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Agrobacterium tumefaciens and Agrobacterium rhizogenes-mediated transformation of potato and the promoter activity of a suberin gene by GUS staining

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Dear Editor,

We are hereby replying the comments raised by the Reviewers (and the Editor) for the manuscript JoVE59119 entitled: "*Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*-mediated transformation of potato and the promoter activity of a suberin gene by GUS staining" by Sandra Fernández-Piñán, Jennifer López, Iker Armendariz, Pau Boher, Mercè Figueras and Olga Serra.

We have structured the Reviewers' comments in a question and answer (in blue) format in order to facilitate the identification of the points raised by them.
The original manuscript has been extensively revised and modified according to the reviewers' comments in order to clarify their questions and to increase the general quality and readability of the manuscript. The authors express their gratitude to the Reviewers for their time and for providing helpful comments, which have clearly contributed to the improvement of our original submission. We are confident that, following this revision, the manuscript is clearer and we hope that it will be finally considered acceptable for publication.

Best regards,

Olga Serra
Universitat de Girona.

TITLE:

Agrobacterium tumefaciens and *Agrobacterium rhizogenes*-Mediated Transformation of Potato and the Promoter Activity of a Suberin Gene by GUS Staining

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KEYWORDS:

Agrobacterium rhizogenes, *Agrobacterium tumefaciens*, *Solanum tuberosum*, suberin, root, potato

SUMMARY:

Here, we present two protocols to transform potato plants. The *Agrobacterium tumefaciens* transformation leads to a complete transgenic plant while the *Agrobacterium rhizogenes* produces transgenic hairy roots in a wild type shoot that can be self-propagated. We then detect promoter activity by GUS staining in the transformed roots.

ABSTRACT:

Agrobacterium sp. is one of the most widely used methods to obtain transgenic plants as it has the ability to transfer and integrate its own T-DNA into the plant's genome. Here, we present two transformation systems to genetically modify potato (*Solanum tuberosum*) plants. In *A. tumefaciens* transformation, leaves are infected, the transformed cells are selected and a new complete transformed plant is regenerated using phytohormones in 18 weeks. In *A. rhizogenes* transformation, stems are infected by injecting the bacteria with a needle, the new emerged transformed hairy roots are detected using a red fluorescent marker and the non-transformed roots are removed. In 5-6 weeks, the resulting plant is a composite of a wild type shoot with fully developed transformed hairy roots. To increase the biomass, the transformed hairy roots can be excised and self-propagated. We applied both *Agrobacterium* mediated-transformation methods to obtain roots expressing the *GUS* reporter gene driven by a suberin biosynthetic gene promoter. The GUS staining procedure is provided and allows the cell localization of the

promoter induction. In both methods, the transformed potato roots showed GUS staining in the suberized endodermis and exodermis, and additionally, in *A. rhizogenes* transformed roots the GUS activity was also detected in the emergence of lateral roots. These results suggest that *A. rhizogenes* can be a fast alternative tool to study the genes that have a role in roots.

INTRODUCTION:

Aside from economic interest, the generation of transgenic plants has its own relevance in research to demonstrate the ultimate function of genes and to better understand plant physiology and development. The most widely used method for plant DNA insertion is *Agrobacterium*-mediated transformation. *Agrobacterium tumefaciens* is able to generate crown galls in the infected tissue of many plant species by the action of its tumour-inducing (Ti) plasmid. The plasmid contains a T-DNA region with a set of genes that will be integrated in the plant genome and induce tissue dedifferentiation[1,2]. The exchange of these genes within the T-DNA by the transgene has allowed the generation of specific plant modifications avoiding phenotypic effects[3]. To promote the transgene cloning into the T-DNA, the T-DNA region has been excised in an independent plasmid called a binary plasmid, while the rest of the genes of the Ti plasmid (the virulence genes that allow the T-DNA transfer and insertion mechanisms) have been placed in a helper plasmid. For plant biotechnology research, transformation by *A. tumefaciens* has several advantages: it does not need expensive devices, is able to generate both stable and transient plant transformation, and integration of low numbers of gene copies into the chromosome[4]. However, for most plants, but not Arabidopsis, the generation of stable transformants requires plant regeneration from a single or a few cells using exogenous phytohormones, making this process laborious and time consuming. *A. rhizogenes* is also able to modify the plant genome, but in this case, it yields hairy roots or adventitious roots due to the expression of *rol* (root *loci*) genes encoded in the root-inducing (Ri) plasmid[5]. Although less studied than *A. tumefaciens*, *A. rhizogenes* is also used for obtaining transgenic roots. In this case, the *A. rhizogenes* contains the original T-DNA in the Ri plasmid and a binary plasmid with a second T-DNA carrying the transgene. When the infection site is in stems or hypocotyls, a composite plant can be obtained, with new hairy transgenic roots emerging from wild type shoots. Alternatively, hairy transformed roots can grow autonomously in vitro in media with carbon source inputs. The use of *A. rhizogenes* instead of *A. tumefaciens* to produce transgenic tissue is gaining relevance when the root is the target organ, because plant regeneration is not required and hence it is faster and less costly. Previous studies have demonstrated this methodology appropriated for the phenotypic characterization of root specific genes[6–9].

