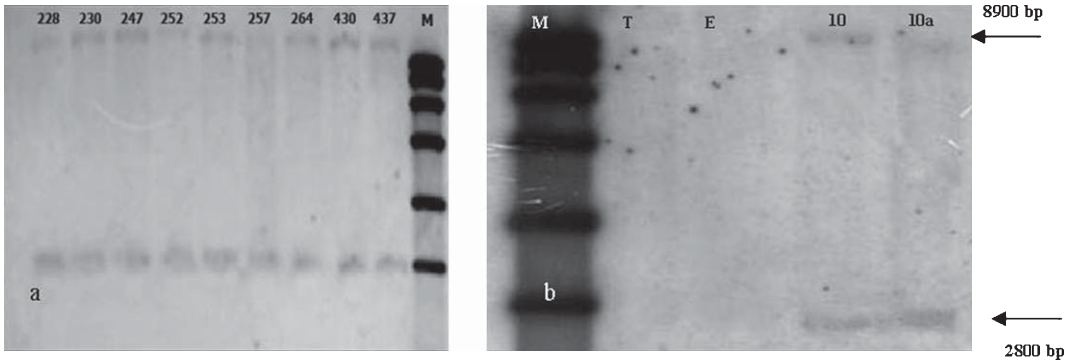

Fig. 2. a) Southern blot analysis of *uidA* gene. (M, DNA Molecular weight marker VII Dig-labelled; b) T, cv Teodora non-transformed; E, cv Egla, non-transformed).

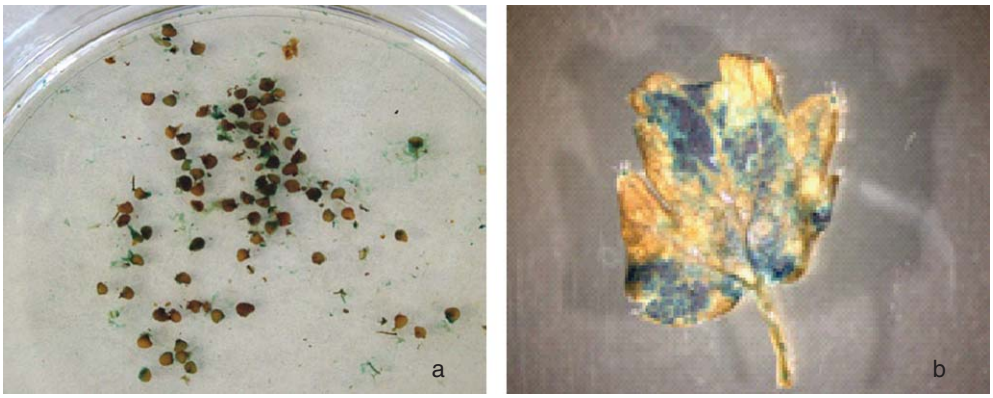

Fig. 3. GUS histochemical assay in achenes (a) and in plants obtained from seeds (b).

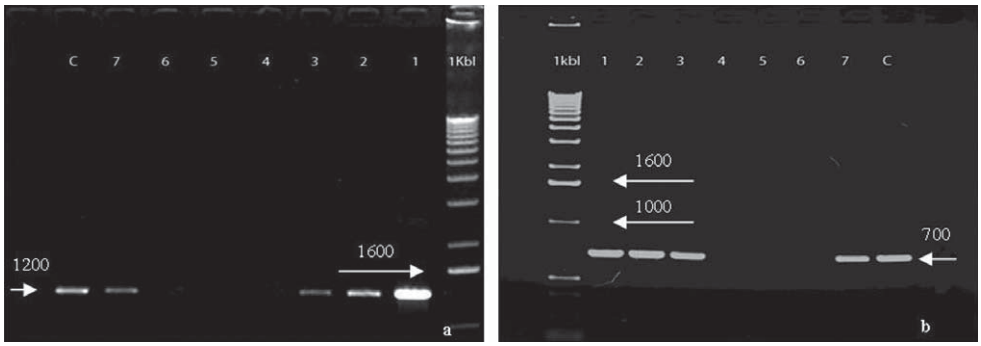

Fig. 4. PCR amplification of *uidA* (a) and *nptII* (b) pair-primers in different cross-pollination plants, derived from germinated seeds. (C, pGV3850 positive control; 1, *F. moscata*; 2, *F. virginiana*; 3, *F. x ananassa* cv Teodora clone 10; 4, *F. x ananassa* cv Capitola; 5, *F. vesca* cv Snovit; 6, *F. vesca* cv Ilaria, 7, *F. vesca* cv Zimbaro.).

cycles of 60 s at 95°C, 60 s at 55°C and 2 min at 72°C and a final step of 10 min at 72°C using 25 ng total DNA, 1X PCR buffer (Qiagen), 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP and dTTP, (Invitrogen); 0,4 mM of each forward and reverse primer and 1U Taq DNA polymerase (Qiagen). Primers sequences were: 5′-GGT GGG AAA GCG CGT TAC AAG (forward) and 5′-TGGCGGAAGCAACGCGTAAAC (reverse) for *uidA* (1200 bp) (Fig. 4a) and 5′-GAC TGG GCA CAA CAG ACA ATC (forward) and 5′-ATC GGG AGC GGC GAT ACC GTA (reverse) for *nptII* (700 bp) (Fig. 4b). Fragments were separated by electrophoresis on 1.2% agarose gel in TBE (Tris-Borate EDTA) 0.5X and stained in ethidium bromide.

