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Transformation of a Strawberry Cultivar Using a Modified Regeneration Medium

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Abstract. **A protocol for shoot regeneration of strawberry (*Fragaria* ×*ananassa* Duch. 'Pajaro') leaf disks was developed. In Murashige and Skoog basal medium with 3% of sucrose (w/v), BA (1 mg·L^{-1}), and 2,4-D (0.1 mg·L^{-1}), 70% of the cultivated leaf explants regenerated plants. This regeneration system was used for genetic transformation of strawberry with *Agrobacterium tumefaciens* strain LBA 4404 carrying the binary vector plasmid pBI121 that contains the *npt* II (neomycin phosphotransferase) and *uid*A (ß-D glucuronidase) genes. A transformation rate of 6.6%, calculated as the number of leaf disks able to regenerate kanamycin-resistant plants/total leaf disks infected, was obtained. The integration of both marker genes was evaluated in each transformed line by PCR (polymerase chain reaction) amplification of the *npt* II and *uid*A genes. High expression levels of the *uid*A gene were found in leaves, flower, and fruits of the transgenic lines. The protocol here reported may represent a way to conduct transformation research in strawberries. Chemical names used: benzyladenine (BA); 2,4-dichlorophenoxyacetic acid (2,4-D).**

The development of an efficient and reliable transformation procedure is the first step in the genetic manipulation of cultivated plant species at the molecular level. The availability of micropropagation and regeneration systems coupled with the susceptibility to infection by *Agrobacterium* sp. (Uratsu et al., 1991) makes the strawberry well suited for genetic transformation studies. The clonal propagation of strawberry provides an added advantage for stable transfer of genes associated with desirable agronomic traits to commercially important genotypes.

In the past decade various experimental methods have been devised to accomplish the transformation of cultivated strawberries and the wild strawberry *Fragaria vesca* L. (Barceló-Muñoz et al., 1998; El-Maunsouri et al., 1996; Graham et al., 1990; James et al., 1990; Jelenkovic et al., 1986; Mathews et al., 1995; Nehra et al., 1990a, 1990b; Nyman et al., 1992). However, transformation success is highly dependent on the particular strawberry genotype used, and a protocol developed for one cultivar is not necessarily applicable to others.

Fragaria ×*ananassa* cv. Pajaro is an excellent strawberry cultivar. From 1980 to 1990 it was one of the most commercially important cultivars in Argentina, but planting acreage has decreased due to susceptibility to a local strain of *Colletotrichum acutatum* (Ramallo et al., 2000). Nevertheless, this cultivar is still being used as important parental line in breeding programs (Craig Chandler, pers. comm.).

In this report, we describe the development of a high frequency *Agrobacterium*-mediated genetic transformation protocol for the introduction of two marker genes into the cultivar Pajaro of strawberry using a leaf disk regeneration system. We were successful to accomplish the genetic transformation with a protocol that provides strawberry researchers the chance to conduct transformation research without infringing the U.S. patents 6,274,791 and 5,750,870. The first protects a method for strawberry transformation mediated by *A. tumefaciens* using glucose or fructose in the regeneration medium and the second patent protects an iterative procedure for recovery of uniformly transformed plants. The protocol described here uses sucrose in the regeneration medium and a one-step-selection-procedure in rooting medium with kanamycin to recover uniformly transformed plants. The transgenic plants obtained with our protocol showed high expression levels of the *uid*A reporter gene.

Materials and Methods

Plant material and culture conditions. *In vitro* stock plants of 'Pajaro' proliferating in multiplication medium with 2.21 µM of kinetin (6-furfurylaminopurine) were used. Leaf disks of 4 × 4 mm with the adaxial surface in contact with the medium were used as explants. The basal medium contained: 3% (w/v) of sucrose and 0.22 % (w/v) of Phytagel (Sigma, St. Louis), vitamins and mineral salts according to Murashige and Skoog (1962). The pH of all media was adjusted to 5.7 with sodium hydroxide 0.1 N and autoclaved for 20 min at 121 °C. Culture conditions were 25 ± 2 °C temperature and a 16-h photoperiod under 40 µmol·m^{-2}·s^{-1} irradiance.

The effect of growth regulator balance on the morphogenetic response was studied. The growth regulators BA and 2,4-D were used in combination of 0, 1, 2, 3, 4 mg·L^{-1} and 0, 0.1, 0.2, 0.3 mg·L^{-1} , respectively. In regeneration experiments, the number of regenerating disks was evaluated after 8 and 16 weeks of culture.