The potato (*Solanum tuberosum*) is the fourth most important crop in the world according to the Food and Agriculture Organization of the United Nations (FAO) since the tuber has nutritional relevance for human consumption for being a good source of vitamins and minerals. For that reason, potato has been placed in the spotlight of agricultural biotechnology and also is considered as a good biological model for genetic and developmental studies[10,11]. Potato transformation significantly contributed to the understanding of molecular mechanisms underlying suberized tissues through the characterization of genes involved in suberin and wax biosynthesis[12–17], suberin monomer transport[18] and transcription regulation[19]. The suberin

feruloyl transferase gene, *FHT*, is one of these characterized biosynthetic genes; its downregulation gives rise to a strong impairment of the periderm protection, which is correlated with a strong decrease in ferulate esters of suberin and waxes in potato tubers[14]. Concomitantly, in roots and seeds of Arabidopsis, the knockout of its putative orthologue (*ASFT/RWP1*) also demonstrated its role in producing alkyl ferulates in suberin[20,21]. In potato, the *FHT* transcriptional reporter line and the FHT antibody showed that the promoter activity and the protein are located in the exodermis, the endodermis, the phellogen of the periderm and in wounded tissues[15].

In this work, we detail a protocol using *A. rhizogenes* to produce transgenic hairy roots that are maintained in a wild type stem, generating composite potato plants, or excised to grow autonomously in vitro. We also provide the protocol using *A. tumefaciens* to obtain complete transgenic potato plants. As a case study, *A. rhizogenes* and *A. tumefaciens* transformed with the same binary vector are used to obtain roots with the *FHT* promoter driving *GUS* reporter gene expression. The results are reported and compared.

PROTOCOL:

The *A. rhizogenes* transformation protocol was adapted and modified from Horn et al.[7] and the genotype tested was *S. tuberosum* ssp*. tuberosum* (cv. Désirée). The *A. tumefaciens* transformation protocol was adapted and modified from Banerjee et al.[22] and the genotypes tested were *S. tuberosum* ssp*. tuberosum* (cv. Désirée) *and S. tuberosum* ssp. *andigena*. The main steps of both procedures are summarized in **Figure 1 and Figure 2**, respectively.

NOTE: In all the steps of the procedure performing in vitro transfers, do so rapidly, and when possible, maintain the plates or pots closed, thus minimizing plant exposure to the air to avoid wilting and contamination. Otherwise stated, all the plant incubations were done in cabinets under long day conditions of 12 h of 24 °C light/ 12 h of 20 °C dark and 67 µmol m^{-1} s^{-1}. Otherwise stated, perform all the bacteria manipulation and in vitro plant transfers in aseptic conditions in a laminar flow hood. All the media recipes for *Agrobacterium* and in vitro plant cultures are provided in **Table 1**.

CAUTION: Deposit all material contaminated with genetic modified plants to the appropriated waste container.

[Place Table 1 here]

1. *Agrobacterium* cultures used for transformation

NOTE: The strain used for *A. rhizogenes* was the C58C1:Pri1583[7] (kindly provided by Dr. Inge Broer) and that for the *A. tumefaciens* was the GV2260. Both bacteria were transformed with the binary vector carrying the transgene. *A. rhizogenes* was transformed with PK7GWIWG2_II-RedRoot (VIB-Department of Plant Systems Biology at Universiteit Gent) that contains a T-DNA

carrying a selectable marker to monitor the hairy root formation. To compare the transformed roots generated by both *Agrobacterium*, *A. rhizogenes* and *A. tumefaciens* were transformed with the binary vector pKGWFS7 which contains a T-DNA carrying the *FHT* promoter driving the *β-glucoronidase* (*GUS*) reporter gene and the Kanamycin resistance as a selective marker[15].

1.1. Pick a colony of *Agrobacterium* and grow it overnight (O/N) in 5 mL of YEB medium supplemented with antibiotics (**Table 1**) in a 50 mL centrifuge tube at 28 °C with shaking at 200 rpm.

1.2. For *A. tumefaciens* transformation, measure the optical density, which must be OD_{600} = 0.6-1.

1.2.1. If the optical density is higher, make a subculture lowering it to OD_{600} = 0.3 with fresh media and wait until the culture reaches OD_{600} = 0.6-1.

1.3. Centrifuge 1 mL of *Agrobacterium* culture at 3,000 x *g* in a bench-top centrifuge for 10 min at room temperature.

1.4. Remove the supernatant by pipetting, and resuspend cells in 1 mL of fresh YEB medium without antibiotics. Repeat this step twice to ensure the complete removal of antibiotics.

1.4.1. For *A. tumefaciens* transformation, in the last resuspension add the appropriate YEB volume to obtain a final optical density of OD_{600} = 0.8.

1.5. Keep cells on ice while preparing the plants to be infected.

2. Plant material for transformation

2.1. Make one or two node stem cuttings either containing the apical or the auxiliary buds from sterile *in vitro* potato plants (donor plants); grow them in solid 2MS medium in pots for 3 to 4 weeks (**Figure 1A** and **Figure 2A**).