3. Results and discussion

3.1. Characterisation of transgenic plants

In order to characterize the transgenic clones obtained [13] as well as the donor plant (clone 10), Southern hybridisations were carried out using *nptII* and *uid-A* labelled probes. Results for *Eco*RI-*Pst*I digestion are reported in (Fig. 1a, b). A double digestion was necessary because it was not possible to hybridise *EcoRI*-digested DNA from transgenic plants with dig-labelled *nptII* probes (data not shown). Hybridisation showed four fragments, ranging from 8.9 kb to 2.8 kb in length, and confirmed the integration of transgenes. Normally, to estimate *nptII* copy number, bands of different sizes are considered independent integration events and transgene copy number is estimated from

the number of fragments exhibiting comparable hybridisation intensity. Because we used two restriction enzymes to digest the DNA, and one of these, *Pst*I, showed the ability to cut within *nptII* gene, it would be inappropriate to estimate of *nptII* copy number as above. To better understand this aspect, a further Southern hybridisation using *uid-A* probe was performed (Fig. 2a, b) and it produced two fragments of 8.9 kb and 2.8 kb in length. When plants are transformed by two different genes and are analysed by Southern hybridisation using probes from both of these genes, results confirm the same copy number of genes, as shown in apple [18] and peas [19]. In our case, the *Pst*I-DNA digestion could cause the breaking of the T-DNA cassette and, consequently, the hybridisation could generate "false positives". Finally, at least one un-truncated transgene copy was integrated in strawberry plants, without excluding the possibility of multiple copy integration, as reported in C*itrus* [20, 21], in *Festuca* [22] and strawberry [23, 24]. For these species expected fragments confirmed the insertion of one copy of the transgene, while the presence of different transgenic lines showed additional bands of higher molecular weight, which suggest that rearrangements had affected the T-DNA cassette.

Our data also confirm the stability of transgenes, obviously not in terms of inheritance because we would need to go through at least one sexual generation to state this, but only in terms of "stabilitise" the transgenes in the regenerated plants. In fact, hybridisation results from transgenic clones 10 and further regenerations (10a, 228, 230, 247, 252, 253, 257, 264, 430 and 437) obtained from the stipules of transformed plant [13] show the same band pattern. The use of this procedure is justified by the fact that crown galls induced by *A. tumefaciens* are chimeric tissue. In fact the hormone (auxin and cytochinin) -synthesizing enzymes coded by the T-DNA genes, modify both transformed and non-transformed cells [25–27]. Therefore the tissue transformed by disarmed *A. tumefaciens* (but also any other transformation procedure) could harbour both transformed and non-transformed cells. As a result, the regenerated plants could be chimeric because of the foreign genes. The extent of chimeric tissues, in relation to the proportion of transformed and non-transformed cells, would depend on the effective number of transformed cells in a given area. Thus, repeating the regeneration using transgenic tissue as explants and adding kanamycin (selective agent) to the medium, it is possible to stabilise transgenes in the regenerated plants.

3.2. In vivo pollination and evaluation of transgenes transmission to the progenies

In the donor plant, pollen viability and germinability ranged from 20% to 95% and from 4.3% to 55.7%, respectively. Pollen used in pollinations had germinability and viability over 50%. Achenes were collected in varying number from each cross (Table 1) except *F. chiloensis,* though the perennial fruit crop of strawberry (*F. x ananassa*) is a hybrid of the two species *F. virginiana* and *F. chiloensis*. This was probably due to the low number of pollinated flowers present in *F. chiloensis*, as reported in Table 1. The results of the GUS histochemical assay performed on the first group of achenes (directly analysed) were showed in Table 2. In all of crosses the achenes collected were GUS positive except for *F. vesca* cv Zimbaro and *F. moscata*. In *F. moscata*, despite having collected 320 achenes, (52 for the first group and 133 which produced seed-derived plants, as reported in Table 3), transgene expression was not observed. This lack of expression could be due to the different ploidy level (6*n*) of this species with respect to the donor plant (8*n*). Evaluation of transgenic transmission in *F. vesca* showed different results: for the cultivars Ilaria and Snovit we detected GUS activity in achenes but not in seed-derived plants. This evidence was also confirmed by

