

Research Report

GFP transient expression and silencing in *Fragaria x ananassa*

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Abstract.

BACKGROUND: Stable transformation, transient expression, and post-transcriptional gene silencing (PTGS) are powerful methodologies that allow exploration of gene function.

OBJECTIVE: We aimed to apply these methodologies to strawberry leaves. Methods: the binary vectors pBIN19-*sgfp*, pBICdsGFP and pBIN61-P19 were transferred into *A. tumefaciens* EHA105 supervirulent strain by electroporation. The *sgfp* gene silencing was carried out in stably transformed GFP (green fluorescent protein) *F. x ananassa* Duch. cultivar ‘Pájaro’ strawberry plants by agroinfiltration. GFP-fluorescence was observed using a stereomicroscope (507 nm).

RESULTS: We attained a GFP transgenic *F. x ananassa* plant that expresses the functional protein in all the tissues during a complete and normal life cycle. *In planta sgfp* transient expression and silencing have also been achieved in *F. x ananassa* cv. ‘Pájaro’ leaves of wild type and GFP transgenic plants, respectively. *Agrobacterium*-mediated transient expression was visualized as high intensity green fluorescent spots as early as 7 days post-agroinfiltration (dpa), peaking between 10 and 14 dpa and persisting as long as 24 dpa. A knockdown GFP phenotype was achieved by silencing using a dsGFP hairpin.

CONCLUSION: This work contributes significantly to the reverse genetics field in strawberry, might help to gain knowledge in the analysis of functional promoters and thereby allow protein expression and silencing of genes. This will help to develop resistant plants expressing plant defense elicitors or silencing pathogen receptors and/or negative regulators of plant defense.

Keywords: *Fragaria x ananassa* Duch., green fluorescent protein, *in planta* monitoring, post-transcriptional gene silencing, strawberry, transgenesis, transient expression

1. Introduction

Reverse genetics is a powerful methodology that allows exploration of gene function through modifying a known gene sequence or expression to further analyse its phenotypic effects. For this purpose several approaches

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have been developed in plants, including stable and transient transformation, mutagenesis, and silencing [1]. *Agrobacterium*-mediated transformation is the most common methodology for delivering genes of interest in plants [2]. The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has been used as a universal reporter in a broad range of biological systems [3]. However, the use of GFP in higher plants has been limited by aberrant splicing of the corresponding mRNA and by protein insolubility. Therefore, a new synthetic GFP gene sequence (*sgfp*) with optimal human codons in plant cells was attained, resulting in a more soluble version of the protein GFP [4]. Moreover, a site-directed mutagenesis in the chromophore (the replacement of the serine position 65 with a threonine -S65T-) was also introduced into *sgfp* to create *sgfp(S65T)*, resulting in enhanced brightness, faster chromophore formation and slower photobleaching [4]. The *sgfp(S65T)* gene, hereafter in the study referred to as *sgfp*, became a very useful marker gene for establishing efficient transformation protocols and monitoring promoter or protein functionality in plants. In general, the level of *sgfp* expression peaks between 2 and 3 days post-agroinfiltration (dpa), and declines rapidly thereafter due to an intrinsic post-transcriptional gene silencing process (PTGS), as described in leaves of Arabidopsis [5], tobacco [6] and potato [7], or as for lettuce and tomato fruits [5], and even strawberry fruits [8].

PTGS is an interesting strategy that has been successfully used in plants for studying gene silencing. One approach is based in the introduction of plasmid constructs encoding a double-stranded RNA (dsRNA) homologous in sequence to the gene of interest [9]. As a result, the target gene transcripts are degraded leading to a knockout or knockdown phenotype [10]. During the evolutionary race between plants and viruses, the latter have evolved proteins that suppress PTGS, thereby allowing plant infection [11–13]. Among them, the protein P19 encoded by the tomato bushy stunt virus (TBSV) is a very effective suppressor that lets the virus evade the host silencing response restoring high levels of the transiently expressed protein [14, 15, 16].

Strawberry, including the cultivated octoploid *Fragaria x ananassa* Duch. and the wild diploid *F. vesca*, is considered a plant model system for genetic and molecular studies in the *Rosaceae* family due to its numerous advantages, such as the availability of the complete genome sequences [17–20]. Particularly for *F. x ananassa* fruits, reverse genetics has been successfully applied to investigate the function of a target gene using stable transformation, transient expression, and PTGS. New protocols were adjusted using mainly *GUS* as the marker gene rather than *GFP*, and beneficial traits such as disease resistance and fruit quality have been improved [8, 21–28]. However, there are few studies on transient expression and silencing in strawberry leaves, which are an interesting tissue to apply reverse genetics techniques using genes encoding pathogen effectors and defense-inducing elicitors. In fact, there is only one study reporting transient expression of yellow fluorescent protein (YFP) in detached leaves of *F. vesca* [29] and another about virus-induced gene silencing (VIGS) of a *F. x ananassa* cultivar ‘Benihoppe’ *phytoene desaturase* (*PDS*) gene [30]. Therefore, the objectives of the present study were to develop 1) a stable *F. x ananassa* cv. ‘Pájaro’ GFP transgenic line, 2) an *Agrobacterium*-mediated methodology for transient expression of *sgfp* in strawberry leaves, and 3) a PTGS-based tool using a *dsGFP* hairpin.