3. Plant transformation using *A. rhizogenes* (Figure 1)

NOTE: This procedure allows the obtaining of transformed hairy roots. To evaluate the transgene expression, a negative control is needed. To prepare the negative control, follow the procedure using an *A. rhizogenes* strain either untransformed or transformed with the empty vector that includes the transformation marker.

3.1. Use fresh media plates; alternatively, the plates can be kept at 4 °C with the lid side up, tightly sealed with transparent film to avoid media dehydration. To prepare the square media plates, incline them ~15°, fill with 40 mL of MS, and let solidify. This will help the aerial part of the plant to minimize the contact with the medium.

3.2. Transfer very carefully a donor plant from the 2MS medium to a 120 mm x 120 mm square plate.

3.3. Inject to one stem internode 5 µL of the *A. rhizogenes* culture using a surgical needle and repeat it twice per plant in different internodes.

NOTE: Consider each injection as an independent transformation event (**Figure 1B**).

3.4. Transfer immediately the entire plant to a square plate with solid MS medium supplemented with 0.1 mM acetosyringone. Accommodate 2 plants per plate.

NOTE: The 1 M stock of acetosyringone is prepared in DMSO and can be stored at -20 °C.

3.5. Seal the plate using surgical tape and arrange it vertically inside a growth cabinet for 2 weeks.

3.6. Excise the native roots of the plant, and transfer the plant to a new square plate with MS medium supplemented with cefotaxime sodium [500 µg/mL] to kill *A. rhizogenes*. The new hairy roots will start to appear at this time (**Figure 1C**,**1D**). They can be checked by red fluorescence when using a DsRed transformation marker (**Figure 3D**).

3.7. To obtain a composite plant (**Figure 1E**), let the transgenic hairy roots grow for 3-4 weeks in MS medium supplemented with cefotaxime sodium [500 mg/mL] (change the medium every week).

3.8. Depending on the purpose, propagate the transgenic hairy roots and the negative controls as follow.

3.8.1. Transfer the whole composite plant to a hydroponic (**Table 2**) or soil medium to allow for massive development.

3.8.2. To individually propagate the transformed hairy roots, using a scalpel cut the roots expressing the red fluorescent transformation marker (DsRed protein) when they are 4-8 cm long (**Figure 1E**) and transfer them into a Petri dish with Gamborg B5 solid medium supplemented with 2% sucrose and cefotaxime sodium [500 mg/mL]. Seal the plates with laboratory film and grow them in the dark at 20 °C.

NOTE: The roots can be manipulated under a stereomicroscope equipped to detect the fluorescence (see **Table of Materials**).

3.8.3. For biomass production (i.e., gene expression analysis), cut a 5 cm long hairy root and propagate it in a 150 mL Erlenmeyer flask with 20 mL of Gamborg B5 liquid medium

supplemented with 2% sucrose and cefotaxime sodium [500 µg/mL]. Grow it for 6 weeks in the dark at 20 °C and 60 rpm.

[Place Figure 1 here]

4. Plant transformation using *A. tumefaciens* (**Figure 2**)

NOTE: This procedure allows the obtaining of transformed plants. To evaluate the transgene's effect, a negative control is needed. One option is to follow the procedure using an *A. tumefaciens* transformed with the empty vector. Alternatively, wild type plants can be used.

4.1. Place a leaf from the 3-4-week old plants in a Petri dish. Using a scalpel exclude the petiole and make transverse cuts (1-3 depending on the leaf size) from the center of the leaf to the edges avoiding cutting them off (**Figure 2B**).

4.2. Immediately place the leaf floating on 10 mL of fresh 2MS liquid media in a Petri dish with the abaxial side up and close the plate. Repeat the step accommodating up to 15 leaves for cv. Désirée and 25 leaves for ssp. a*ndigena* (depending on the leaf size).

4.3. Immediately add 80 µL of *A. tumefaciens* culture at OD_{600} = 0.8 in the liquid media and homogenize the plate manually for 1 min to distribute the bacterial solution.

4.4. Carefully seal with sealing film, cover with aluminum foil and incubate for 2 days in a dark chamber at 24 °C to let the transformation occur.

4.5. Transfer the leaves keeping abaxial side up to CIM medium (**Figure 2B**) and incubate them for one week in a growth cabinet.

4.5.1. Scrape the CIM medium with the tweezers so that the leaves can be better accommodated on the media.

4.6. Transfer the leaves keeping abaxial side up to SIM medium (**Figure 2C**) and incubate them in a growth cabinet, refreshing the medium every 7-10 days, until the shoots are about 2 cm tall (**Figure 2E**).

4.6.1. Scrape the SIM medium with the tweezers so that the leaves can be fully surrounded by the media. When the emerged shoots reach the lid, work with tall Petri dishes.

NOTE: The callus will form after 2-3 weeks in SIM medium (**Figure 2D**) and the shoots after 6-7 weeks. The shoots will be considered as independent transformation events when they emerge from callus formed from independent wounds. The shoots will form after 6-7 weeks in SIM medium (**Figure 2E**).