Bacterial strain and plasmids. *Agrobacterium tumefaciens* strain LBA 4404 containing the non-oncogenic plasmid pAL 4404 and the binary vector pBI121 (Jefferson et al., 1987) was used in transformation experiments. The pBI121 plasmid, containing the phosphotransferase gene (*npt* II) for kanamycin resistance and β-glucuronidase gene (*uid*A) as a reporter gene, was introduced into *Agrobacterium* by the "freeze-thaw" transformation method (Holsters et al., 1978).

Transformation procedure and characterization of transformants. Transformation experiments started by pre-culturing, prior to *Agrobacterium* infection, 60 leaf disks for 4 d in antibiotic free regeneration medium. Three identical and independent transformation experiments were performed. Incubation was carried out in petri dishes containing 25 mL of regeneration medium (Basal medium supplemented with 1 mg·L^{-1} of BA and 0.1 mg·L^{-1} of 2,4-D). After this induction period explants were inoculated with an overnight culture of *Agrobacterium,* grown at 28 °C in Luria Broth base (LB) medium containing 50 mg·L^{-1} kanamycin, diluted in MS medium at OD_{600} = 2.0 and gently shaken for 15 min. Infected explants were blot-dried on sterile filter paper and co-cultivated in the dark for 2 d at 25 °C. The leaf disks were then transferred to a regeneration medium supplemented with 25 mg·L^{-1} of kanamycin and 500 mg·L^{-1} of cefotaxime (selection medium) under the culture conditions mentioned above and transferred intact to fresh medium every 30 d. Cefotaxime was used to inhibit growth of *A. tumefaciens*.

The shoots that regenerated on selection medium were transferred after 18 weeks to a rooting medium (MS free of growth regulators supplemented with 25 mg·L^{-1} of kanamycin). The shoots were scored for root formation after 4 weeks of culture.

The GUS [β-glucuronidase (EC 3.2.1.31)] activity was determined in leaves, roots, fruits and flowers of transformed lines by histochemical and fluorimetric assays according to Jefferson et al. (1987). The histochemical detection used X-Gluc (4-chloro-3-indolyl-ß-D-glucuronide) as substrate prepared essentially as described by Stomp (1992). Histochemical reaction was performed by incubating plant tissues in 200 µL of X-Gluc solution at 37 °C until the development of the blue color (2–12 h). For fluorimetric assays, 20 mg of young fully expanded leaves, petals and fruit organs were homogenized separately in the following extraction buffer: 50 mM $NaHPO_4$ pH 7; 10 mM 2-mercaptoethanol; 10 mM Na_2EDTA; 0.1%

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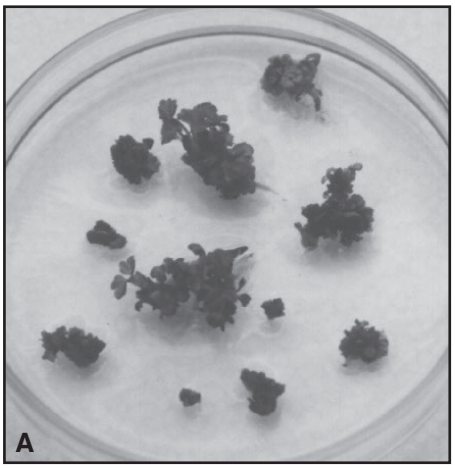
sodium lauryl sarcosine; 0.1% triton X-100. The homogenate obtained was then centrifuged (5000 *g*) at 4 °C for 10 min and the supernatant collected; 5–10 µL of extract was incubated at 37 °C for 0, 5, 10, and 15 min with 2 mM of MUG (4-methylumbelliferyl ß-D-glucuronide) dissolved in extraction buffer. The time course assay (in triplicate) was performed from two independent extractions of each transformed plant. At the end of the incubation time, the reaction was stopped by adding 2 mL of sodium carbonate 200 mM and the fluorescence register in a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech, San Francisco). Total soluble protein was determined by using the BIO-RAD Protein Assay (BIO-RAD Laboratories, Hercules, Calif.).