2. Materials and methods

2.1. Plant material

Strawberry plants of *F. x ananassa* Duch. cv. ‘Pájaro’ were micropropagated at the Strawberry BGA (Banco de Germoplasma Activo) at Universidad Nacional de Tucumán, Argentina. Strawberry plants that were not stably transformed and thus maintain its wild genetic background are hereafter referred to as wild type (WT) plants. Briefly, runner meristems obtained from healthy strawberry WT plants were implanted into initiation medium (IM) containing macroelements according to the N30K formulation [31], microelements and vitamins (Neolab, Argentina) according to MS basal medium [32], 100 mg/L myoinositol, 3% (m/v) sucrose, hormones (Neolab, Argentina): 1 mg/L butyric indol acid (IBA), 0.1 mg/L benzylaminopurine (BAP) and 0.1 mg/L gibberellic acid

(GA3), and 2.1.1 g/L Phytigel™ (Sigma-Aldrich, St. Louis, MO, USA). After one month, emerging shoots were transferred into multiplication medium (MM), which differed from IM by using 0.3 mg/L BAP as unique hormone, and replacing Phytigel™ with 6 g/L agar (Sigma-Aldrich, St. Louis, MO, USA). They were transplanted into fresh MM every month and the process was repeated a maximum of five times to minimize the possibility of somaclonal variation. For genetic stable transformation, *in vitro* plant material that was previously transplanted into fresh MM for five times, was transplanted into a modified MM supplemented with 0.5 mg/L kinetin (Neolab, Argentina) instead of BAP, and maintained for one month prior to assays. Etiolated plants were used to investigate whether a chlorophyll masking phenomenon was taking part in GFP stably transformed *in vitro* plants. For this assay, one-month-old *in vitro*-grown plants were maintained in rooting medium, prepared with half strong salts and vitamins according to MS, 100 mg/L myoinositol, 4% (m/v) sucrose and 6 g/L agar (Sigma-Aldrich, St. Louis, MO, USA), and then kept in darkness for one month to eliminate chlorophyll. All meristems, explants and plants grown and kept *in vitro* were incubated in a growth chamber under controlled conditions in a 16 h light cycle (white fluorescent light, 350 $\mu\text{mol}/\text{m}^2\cdot\text{s}$) and $24^\circ\text{C} \pm 2^\circ\text{C}$.

For transient expression and silencing assays, three-month-old plants grown in soil were used. To obtain these plants, first, *in vitro* shoots proliferated in MM were transferred into rooting medium, and after one month these plantlets were transplanted into a humus/perlite soil mixture (2/1, m/m) and kept in a growth chamber under controlled conditions in a 16 h light cycle (white fluorescent light, 350 $\mu\text{mol}/\text{m}^2\cdot\text{s}$) and $24^\circ\text{C} \pm 2^\circ\text{C}$. For the first week these plants were covered with a transparent bag to maintain 100% relative humidity (RH); then were maintained uncovered for another week, and finally were moved to a greenhouse under semi-controlled conditions in a 16 h natural light cycle, $24^\circ\text{C} \pm 4^\circ\text{C}$ with 70% RH.

2.2. Plasmids and *Agrobacterium tumefaciens*

The binary vectors pBIN19-*sgfp*, pBICdsGFP, and pBIN61-P19 were transferred into *A. tumefaciens* EHA105 supervirulent strain [33] by electroporation following instructions of the manufacturer (MicroPulser Electroporator, BioRad, USA). pBIN19-*sgfp* expresses the marker gene *sgfp* which encodes the modified sGFP protein (hereafter referred as GFP) [4] (Online Resource 1A). pBICdsGFP expresses the self-complementary *sgfp* gene separated by an intron sequence, codifying for a RNA GFP hairpin (dsGFP) [34] (Online Resource 1B). pBIN61-P19 expresses the *orf4* gene from tomato bushy stunt virus (TBSV) that encodes the silencing suppressor P19 [15] (Online Resource 1C). Transformed clones were evaluated by restriction site analysis. Plasmid DNA was extracted from three isolated colonies per construct using PureYield Plasmid Miniprep System (Promega, USA) and digested with the following restriction endonucleases: *EcoRI* and *HindIII* for pBIN19-*sgfp* and pBIN61-P19 constructs, or *HindIII* and *SalI* for pBICdsGFP (Thermo Fisher Scientific, USA). The fragments were examined by electrophoresis on a 1% (m/v) agarose gel (Biodynamics SRL, Argentina) and stained with ethidium bromide (Thermo Fisher Scientific, USA) according to [35]. Since the expected bands were visualized for pBIN19-*sgfp* (1.5 kb and 12 kb), pBIN61-P19 (764 bp, 411 bp, 290 bp and 11.7 kb) and pBICdsGFP (666 bp, 2.3 kb and 11.7 kb) it was confirmed the correct plasmid transference.

2.3. Plant stable transformation

F. x ananassa stable transformation was carried out with *A. tumefaciens* strain EHA105 containing the pBIN19-*sgfp* plasmid, according to [36] with minor modifications. One cm^2 -leaf disks were excised from 'Pájaro-WT' *in vitro* plants cultured during one month in the modified MM, placed abaxial side up and cultured on shoot regeneration medium (SRM), a basal MS medium supplemented with 3% (m/v) sucrose, 1 mg/L BAP, 0.1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) (Neolab, Argentina), and 2.2 g/L Phytigel™ (Sigma-Aldrich, St. Louis, MO, USA), for four days prior to inoculation. The leaf explants were dipped into 25 ml of EHA105(pBIN19-*sgfp*) bacterial culture for 15 min at room temperature with gently shaking every 2 min. The bacterial culture

grown at $OD_{600} = 2$ was diluted with liquid MSO medium (MS basal salts without sugar) at the ratio of 1 : 10 LB broth:liquid MSO. Explants were blotted dry on autoclaved filter paper N°1 (Whatman), placed abaxial side up on SRM, and maintained in darkness at 20°C. After two days, the explants were transferred to selection medium (SM) which was similar to SRM but supplemented with 25 mg/L kanamycin (Kn) (Sigma-Aldrich, St. Louis, MO, USA) and 500 mg/L cefotaxim (Cf) (Klonal Laboratorios, Argentina). They were subcultured to fresh SM every seven days for two times and thereafter every fourteen days. In addition, leaf explants without *Agrobacterium* inoculation were cultured in SRM (regeneration control) or in SM (selection control). Glass Petri dishes (100×25 mm) with 25 ml of all media were used. After four months, transgenic shoots regenerated from each leaf explant were transferred into selective rooting medium (SRM'), similar to the rooting medium described above but containing 25 mg/L Kn. After one month, Kn-resistant rooted plants were transferred into a sterilized humus/perlite soil mixture (2/1, m/m) and maintained in greenhouse. All the explants used in this study were incubated in a growth chamber under controlled conditions as mentioned in 2.1 sub-section. Treatments were repeated five times with ten explants per treatment ($n = 50$) and replicated three times independently. Each of the two transgenic lines attained were vegetatively multiplied to provide sufficient plant material for the studies.