4.7. Cut three shoots emerged from each callus (considered the same transformation event) (**Figure 2E**), transfer them to culture flasks with MG medium supplemented with cefotaxime sodium [250 mg/L] to allow rooting, label the subset with a number and incubate in a growth cabinet for 3-4 weeks or until the shoots are vigorous (**Figure 2F**).

NOTE: When cutting the shoots, remove well the callus otherwise the root will not form.

4.7.1. Repeat the step as many times as independent lines are needed. Up to 5 different transformation events can be placed in a culture flask with a diameter of 8 cm to work in full confidence that the plants from different events are not mixed.

4.8. Select the most vigorous plant of each event, cut the apical segment of the shoot with 3-4 internodes and place it in a new culture flask with 2MS medium supplemented with cefotaxime sodium [250 mg/L].

NOTE: In 3-6 weeks the plant will grow efficiently, developing a vigorous shoot and roots.

4.8.1. Bring back to the chamber the non-selected shoots until the plant selected has fully developed.

4.9. Make stem segments from the plant with at least one internode or with the apical bud and transfer them to new 2MS medium supplemented with cefotaxime [250 mg/L]. Incubate them in the growth cabinet.

4.9.1. Replicate every 3-4 weeks to establish the in vitro transformed lines.

NOTE: The cefotaxime sodium is needed in at least three subsequent transfers to 2MS medium to be sure to kill the *A. tumefaciens*; afterwards, if *A. tumefaciens* overgrowth is observed, transfer plant lines again to 2MS media supplemented with cefotaxime sodium.

4.10. To characterize the plant phenotype, transfer plants to soil for their full characterization or to hydroponics for root inspection.

4.10.1. Keep the tubers produced in soil to propagate and maintain the established lines.

[Place Figure 2 here]

5. Hydroponic culture

5.1. Prepare the Hoagland's solution to a half strength (0.5x) (**Table 2**) in a 10 L bucket.

5.2. Immerse an aquarium pump to maintain homogeneity and proper oxygen conditions.

5.3. Cover the walls of the bucket with aluminum foil to grow roots in dark conditions.

5.4. Avoiding root damage, transfer in vitro plants to hydroponic culture.

NOTE: Remove any remaining in vitro medium from roots to avoid microorganism proliferation during incubation by shaking carefully the roots immersed in water.

5.5. Cover the plants with transparent film like a glasshouse to allow adequate acclimatization and incubate in the growing chamber.

5.6. Make holes in the film after 3 days and remove it completely one week after.

5.7. Replace with fresh media every 10 days.

[Place Table 2 here]

6. *GUS* histochemical reporter gene assay

NOTE: In our case the GUS analysis was performed with roots of 2-3 weeks grown in hydroponics.

6.1. Fix the roots with 90% chilled acetone (v/v) and incubate it for 20 minutes on ice.

6.2. Perform two washings with distilled water.

6.3. Add fresh GUS staining solution (**Table 3**) and apply vacuum (-70 Pa) for 20 min.

6.4. Incubate at 37 °C in dark to protect the photosensitive GUS for 4 h or until a blue color is visible.

NOTE: The presence of ferri- and ferrocyanide in GUS solution minimize the diffusion of reaction products and provide more precise localization.

CAUTION: Use a fume hood and wear protective clothing when handling the toxic cyanide derivatives in GUS solution (the potassium ferricyanide and potassium ferrocyanide). The GUS substrate and the disposal material should be disposed safely.

6.5. Remove GUS staining solution and discard it in appropriate containers.

6.6. Perform two washings with ethanol 70% (v/v).

6.7. Observe under a bright field microscope.

NOTE: GUS staining is stable for a few weeks; however, during the first week, the GUS signal is clear and diffuses less into neighboring cells. For longer storage, seal the tube and store it at 4 °C.

[Place Table 3 here]

REPRESENTATIVE RESULTS:

***Agrobacterium rhizogenes*-mediated potato transformation**

In this manuscript the step-by-step procedure set up to obtain transformed root with *A. rhizogenes* is presented. **Figure 1** presents an overview of the assay, which altogether takes around 5-6 weeks (from injection of *A. rhizogenes* to obtain fully developed hairy roots). Then, the plant can be studied as a composite (wild type shoot, transgenic root) or the transgenic hairy root clones can be excised and grown autonomously in solid Gamborg B5 medium supplemented with 2% sucrose. Alternatively, the hairy roots can be massively propagated using the liquid Gamborg B5 media. The procedure presented has been carried out with *S. tuberosum* spp. *tuberosum* (cv. Désirée).

The method to monitor the procedure and to obtain potato transgenic hairy roots has been validated using a binary vector with the DsRed as a transformation marker (PK7GWIWG2_II-RedRoot from VIB-Department of Plant Systems Biology at Universiteit Gent). This allowed the easy distinction of transgenic hairy roots from non-transgenic by the red fluorescence. According to that, in **Figure 3** the transformed hairy roots exhibited red fluorescence when illuminated with green light. The negative control using the untransformed *Agrobacterium* showed no red fluorescence (**Figure 3C**), overall indicating the suitability of the DsRed transformation marker to identify the transgenic hairy roots (**Figure 3D**). Other transformation markers such as antibiotic resistance can be used as described by other authors[23,24]; however, the antibiotics in the media can produce a growth delay in transgenic roots containing the marker.