DNA isolated from leaves of transformed and control plants using the methods of Mercado et al. (1999) was tested for the presence of the *npt*II and *uid*A marker genes by PCR (polymerase chain reaction) according to Haymes and Davis (1998). Additionally the presence of *Agrobacterium* contamination in transformed plants was tested by PCR amplification of a fragment of the gene *vir*D1 contained in the Ti plasmid (Lip-Joao and Brown, 1993). Primers used were (5´-3´): TCCAGATCATCCTGATCGACAAG (forward) and CAAGATGGATTCCACGCAGGTTC (reverse) for the *npt*II gene; CAACGCTGACATCAC (forward) and ACTGGCAGACTATCC (reverse) for the *uid*A gene; ATGTCGCAAGGCAGTAAGCCCA (forward) and GGAGTCTTTCAGCATGGAGCAA (reverse) for *vir*D1 gene. Expected sizes of the *npt*II, *uid*A, and *vir*D1 amplification products are 503, 230, and 437 bp, respectively.

The PCR reaction volume was 20 µL, containing: 0.5 units of *Taq* polymerase (Promega, Madison, Wis.), 1× *Taq* buffer, 1.5 mM $MgCl_2$, 0.1 mM each dNTP, 0.4 µM of the corresponding *nptII, uid*A, or *vir*D1 primers and 50 ng of the plant DNA. In control experiments 10 ng of the plasmid (pBI121) or *Agrobacterium tumefaciens* DNA used. An PTC-100 thermocycler (MJ-Research, Watertown, Mass.) was used for the PCR reactions using the following step cycle program: 94 °C, 50 s; 60 °C, 1 min and 72 °C, 2 min for 35 cycles linked to a final 72 °C 5 min extension.

In order to evaluate the heredity of foreign genes into the progeny, two independent crosses between a transformed line and a non transformed 'Pajaro' plants were made. The hybrid seeds were aseptically germinated to obtain R1 seedlings and tested for kanamycin resistance and GUS activity. Surface sterilized seeds were transferred to petri dishes containing basal medium added with 50 mg·L^{-1} of kanamycin and incubated for 6 weeks under conditions mentioned above. Seeds germinated in the first two weeks and kanamycin resistances were scored 4 weeks later. Leaf sections of the newly germinated seedlings were incubated separately in a 96-well microtiter plate with 100 µL X-Gluc substrate solution (Jefferson et al., 1987) per well. Microtiter plates were then incubated overnight at 37 °C in the dark. After incubation in the X-Gluc, GUS activity was indicated by the blue color of the tissues.



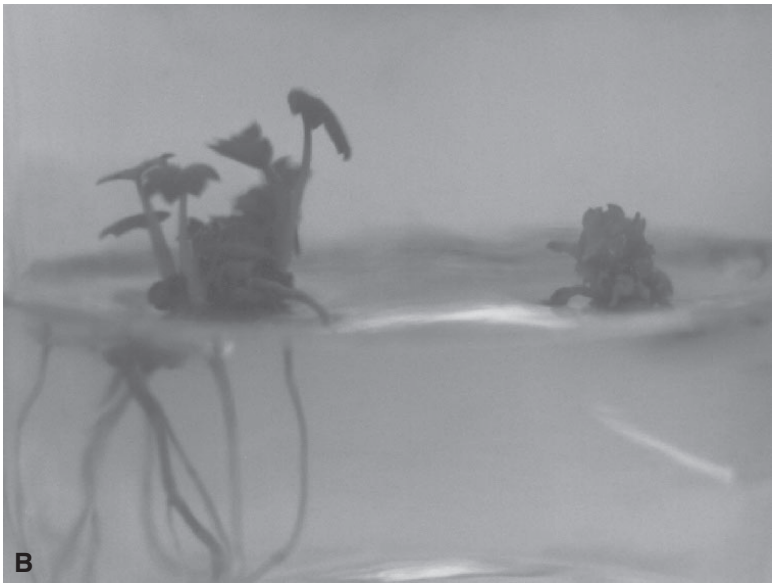


Fig. 1. (**A**) Regeneration of strawberry plants *Fragaria* ×*ananassa* cv. Pajaro from leaf disks in selection medium; (**B**) transformed (*left*) and control shoots (*right*) in rooting media containing 25 mg·L^{-1} of kanamycin.