2.4. Transient expression assays

A. tumefaciens strains EHA105 containing the pBIN19-*sgfp*, pBICdsGFP and pBIN61-P19 plasmids were agroinfiltrated in 'Pájaro-WT' leaves according to an adapted protocol from [27]. Briefly, bacteria were grown in 50 ml LB containing 5 g/L sucrose, 2 mM MgSO₄ and 10 mM morpholine ethanesulphonic acid (MES, pH 5.6) (Sigma-Aldrich, St. Louis, MO, USA), 25 mg/L nalidixic acid, 50 mg/L Kn and 20 μmol/L acetosyringone (Neolab, Argentina), at 28°C and 135 rpm. When the cultures reached $OD_{600} = 1$ were centrifuged (5.000 rpm, 4°C) and pellets resuspended in the infiltration solution (IS), a MS modified medium with MS salts, 10 mM MES, 20 g/L sucrose, 200 μmol/L acetosyringone (pH 5.6) and incubated at 20°C with 135 rpm until $OD_{600} = 0.8$. 500 μl of any bacterial suspension were carefully infiltrated in the abaxial side of leaves with the help of a 3 ml-syringe without needle. Infiltrated areas (2 mm diameter) were slightly marked with blue ink and excess of bacteria was removed with tissue paper (Online Resource 2). The wild type bacterium EHA105 (without plasmid, denoted as EHA105-WT) and the IS were used as controls. Plants were maintained in a growth chamber under controlled conditions as mentioned in 2.1 sub-section, but at 20°C ± 2°C.

2.5. Silencing and suppression of silencing assays

The *sgfp* gene silencing was carried out in stably transformed GFP *F. x ananassa* plants by agroinfiltration according to [37]. With a similar technique as for transient expression assays, leaves were infiltrated with a co-culture of *A. tumefaciens* EHA105(pBIN19-*sgfp*) + EHA105(pBICdsGFP), from now on denoted as pBIN19-*sgfp* + pBICdsGFP, for silencing studies. EHA105-WT and the IS were used as controls. The suppression of intrinsic silencing was studied in WT plants by agroinfiltration of a co-culture of EHA105(pBIN19-*sgfp*) + EHA105(pBIN61-P19), from now on denoted as pBIN19-*sgfp* + pBIN61-P19. Plants were maintained in a growth chamber under controlled conditions as mentioned in 2.4 sub-section.

2.6. Analysis of GFP expression

GFP was observed using a stereomicroscope (Leica MZ16 F, Leica Microsystems, Germany) equipped with an external lamp set with a 484 nm excitation filter. Detection of green fluorescence (507 nm) was indicative of GFP protein expression. Pictures were taken with a digital camera (COOLPIX 4300, Nikon) and saved as.TIF files for further quantification using ImageJ 1.44 software. Fluorescence values were corrected as follows: for GFP transient expression assays, fluorescence (pBIN19-*sgfp*) – basal fluorescence (mean between EHA105-WT and IS); for silencing assays, fluorescence (pBIN19-*sgfp* + pBICdsGFP) – basal

fluorescence (mean between EHA105-WT and pBICdsGFP), and for suppression of silencing assays, fluorescence (pBIN19-*sgfp* + pBIN61-P19) – basal fluorescence (mean between EHA105-WT and dsGFP). Means \pm standard errors (SEs) were calculated from fluorescence corrected values and represented in each graph.

2.7. Image processing

Each of the digital images was processed using ImageJ 1.44 software (NIH) (<https://imagej.nih.gov/ij/>), an open source, Java-written image processing program. Digital image files in.TIF format were open in ImageJ, and image settings were adjusted by selecting IMAGE – TYPE – 16-bit; EDIT – INVERT; PROCESS – SUBTRACT BACKGROUND where “Rolling ball radius” was set in 50.0 pixels and “light background” was selected. In IMAGE – ADJUST – THRESHOLD “B&W” was set in the right window and the bar was dragged to modify the black coloration as faithfully as the green fluorescence in the original picture. Quantification parameters “Area”, “Area fraction” and “Display label” were set in ANALYSE – SET MEASUREMENTS and after selecting MEASURE – ANALYSE a Summary table appeared from which “%Area” values were chosen to present the results. This parameter represents the percentage of black-colored area from the total area of the image, given in pixels.

2.8. Statistical analysis

For green fluorescence analysis in transient expression and silencing assays one leaf (including the three leaflets) of three plants was infiltrated at two points on each side of the central vein ($n = 36$). For suppression of *sgfp* silencing assays one leaf (including the three leaflets) of one plant was infiltrated at two points on each side of the central vein ($n = 12$). Fluorescence values in transient expression assays in WT plants (Fig. 2), and in silencing assays in GFP stably transformed plants (Fig. 4) were analyzed via Kruskal-Wallis test (non-parametric data). Fluorescence values in suppression of intrinsic *sgfp* silencing in WT plants were analyzed via ANOVA followed by LSD-Fisher test (parametric data). In all figures significant differences are indicated with different letters ($P \leq 0.05$). Statistical analyses were performed using the InfoStat 2013 software [38].