[Place Figure 3 here]

***Agrobacterium tumefaciens*-mediated potato transformation**

The second protocol described in this manuscript is set up to obtain, step-by-step, a complete potato plant transformed with *A. tumefaciens.* **Figure 2** presents an overview of the procedure, which altogether takes between 15-18 weeks (from leaf infection with *A. tumefaciens* to obtaining fully regenerated plants). The most time-consuming part of the procedure is the plant regeneration by organogenesis. This particular step makes this method more laborious than using *A. rhizogenes*. The procedure has been carried out with *S. tuberosum* spp. *tuberosum* (cv. Désirée) and *S. tuberosum* ssp. *andigena*, the former being less dependent on short-day conditions to induce tuberization.

In the *A. tumefaciens*-mediated transformation, in contrast to the *A. rhizogenes*-mediated transformation, the regenerated plants are completely transgenic organisms. However, though

the transgenic plants are regenerated in a kanamycin selective media, not all the lines efficiently express the transgene. Hence, validation of the transgene expression is needed.

Comparison of the promoter *FHT* activity in roots obtained using *A. tumefaciens* and *A. rhizogenes*

The aforementioned procedures were applied to produce roots expressing the *GUS* gene under the promoter of *FHT* gene. The complete transformed plants with *A. tumefaciens* were previously reported[15], using the binary vector pKGWFS7 containing the *FHT* promoter. Now, this binary vector has been used to produce new transformed hairy roots to compare the tissues where the promoter is active and therefore to test the hairy root system as a tool to study promoter activation.

Figure 4 shows GUS staining in roots of transgenic plants obtained by *A. tumefaciens* (**Figure 4A,B**) and transgenic hairy roots obtained by *A. rhizogenes* (**Figure 4C,D,E,F**), respectively. As can be seen, roots transformed with *A. tumefaciens* and grown in vitro show blue staining in the endodermis (**Figure 4A**), a cell layer between the cortex and the stele. In more developed roots, the blue labelling is patchy in the external layer corresponding to the exodermis (**Figure 4B**). In transformed hairy roots grown in hydroponics, the GUS marker was specifically located in the endodermis (**Figure 4C,E**), in the emergence of lateral roots (**Figure 4D,E**), in the wounded areas (**Figure 4E**) and in the exodermis (**Figure 4F**). The roots showed no GUS stain in negative controls that were either hairy roots without the *PromFHT:GUS* T-DNA cassette or wild type roots.

[Place Figure 4 here]

FIGURE AND TABLE LEGEND:

Figure 1: **Timeline to obtain potato transgenic hairy roots using *A. rhizogenes*.** The cumulative weeks to reach each stage of the transformation process and the subsequent steps to grow the hairy roots are shown. Representative images of different stages are depicted: the initiation of the process using 3-week-old in vitro plants (**A**), then infection of the plants by injecting *A. rhizogenes* (**B**), the formation of the proliferative tissue (**C**, arrows) with emerging hairy roots (**D**), and the developed hairy roots expressing the red fluorescent transformation marker DsRed (**E**).

Figure 2: **Timeline to obtain potato transformed plants using *A. tumefaciens*.** The cumulative weeks to reach each stage of the transformation process and the subsequent steps to grow the plants are shown. Representative images of different stages are depicted: the initiation of the process using leaves from 3-week old in vitro plants (**A**), the transfer of the wounded and infected leaves to the CIM media (**B**), the leaves when transferred to SIM media (**C**), the visualization of the callus around the wounded areas after 2-3 weeks in SIM media (**D**), the shoot formation after 9-11 weeks in SIM media (**E**), and the shoots after being transferred to MG media (**F**).

Figure 3: **Fluorescent transgenic hairy roots of potato (cv. Désirée) transformed by *A. rhizogenes*.** The hairy roots were obtained using a non-transformed *A. rhizogenes* (strain C58C1: pRI1583) (**A** and **C**) and with *A. rhizogenes* (strain C58C1:pRI1583) transformed with the empty vector pK7GWIWG2- (II) Red-Root carrying a DsRed transformation marker (**B** and **D**). The hairy roots are formed in both infections (**A, B**) but red fluorescence is only observed in hairy roots transformed with the *A. rhizogenes* containing the binary vector. The images were taken with a stereomicroscope equipped with a lamp and a specific filter to visualize the red fluorescence.

Figure 4: **Histochemical observation of transgenic potato roots expressing the *GUS* reporter gene driven by the promoter of *FHT*.** The roots from complete transgenic plants obtained by *A. tumefaciens* (*S. tuberosum* ssp. *andigena*) transformation (**A-B**) show blue staining in the endodermis (**A**) and exodermis (**B**). The transgenic hairy roots obtained by *A. rhizogenes* (*S. tuberosum* ssp. *tuberosum* cv. Désirée) transformation (**C-F**) display GUS staining in the endodermis (**C** and **E**), in the lateral root emergence (**D** and **E**), in the wound-healing zone (**E**) and in the exodermis (**F**). Endodermis (EN); Exodermis (EX); Xylem (XL); Primordia of a lateral root (LR). The red arrow indicates the wounded area.