Results and Discussion

Plant regeneration from leaf disks. Evaluation of the regeneration showed no regeneration when BA or 2,4-D was absent in the media. In other strawberry cultivars, auxins and cytokinins were required to induce the regeneration of plants from leaf disks. Liu and Sanford (1988) observed different requirement of cytokinins and auxin levels to induce regeneration from leaf disks *Fragaria* ×*ananassa* cv. Allstar depending on the source of explants. These authors reported that 2.5 mg·L^{-1} of BA and 0.5 mg·L^{-1} of IBA (indole-3 butyric acid) induced 79% of regeneration from leaf disks coming from *in vitro* culture, while leaves of plants grown in greenhouse regenerated at 34% with 3 mg·L^{-1} of BA and 0.1 mg·L^{-1} of IBA. Nehra and Stushnoff (1989) found that BA at 2.25 mg·L^{-1} and IAA (indole-3 acetic acid) at 1.75 mg·L^{-1} induced 93% of regeneration when leaf disks of *Fragaria* ×*ananassa* cv. Redcoat grown in greenhouse were used. Barceló et al. (1998) used 1 mg·L^{-1} and 0.5 mg·L^{-1} of BA and IBA, respectively, for shoot regeneration from leaf disks of *Fragaria* ×*ananassa* cv. Chandler and observed that higher levels of hormone in the media decreased the regeneration efficiency. Our results show a yield of 70% of shoot regeneration when in vitro disks of *Fragaria* ×*ananassa* cv. Pajaro were cultivated with BA and 2,4-D at 1 mg·L^{-1} and 0.1 mg·L^{-1}, respectively. The leaf disks started to regenerate after 8 weeks and 8 weeks later formed a cluster of 5-10 shoots from each regeneration site. This media was used in later transformations experiments. The higher hormone concentrations used in experiments with *Fragaria* ×*ananassa* cv. Pajaro had negative effects on the regeneration process, 0.2–0.3 mg·L^{-1} of 2,4-D and 2-4 mg·L^{-1} of BA induced callus formation but no plant regeneration.

Genetic transformation. In three independent experiments, a total of 180 leaf explants were co-cultivated with the *Agrobacterium* strain LBA4404 carrying the plasmid pBI121 and cultured in the medium for plant regeneration with 25 mg·L^{-1} of kanamycin. After 8 weeks, four leaf explants from each independent experiment showed kanamycin resistant