3. Results

3.1. *F x ananassa* stable transformation

In vitro propagated *F. x ananassa* cv. ‘Pájaro’ plants were used as leaf explant donors for further stable genetic transformation assays. Briefly, plants were micropropagated from apical meristems (Online Resource 3B) extracted from runners (Online Resource 3A), after four weeks emerged shoots (Online Resource 3C), which were transferred to MM where proliferated forming clump shoots (Online Resource 3D). These new shoots were transferred to the modified MM supplemented with kinetin, and after one month served as leaf explant donors for stable transformations assays. Eight out of fifty leaf discs transformed with pBIN19-*sgfp* induced calluses after three months post *Agrobacterium*-mediated stable transformation (Online Resource 4A), and only two of them finally regenerated shoots. Shoots formed from each callus were considered as independent transformation events. After transferring shoots to SM supplemented with kn 25 $\mu\text{g/ml}$, two kn-resistant lines were obtained, representing 4% transformation efficiency. Calluses from leaf explants cultivated in SRM (regeneration control) grew faster than the transformed ones (Online Resource 4B) with 60% of efficiency. Whereas non-transformed leaf discs cultured in SM (negative control) did not develop calluses and died after 4 months (Online Resource 4C).

The expression of GFP protein was analysed *in planta* during the different developmental stages of both transgenic lines. Only one of them presented strong and uniform green fluorescence in all tissues observed;

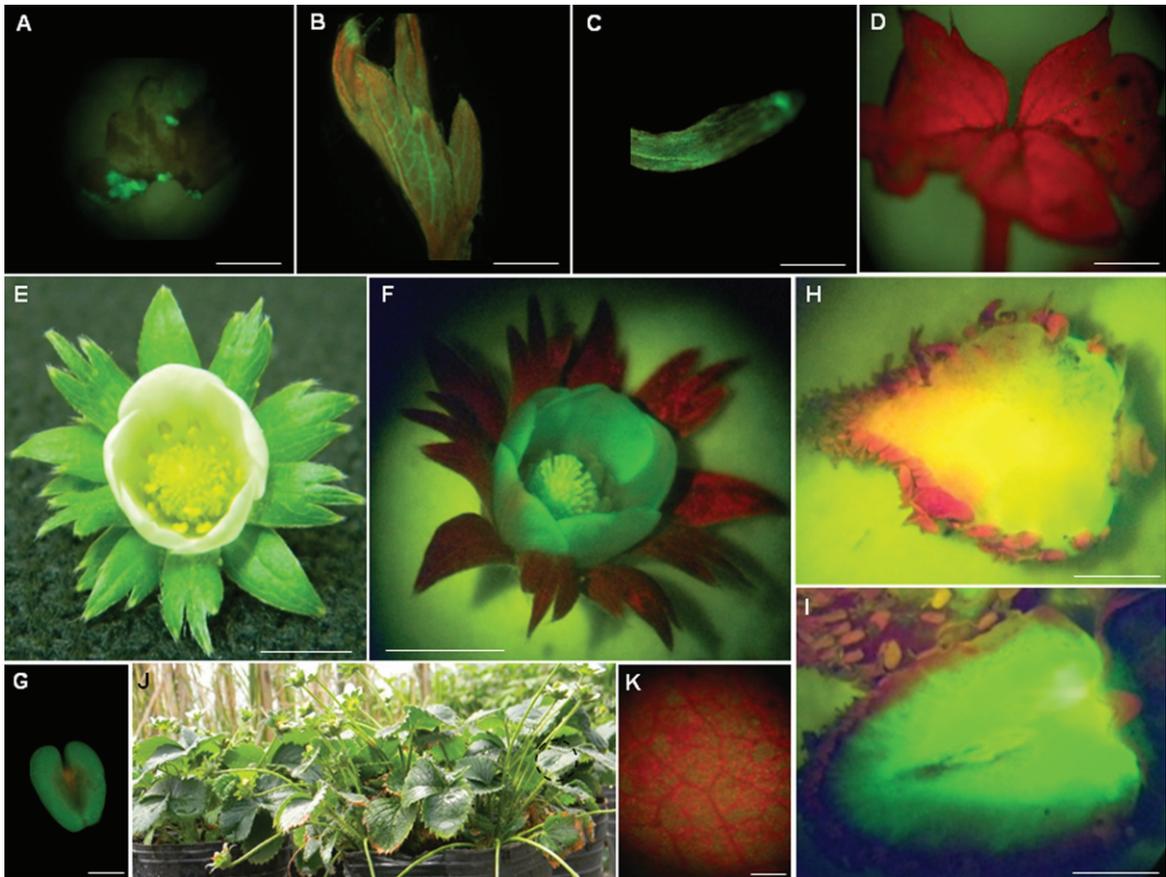


Fig. 1. Expression of GFP protein from transformed callus to strawberry fructification. A) A transformed callus expressing GFP protein; bar = 3 mm. B) A fluorescent non-expanded leaf, bar = 3 mm; C) fluorescent root, bar = 1 mm and D) expanded non-fluorescent leaf, bar = 3 mm. Normal flower with its calyx, non-expanded petals, and numerous stamens inserted into the receptacle observed under white (E) and UV light (F); bars = 7 mm. G) A fluorescent stamen observed under UV light; bar = 1 mm. View of a longitudinally-cut white (H) and red (I) fruit observed under UV light, bars = 9 mm. J) Vegetative multiplication of stably transformed GFP strawberry plants in greenhouse. K) Foliar tissue from these plants displayed green fluorescence partially masked by red fluorescence from chlorophyll. One representative image of each developmental stadium was used for illustration.

hence, it was chosen for further analysis and studies (Fig. 1). During calluses formation in the leaf tissue of the selected transgenic line, a homogenous and strong green fluorescence was observed in the entire cellular mass (Fig. 1A). Intense fluorescence was observed in proliferating shoots (Fig. 1B) and roots (Fig. 1C) of *in vitro* plants, but it almost disappeared in totally expanded leaves (Fig. 1D). Transgenic flowers presented normal aspect with its calyx and sepals, the corolla with five white petals, and numerous stamens inserted at the base of the receptacle (Fig. 1E). Under UV light, green fluorescence was visualized in the petals, receptacle and stamens (Fig. 1F). Zoom view of a fluorescent stamen (Fig. 1G). Likewise, the fleshy receptacle tissue of white (Fig. 1H) and red (Fig. 1I) fruits displayed intense fluorescence. The vegetatively multiplied T0 progeny of transgenic plants grew normally in greenhouse and produced numerous runners (Fig. 1J). Foliar tissue of these plants displayed green fluorescence partially masked by red fluorescence from chlorophyll (Fig. 1K).