Table 2
GUS activity performed directly on achenes

Cross	Achenes		
	Analysed	GUS+	%
cv Teodora clone 10	362	176	48.6
cv Capitola	21	8	38.1
F. virginiana	86	51	59.3
F. moscata	52	0	0
F. vesca cv Snovit	74	6	8.1
F. vesca cv Ilaria	128	14	10.9
F. Vesca cv Zimbaro	45	0	0

Table 3
Evaluation of transgene transmission in plants derived from aseptically germinated achenes (second lot of achenes). For each plant we performed PCR for *uidA* and *nptII* genes, selection on kanamycin and GUS histochemical assay

Cross	No. of plants	PCR		Selection on kanamycin		GUS activity in plants	
	analysed	*uidA*	*nptII*	Resistant plants	%	GUS+	%
cv Teodora clone 10	20	20	20	20	100	20	100
cv Capitola	8	2	2	2	25	2	25
F. virginiana	16	8	8	8	50	8	50
F. moscata	133	0	0	0	0	0	0
F. vesca cv Ilaria	24	0	0	0	0	0	0
F. vesca cv Snovit	12	0	0	0	0	0	0
F. Vesca cv Zimbaro	221	1	1	0	0	1	0.5

selection on kanamycin and PCR analysis for *nptII* and *uidA* genes (Table 3). In *F. vesca* cv Zimbaro GUS activity was not detected in achenes but, analysing plants obtained from the second group of achenes, we could detect GUS activity in one sample (Table 2). PCR analysis produced expected fragments for *uidA* and *nptII* genes also if the same plant, when growth on kanamicyn, showed the typical chlorotic symptoms due to the lack of *nptII* gene. Some potential causes of the lack of *nptII* gene expression could include methylation or gene silencing phenomena affecting the *nptII* gene. Also, even if cross-pollination recipient plants were previously emasculated and the resulting flowers were isolated by using a bag, we can not exclude cross-contamination. To confirm these results further investigation by Southern hybridisation analysis should be done.

The results obtained in Ilaria and Snovit varieties could be due to "false positive" or "background" activities when the GUS assay was performed. Use of the GUS-fusion system in the study of foreign gene expression and in crop plant engineering [28–30] is based on the assumption that there was no detectable intrinsic GUS activity in higher plants [17]. In the last years researchers focused their attention on intrinsic GUS activities in plants. The presence of GUS was evaluated in different species, included pear, corn, pea, tobacco and also in different tissues as fruit walls, seed coats, immature and mature embryos, seedlings and plant at flowering stage [31]. In all of these species intrinsic GUS activity, which was widely distributed in different tissues, was found. The authors also indicated that the germination process could decrease GUS activity. Recently, similar results were obtained in *Arasbidopsis taliana*, rice, corn, *Brassica* juncea [32] and tobacco [32, 33]. Also in these species, as wild-type crops, the ubiquitous presence of GUS was detected and it was higher in young tissues than the older or mature tissues. Our results can be explained considering the evidence above reported; achenes are younger tissue than seed-derived plants and also because the germination process decreases GUS activity [31, 32]. To confirm these hypotheses further investigation must be done, including Southern hybridisation and an evaluation of the ubiquitous presence of GUS in wild-type plants of *F. vesca.*

With regard to self-crossing, GUS expression was detected in 48.6% of achenes included in the first group (Table 2) compared to achene-derived plants, which showed GUS activity in all plants analysed (Table 3). The reporter genes are thought to be dominant genes [34], consequently all progeny express these traits. In our case, we could detect GUS activity in all of the achene-derived plants, though this expression was found in only 176 achenes (48.6%). Two hypothesis could explain these results. First, the donor plant could have not been homozygous, but for this to be true in both of the groups of achenes we would have observe the same percentage for GUS expression. Second, the GUS assay could have been inefficient in detecting expression of the reporter genes when achenes were directly analysed [31, 32].

4. Conclusion

With regard to the stability and integration of transgenes in plant genomes, our results are in agreement with previous results obtained in strawberry [27] which a more rigorous selection protocol can minimize the risk of chimaerism.

As expected, genetic exchange occurred at the intra-specific level (*Fragaria* x *ananassa*, cv Capitola and *F. virginiana*). Amongst the cultivated strawberry and American wild relatives we confirmed that inter-specific exchange may give rise to a number of viable hybrids. However, in the case of the inter-specific cross using *F. vesca* cv Zimbaro as recipient plant, only one resultant hybrid seedling was observed. This latter observation, which could indicate the possibility of an inter-ploidic cross, may be taken as an index of a possible transmission of transgenes in those environments where *F. vesca* L. is a relative crop of cultivated strawberry, but this needs to be further investigated. Furthermore, the results of this study indicate that the choice of tissue used to evaluate GUS gene expression is important. In fact, as shown by *Fragaria vesca* and *Fragaria x ananassa* the results obtained in achenes directly analysed were ambiguous and, consequently, the GUS assay seemed to be less effective in achenes than in leaf tissue. This study also shows the possibility to detect the ubiquitous presence of GUS in wild-type plants of *Fragaria* spp.

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