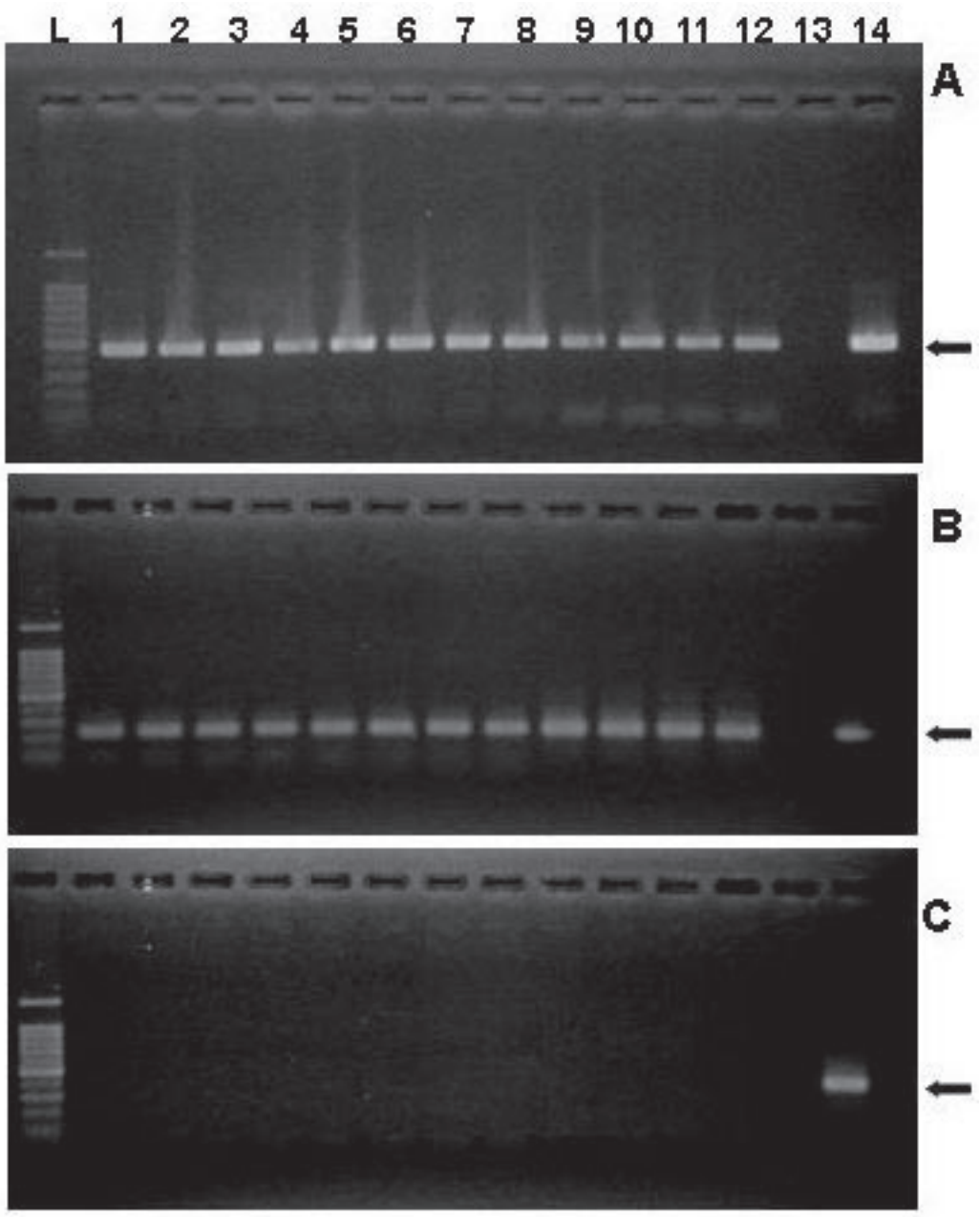


Fig. 2. PCR amplification of the *npt*II (**A**), *uid*A (**B**), and *vir*D1 (**C**) genes electrophoresed in 1.5% agarose gel. Arrows indicate the 503-, 230-, and 437-bp fragments, respectively. Lane L: molecular weight marker; 1–12: transformed plants, 13: untransformed control plant; 14: positive controls from pBI121 plasmid in A and B, and *A. tumefaciens* DNA in C.

calli and, 18 weeks later, all of those formed a cluster of green healthy shoots (Fig. 1A). These primary transformants were transferred to a rooting medium containing 25 mg·L^{-1} of kanamycin to select uniformly transformed plants. After 8 weeks of culture the transgenic shoots formed roots in this medium while the controls shoots (not transformed plants) died (Fig. 1B). The fastest growing and most vigorous rooting plants under antibiotic pressure were selected for further analysis. The transformation efficiency calculated as a number of kanamycin resistance events (coming from separate shoot clusters) with respect to a total number of leaf disks infected was 6.6%. The transformation rate reported for the strawberry cultivar Redcoat was 3% when using calli as explants (Nehra et al., 1990a) and 6.5% for the same cultivar when using a leaf disk regeneration system (Nehra et al., 1990b). However, these plants were not able to root well in the selection medium containing 25 mg·L^{-1} of kanamycin. Other protocols reported for the transformation of *Fragaria* ×*ananassa* cvs. Tristar and Totem, yielded efficiency as high as 12.5% and 58.8%, respectively, but the regenerated plants showed evidence of chimerism, even after an iterative selection procedure in which kanamycin was increased stepwise from 5 to 200 mg·L^{-1} (Mathews et al., 1998). Barceló et al. (1998) and De Mesa et al. (2000) also reported the transformation of the *Fragaria* ×*ananassa* cv. Chandler with an efficiency of 4.22% and 20.7%, respectively, when plants were regenerated in the presence of 25 mg·L^{-1} of kanamycin. The former authors used the *Agrobacterium* mediated transformation protocol (Barceló et al., 1998), the latter used an *Agrobaterium*-coated microparticle bombardment procedure (DeMesa et al., 2000).

Analysis of transformants. The elimination of *Agrobacterium* contamination during the selection process was tested by cultivation of leaf and crown fragments of transgenic plants on antibiotic-free LB medium for 30 days. Results of PCR amplification of the *Agrobacterium vir*D1 gene fragment from DNA of transformed plants were negative confirming the absence of contaminations in transformed plants (Fig. 2). The presence of *npt*II and *uid*A genes in the DNA of transgenic plants was also evaluated by PCR. The expected 230 bp and 503 bp bands corresponding to the *uid*A and *npt*II genes, respectively, were found in

Table 1. ß-glucuronidase activity detected by fluorimetric assay of transformed plants of strawberry *Fragaria* ×*ananassa* cv. Pajaro.

Lines of transformed plants	GUS activity (nmoles of 4-MU produced/ min/mg protein)
AF55-1	88 ± 5
AF55-2	35 ± 2
AF55-3	91 ± 8
AF55-4	150 ± 2
AF56-1	102 ± 8
AF56-2	19 ± 5
AF56-3	151 ± 4
AF56-4	69 ± 8
AF57-1	60 ± 7
AF57-2	38 ± 4
AF57-3	110 ± 8
AF57-4	28 ± 1
Untransformed control	0

all transformed plants and absent in the untransformed control plants (Fig. 2).