To investigate whether the lack of fluorescence in those totally expanded leaves was due to a masking phenomenon of the red fluorescence emitted by chlorophyll, *in vitro*-grown plants were kept in darkness until

chlorophyll disappeared (Online Resource 5A, C). When they were exposed under UV light, an intense green fluorescence was detected in shoots (Online Resource 5B) and roots (Online Resource 5D). In addition to these observations, the fact that green fluorescence was detected in adult leaves of stably transformed GFP plants grown in soil (Fig. 1K) led us to speculate that a gene silencing process was occurring during plant ontogeny process. This hypothesis was reinforced by the fact that non-fluorescent leaves of *in vitro* GFP transgenic plants (Online Resource 6A,) could regenerate fluorescent calluses when cultured in SRM (Online Resource 6B, C).

3.2. *sgfp* transient expression in *F. x ananassa* leaves

In WT plants, green fluorescent spots denoting GFP transient expression were slightly visible for first time 7 days post agroinfiltration (dpa) with pBIN19-*sgfp*. No damages on foliar tissue were recorded just after agroinfiltration, rejecting any possible initial fluorescence emitted as a mechanical response (Online Resource 2). The level of fluorescence highly increased among 10 and 14 dpa (Fig. 2A). Thereafter, the fluorescence decreased being almost imperceptible 24 dpa. By that time leaves started to get necrotic making difficult further fluorescence observations. On the contrary, no fluorescence was observed after agroinfiltration with the controls EHA105-WT or IS at any time analysed. Evaluation of the green spots allowed estimating quantitatively that the maximum GFP expression level was reached between 10 and 14 dpa (Fig. 2B). From these results we speculated that a silencing mechanism was activated after the peak of *sgfp* expression which might be the cause of fluorescence decay observed thereafter. To verify this hypothesis, agroinfiltration with the co-culture pBIN19-*sgfp*+pBIN61-P19 was carried out in WT plants. This procedure caused a significant fluorescence increase in these plants 17 dpa, reaching similar levels as in the transient expressed control pBIN19-*sgfp* (Fig. 3A, B).

3.3. *sgfp* silencing in transgenic *F. x ananassa* leaves

With the aim to verify whether the dsGFP hairpin was actually able to silence the *sgfp* transgene (panels pBICdsGFP and EHA105-WT in Fig. 4A) or not, a similar strategy as in WT plants was carried out but the pBIN19-*sgfp* construct was co-infiltrated to enhance the fluorescent signal over the green constitutive background. This strategy allowed us to clearly observe the transient expressed GFP fluorescence as brilliant green spots protruding over the green background 10 dpa on, and it was specially observed in veins 12 and 14 dpa (panel pBIN19-*sgfp*). To confirm whether a silencing mechanism was induced by the hairpin in strawberry leaves, a co-culture of the pBIN19-*sgfp* + pBICdsGFP was agroinfiltrated. In this case decay in the fluorescence was detected between 12 and 17 dpa (Fig. 4B), mainly in the tissue around veins (see panel pBIN19-*sgfp* + pBICdsGFP in Fig. 4A), in comparison to the control pBIN19-*sgfp*.

4. Discussion

4.1. GFP transgenic plant

With the aim to develop *in planta* transient expression and silencing systems in *F. x ananassa* cv. 'Pájaro' leaves, first a stably transformed GFP strawberry plant was attained. In the present study we report for the first time the generation of a GFP transgenic plant in the octoploid strawberry *F. x ananassa* cv. 'Pájaro' that expresses the protein in all the tissues. Transgenic plants grew normally as denoted by flowers, fruits and runner production. There are few studies reporting stable transformation with *sgfp* gene in strawberry, three performed in the diploid *Fragaria vesca* [39, 40, 41], and just one in the octoploid *F. x ananassa* [42]. An interesting issue observed in this study is that while young and expanding *in vitro* tissues displayed a strong and homogenous green fluorescence, mature leaves did not, as if chlorophyll masking effect was taking place. Fluorescence disappearance was also