Table 1: Media recipes used for growing bacteria and in vitro plants.

Table 2: Half strength Hoagland's solution for growing potato plants in hydroponics.

Table 3: GUS staining solution recipe.

DISCUSSION:

In potato, the most common system to obtain stable complete transgenic plants uses the transformation by *Agrobacterium tumefaciens* strains that require organogenesis using exogenous phytohormones. Although the *Agrobacterium* based protocols has the potential to integrate non-T-DNA vector sequence[25], this methodology is still the easiest and less expensive available to transform potato plants. During last years, the interest in *A. rhizogenes*-mediated transformation has got the attention of researchers for allowing to obtain transgenic roots in shorter periods than using *A. tumefaciens*. The *A. rhizogenes* still preserves the root-inducing (Ri) plasmid that carries a set of genes encoding enzymes for the phytohormone auxin control and cytokinin biosynthesis, and encoding for opines[26]. Once the Ri T-DNA is inserted into the host genomic DNA, the new hormonal balance deregulates the infected cells inducing the formation of proliferating roots, called hairy roots, emerging at the points of infection[27]. When an additional binary vector is used for integrating a foreign DNA, the preservation of the Ri plasmid confers the possibility to obtain transformed hairy roots with no need of organogenesis using exogenously applied phytohormones[28]. The hairy roots can be maintained attached to the wild type shoot generating a composite plant or can be self-propagated. This ability of hairy roots to self-propagate is being exploited to produce in several plants hairy roots as a biological system for mass-producing valuable metabolites or foreign proteins, generating interests in pharmaceutics and even phytoremediation areas (see for review[29,30]). In potato (var. Kufri

Bahar), transgenic complete plants were infected with wild type *A. rhizogenes* strain to produce hairy roots expressing the Hepatitis B surface antigens (HBsAg)[23]. Alternatively, a complete transgenic plant regenerated from hairy roots can be obtained, but potato plant and tubers showed distinct development compared to the untransformed controls. These differences in the phenotype are due to the original Ri T-DNA integrated within the genome[31,32].

In this work, the detailed procedures to obtain transgenic stable hairy roots using *A. rhizogenes* and transgenic stable plants using *A. tumefaciens* are presented (**Figure 1** and **Figure 2**). The fully developed transgenic hairy roots were obtained in 5-6 weeks, while transgenic roots using *A. tumefaciens* needed 15-18 weeks due to the organogenesis requirement from transformed cells, and the selection and propagation of transformed plants. In our hands, the *A. tumefaciens* transformation procedure works efficiently in *S. tuberosum* ssp. *andigena* and ssp. *tuberosum* (cv. Désirée), with a reported transformation efficiency around 35%[22] and 48%[12], respectively. The described *A. rhizogenes* transformation procedure is based on that reported by Horn[7], which showed a high (80–100%) transformation efficiency in cv. Désirée and other three potato cultivars (Albatros, Désirée, Sabina and Saturna).

To present *A. rhizogenes* as an alternative transformation system for potato functional studies and to indicate whether the initial presence of Ri T-DNA was matter of concern, we used both systems to transform potato roots with the same binary vector that contained a T-DNA with a promoter of a suberin biosynthetic gene (*FHT*) driving the expression of the *GUS* reporter gene. The histochemical analysis revealed that in *A. tumefaciens* transformed plants, the activity of the *FHT* promoter was in the inner and outer suberized layers of the roots (endodermis and exodermis, respectively) (**Figure 4A**,**B**) and also in the wounded areas of the leaf, stem and tuber[15]. In *A. rhizogenes* transformed hairy roots, the activity was also detected in endodermis and exodermis and in the wounded root areas (**Figure 4E**). The co-occurrence of the suberin promoter activity in both types of transformed roots indicates that the Ri integrated T-DNA is not affecting the developmental processes related to suberization at least in these root tissues. In transformed hairy roots we also detected activity of the *FHT* promoter in the areas surrounding the emergence of lateral roots (**Figure 4D**,**E**). This agrees with the promoter activity reported by other genes involved in the transport of suberin monomers such as *ABCG11 / WBC11*[33–36] or the regulator *StNAC103*[19]. The potato transformation by *A. rhizogenes* to study a suberin gene has already been reported recently[37] and also in that case hairy roots have allowed to show the promoter activation of *CYP86A33*, a fatty acid ω-hydroxylase. However, the GUS staining in roots was bulk quantified using a fluorometric assay, hence the specific expression in suberized tissues was only presumed.