The transformed plants were screened for GUS activity using the histochemical assay in leaf, petiole and roots. In all tissues analyzed, histochemical staining was uniform (data not shown), suggesting the absence of chimeric plants; we observed no kanamycin-resistant plants that did not show GUS activity. GUS expression level was quantified in leaves of runner plants (R0) by fluorimetric assays. The expression level of the GUS attained in our experiments was between 19 and 151.2 nmoles of 4-MU (4-methylumbelliferone) produced/min/mg protein (Table 1) and the values displayed by each line did not show significant variations after one year from the first assay. The GUS expression values reported by Nehra et al (1990a) range between 0.17 and 8.37 nmoles of 4-MU produced/min/mg protein for plants regenerated from calli and between 0.32 and 0.577 nmoles of 4-MU produced/min/mg protein for plants regenerated from leaf disks (Nehra et al., 1990b). DeMesa et al. (2000) also reported lower values of GUS activity in transformed plants of the cultivar Chandler obtained by bombardment procedure.

We have also quantified the GUS activity levels in flowers and ripe fruits of the transgenic lines 55-4, 56-3, and 57-1 and observed that flowers and fruits showed lower activity than leaf tissues (Table 2). The latter confirms that *uid*A gene is being expressed in different tissue of the plant.

Crosses among untransformed 'Pajaro' plants (mothers) with plants of the transformed line 55-2 were carried out (e.g., backcross) to confirm transmission of the *uid*A gene. Segregant hybrid achenes (351) were evaluated for kanamycin resistance and GUS activity. Of the 351 achenes tested 333 germinated and formed seedlings, 165 were classified as resistant and 168 as susceptible to kanamycin. Histochenical determination of GUS activity showed that all the kanamycin resistant seedlings were GUS positive and the rest were GUS negative. These results indicate that both genetic markers (kanamycin resistance and GUS activity) segregate together; therefore, the gene *npt*II is co-inherited with the *uid*A gene. Furthermore,

Table 2. ß-glucuronidase activity detected in different plant tissues by fluorimetric assay of transformed lines of strawberry *Fragaria* ×*ananassa* cv. Pajaro.

Lines of transformed plants	GUS activity (nmoles of 4-MU produced/ min/mg protein)		
	Leaves	Flowers	Fruits
AF55-4	150 ± 2	20 ± 2	83 ± 5
AF56-3	151 ± 4	45 ± 3	77 ± 4
AF57-1	60 ± 7	6 ± 1	17 ± 1
Untransformed control	0	0	0

the ratio of plants GUS+/GUS- (e.g., 165/168) 1:1 corresponded to the ratio expected for the segregation of a single dominant gene.

The protocol presented in this paper provides a useful alternative for strawberry transformation that avoids potential conflicts with current effective patents. The U.S. patent 5,750,870 protects an *Agrobacterium*-mediated method that uses an iterative procedure to optimize the yield of uniformly transformed plants. The procedure consists on subjecting the primary shoot "regenerants" obtained in the first selection medium to several cycles of tissue isolation and culture on progressively higher antibiotic concentration in the medium, thereby the plants undergo rooting in the presence of kanamycin or hygromycin. With our protocol the recovery of uniformly transgenic plants is made by culture of primary transformants directly in a rooting medium containing kanamycin at 25 $mg \cdot L^{-1}$ and selecting the fastest growing and most vigorous rooting plants. The U.S. patent 6,274,791, on the other hand, protects a method for strawberry transformation mediated by *A. tumefaciens* using glucose or fructose in the regeneration medium whereas the regeneration medium described here uses sucrose.

We would like to emphasize that although the strawberry transformation protocol presented here does not infringe intellectual properties for the reasons mentioned above and because our work was carried out in the frame of an academic research, there are few things that have to be considered carefully before any attempt to used it in commercial breeding program. Even though *Fragaria* ×*ananassa* cv. Pajaro is no longer protected with a patent within U.S territory, restriction may still persist outside the United States. In the latter case, permission should be requested to the Univ. of California at Davis. Likewise, the use of some regulatory sequences and other molecular motives used in cloning and expressions of genes in plants may also be protected by patents [e.g., CaMV 35S (cauliflower mosaic virus) promoter is under Monsanto patent U.S. 5,352,605] and therefore should be thoroughly considered by commercial line breeders.

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