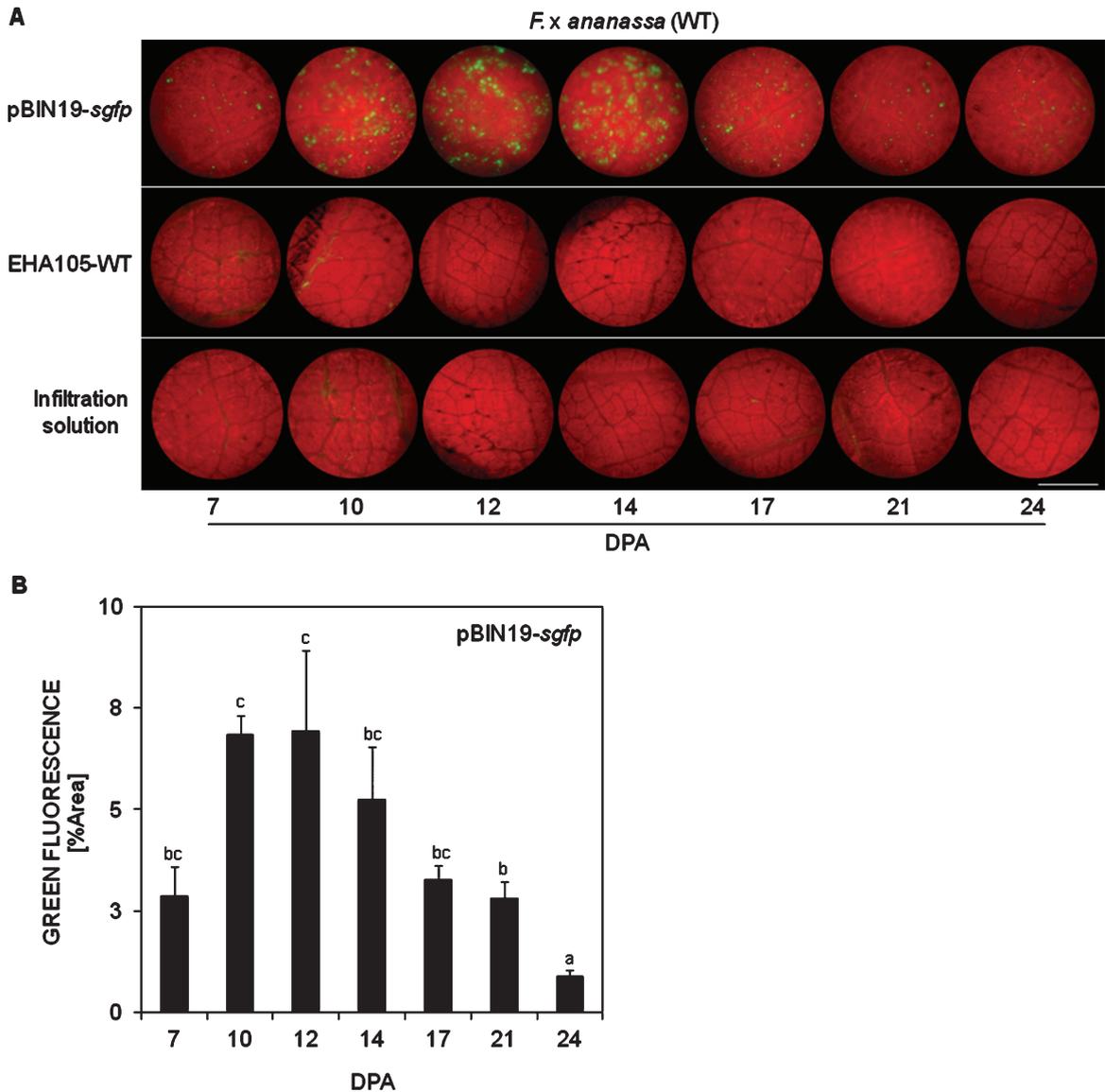


Fig. 2. *In planta sgfp* transient expression in WT *F. x ananassa* leaves. A) Green fluorescence after agroinfiltration with pBIN19-*sgfp* at different days post agroinfiltration (DPA). Infiltrations with EHA105-WT or infiltration solution were used as controls. B) Quantification of green fluorescence. Means \pm SEs are represented from one typical assay ($n = 36$). Two independent assays were performed with similar results. Analysis of non-parametric variance was performed via Kruskal-Wallis test. Different letters represent statistically significant differences ($P \leq 0.05$). Bar = 0.9 mm.

reported in transgenic *F. vesca* plants by [39 and 40], who proposed that green fluorescence could be masked by the intense red fluorescence of chlorophyll, but did not prove it. Here the hypothesis of chlorophyll masking was rapidly discredited since intense green fluorescence was detected in GFP stably transformed *in vitro*-grown etiolated plants and also in mature leaves of GFP transgenic plants grown in greenhouse. It is widely reported that PTGS is involved in regulating the expression of endogenous and foreign genes along the development of