Altogether these results evidence that *A. rhizogenes* transformation is a faster alternative tool to explore the cell type-specific promoter activation of suberin-related genes in roots, which may be extended to studies based on other processes that occur in roots. In agreement, some other functional genetic studies have been successful using *A. rhizogenes* in potato, tomato and eucalyptus to demonstrate the gene function[6–9], to study the hormonal response[38,39] or the promoter activity[40,41]. However, this strategy still may present limitations, especially when

studying tightly-controlled developmental processes that may be deregulated by the Ri T-DNA or when the whole plant or the tubers or other organs different from roots want to be studied. In these situations, the *A. tumefaciens* transformation system is still preferred.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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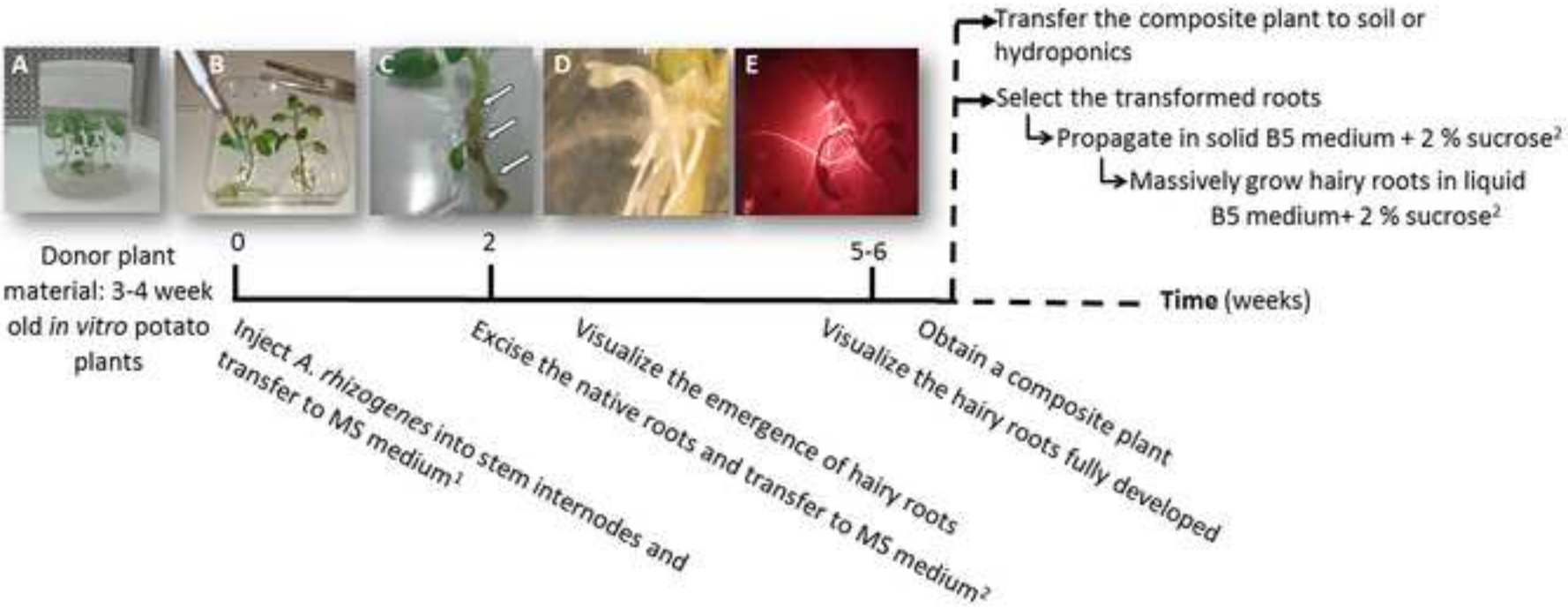
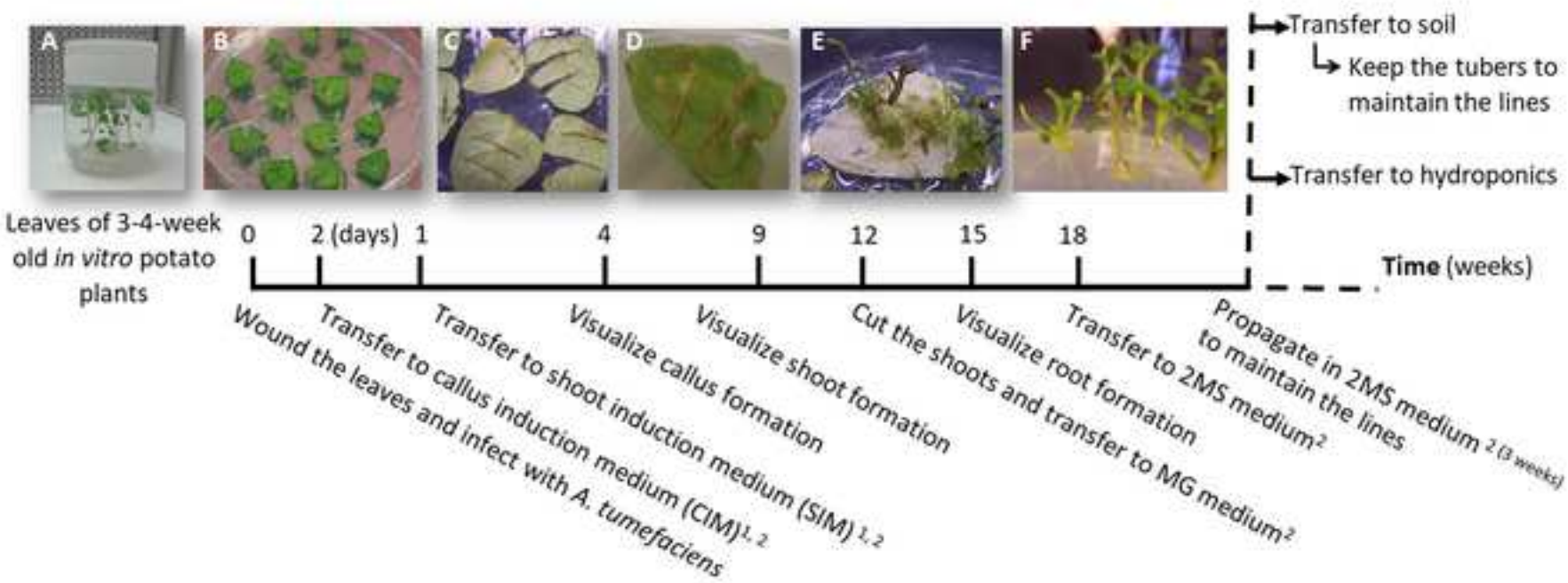
Figure 1