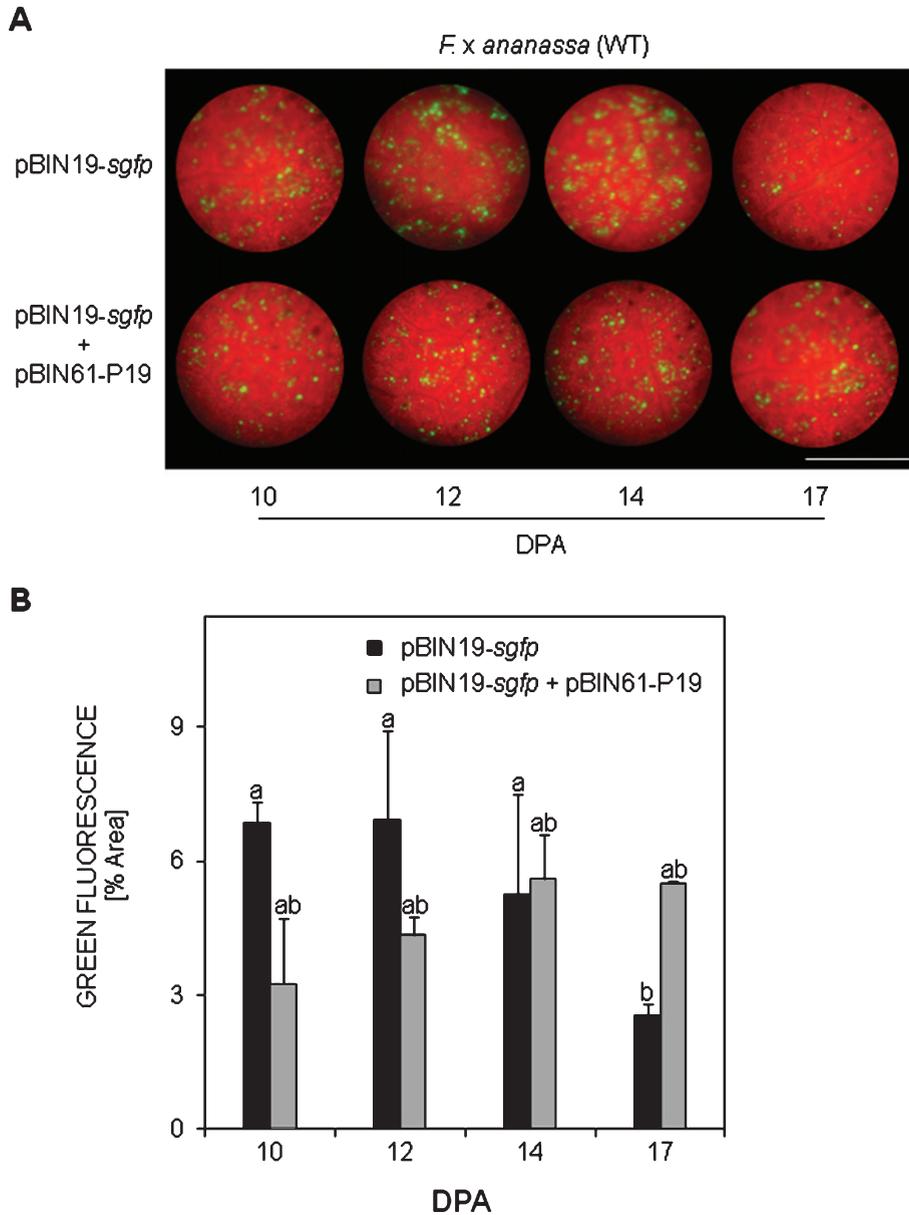


Fig. 3. *In planta* suppression of intrinsic *sgfp* silencing in WT *F. x ananassa* leaves. A) Restoring of green fluorescence by the silencing suppressor protein P19 after agroinfiltration with a co-culture of pBIN19-*sgfp* + pBIN61-P19, compared towards the transient expression positive control pBIN19-*sgfp* at different days post agroinfiltration (DPA). B) Quantification of green fluorescence. Means \pm SEs are represented from one assay ($n = 12$). Analysis of variance (ANOVA) followed by LSD-Fisher test was performed. Different letters represent statistically significant differences ($P \leq 0.10$). Bar = 0.9 mm.

plants through highly specific interactions [43]. According to our observations, it is more likely that a PTGS process occurs during *in vitro* plant ontogeny of *F. x ananassa* plants than a chlorophyll masking effect, being active during a specific stage of the *in vitro* culture and not in undifferentiated cells from regenerating calluses, neither in expanding young tissues nor in mature tissues from plants grown in soil.

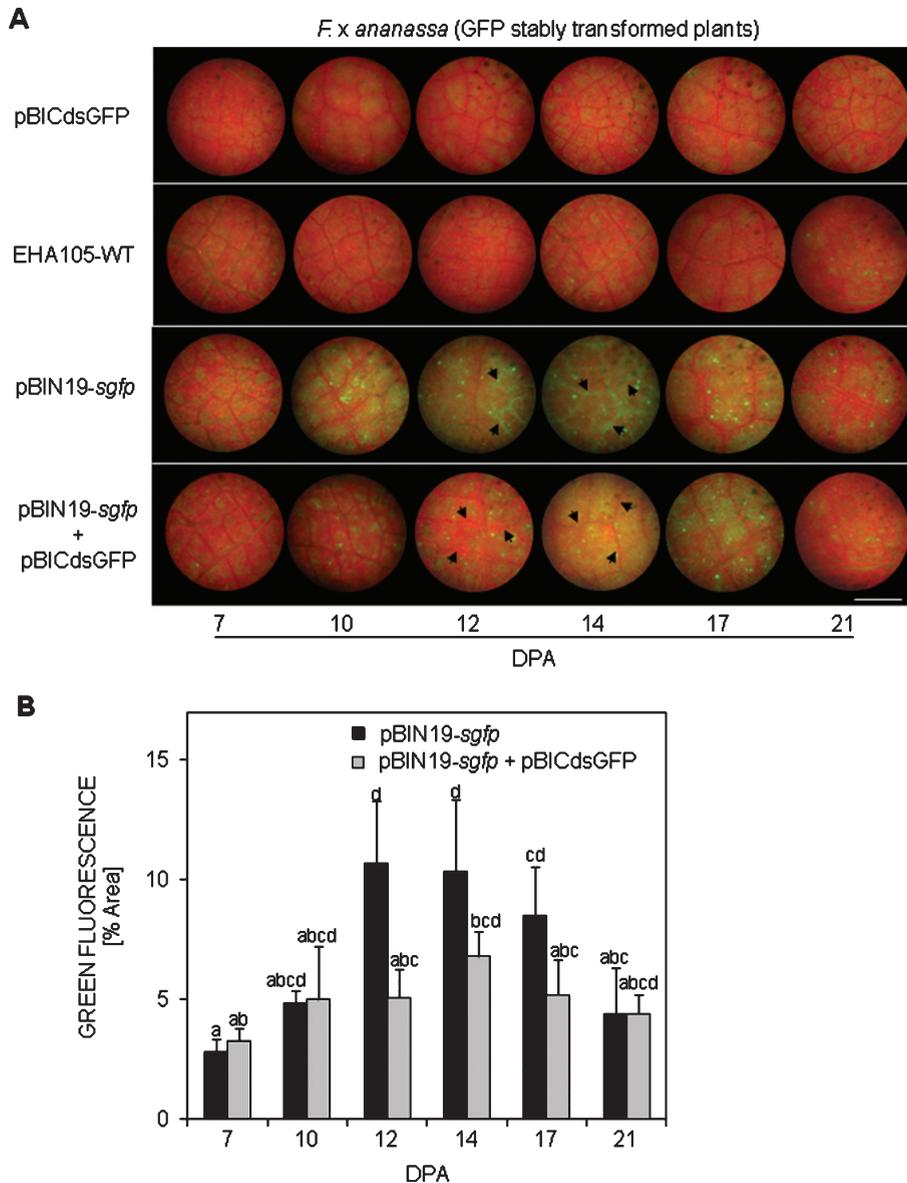


Fig. 4. *In planta sgfp* silencing in stably transformed GFP *F. x ananassa* leaves. A) Residual green fluorescence after pBICdsGFP and EHA105-WT (control) agroinfiltrations, and after silencing (pBIN19-*sgfp* + pBICdsGFP) in comparison to pBIN19-*sgfp* (control) at different days post agroinfiltration (DPA). B) Quantification of green fluorescence. Means \pm SEs are represented from one typical assay ($n = 36$). Two independent assays were performed with similar results. Analysis of non-parametric variance was performed via Kruskal-Wallis test. Different letters represent statistically significant differences ($P \leq 0.05$). Bar = 0.9 mm.

4.2. *sgfp* transient expression

In planta sgfp transient expression was observed for first time 7 dpa in WT *F. x ananassa* leaves. Our finding does not coincide with observations made in numerous plant species in which transient expression of genes

in general, and *gfp* in particular, is detected between 2 and 4 dpa, like reported in leaves of *Arabidopsis* [5], tobacco [6] and potato [7], or as for lettuce and tomato fruits [5], and even strawberry fruits [8]. The difference in transient expression timing between strawberry fruits and leaves could be due to tissues nature, being earlier and clearly visualized in fleshy and soft strawberry fruits than in foliar tissue. Another possibility is that fluorescent compounds such as polyphenols present in *F. x ananassa* leaves could interfere with an early low initial green fluorescence. It is noteworthy that the transient expressed GFP protein was observed as bright and diffuse green fluorescent spots in strawberry leaves, rather than a regular green patch as observed in the well-studied tobacco system [6, 44]. The setup of this simple and reproducible protocol allowed long-term and high levels of *sgfp* expression, and is of great importance since, to our current knowledge, there are no other studies reporting *in planta sgfp* transient expression in *Fragaria x ananassa* leaves. Instead, most of the studies available for strawberry are performed in fruits and use *GUS* despite the drawbacks this marker gene implies [8, 24, 26]. There is only one report about YFP transient expression in leaves of *F. vesca* but was carried out in detached leaves [27]. The current study might contribute to *in vivo* continuous monitoring of functional promoters and protein expression in *F. x ananassa* leaves. For example, defense-inducing elicitors such as the fungal subtilisin-like protein AsES well studied in our laboratory could be transiently expressed in order to evaluate its efficiency to protect against pathogens, similarly as when it is applied exogenously [45, 46, 47]. The results of this study might justify obtaining a pathogen resistant AsES-transgenic strawberry plant.

By the silencing protein suppressor P19 we show that an intrinsic PTGS mechanism could be activated in strawberry leaves and might be the responsible for the rapid decrease of fluorescence after *sgfp* peak expression 17 dpa. Despite new assays should be carried out to confirm this observation, our preliminary results are in agreement with numerous authors who established that PTGS either contributes to, or is the major cause of the rapid decline in transient expression of genes introduced into *Nicotiana benthamiana* and *A. thaliana* [37, 48, 49]. Similarly, PTGS governed the rapid declined of *gfp* expression in Lima bean (*Phaseolus lunatus* L. cv. 'Henderson-Bush') until undetectable levels 72 hpa [50].

4.3. *sgpf* gene silencing

There are no reports about gene silencing in strawberry leaves. All available studies were carried out in fruits in which genes such as mannose-binding lectin (*FaMBLI*) involved in unripe and ripe fruit susceptibility to *Colletotrichum acutatum* [21], glycosyltransferase 1 (*FaGT1*) involved in anthocyanin synthesis [22], chalcone synthase (*CHS*) [23], or the transcription factor *FcMYB1* [26] have been partially or totally silenced. In the present study no reduction of constitutive fluorescence was detected after the RNA hairpin dsGFP was agroinfiltrated in leaves of GFP stably transformed *F. x ananassa* plants. Our results disagree with those achieved in *N. benthamiana* and *A. thaliana* in which *gfp* was clearly silenced by the dsGFP hairpin [48, 49]. To confirm that a silencing mechanism was being activated in strawberry leaves we first enhanced the green fluorescence signal by agroinfiltration of pBIN19-*sgfp* and in this background we evaluated the hairpin capacity to silence the *sgfp* transgene by monitoring fluorescence decay. This strategy allowed us to detect fluorescence reduction of about 30% and 50% along the time, confirming *sgfp* silencing. It is likely there is a threshold that determines the levels of *sgfp* mRNA required for triggering specific degradation of all homologous mRNA in the cytoplasm, as proposed in tobacco by [51]. We can also speculate that there are remaining translated GFP proteins that are so stable that keep emitting fluorescence although the silencing process might had effectively blocked the transduction of *sgfp* mRNA. Interestingly, fluorescence decay was observed mainly in the tissue around veins supporting the idea that in strawberry there is a silencing-mediating diffusible signal that moves from the initial infiltration point through veins, as observed in *N. benthamiana* and *A. thaliana* [48, 49].

In this study we obtained knockdown GFP phenotypes rather than knockouts; thus, we speculate that the expression of dsGFP hairpin alone is not sufficient to induce a complete silencing of *sgfp* in stably transformed GFP *F. x ananassa* leaves. Future studies should be conducted in order to quantify mRNA and siRNA to better understand this phenomenon. It is of great importance to count with an effective silencing method in leaves of

the cultivated strawberry because it might be used for silencing genes associated to the synthesis of allergenic compounds, as well as genes related to pathogen receptors [52] and negative regulators of defense genes.

5. Conclusion

The present study describes for the first time the attainment of a transgenic *F. x ananassa* cv. 'Pájaro' plant expressing GFP protein in all the tissues during the whole life cycle. In addition, efficient and reproducible systems for the continuous monitoring *in planta* of *sgfp* transient expression and silencing are reported for the first time in *F. x ananassa* leaves. These contributions are of great importance for the reverse genetic field in the *Rosaceae* family in general and strawberry in particular because they may be further used for the expression and silencing of genes of potential agronomic interest in agronomical important species.

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Author contributions

VHC, MEI and RHTG performed the experiments, and collected, analyzed and interpreted the data. VHC, MEI, GRV and JCDR conceived and designed the experiments. VHC and MEI wrote the manuscript. JCDR, APC and VHC revised critically the article. All authors approved the final version of the manuscript.

Conflict of interest

The authors have no conflict of interest to report.

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Supplementary material

The supplementary material is available in the electronic version of this article: <http://dx.doi.org/10.3233/JBR-190447>.

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