[1] Supplemented with acetosyringone to induce the *Agrobacterium virulence* (*vir*) genes

[2] Supplemented with cefotaxime sodium to kill *A. rhizogenes*

Figure 2



[1] Supplemented with the plant selectable marker to select the transformed cells
[2] Supplemented with cefotaxime sodium to kill *A. tumefaciens*

Figure 3

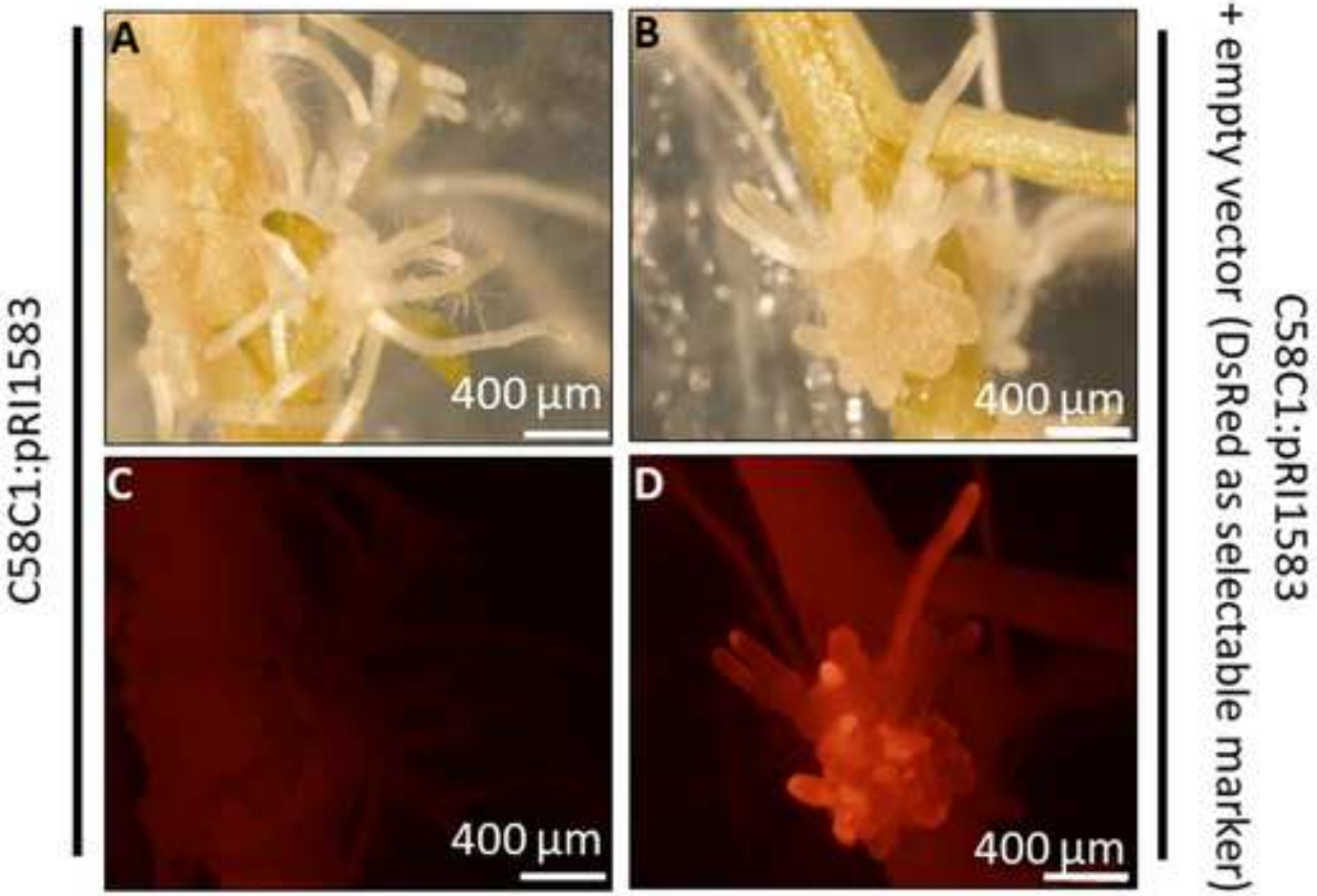


Figure 4

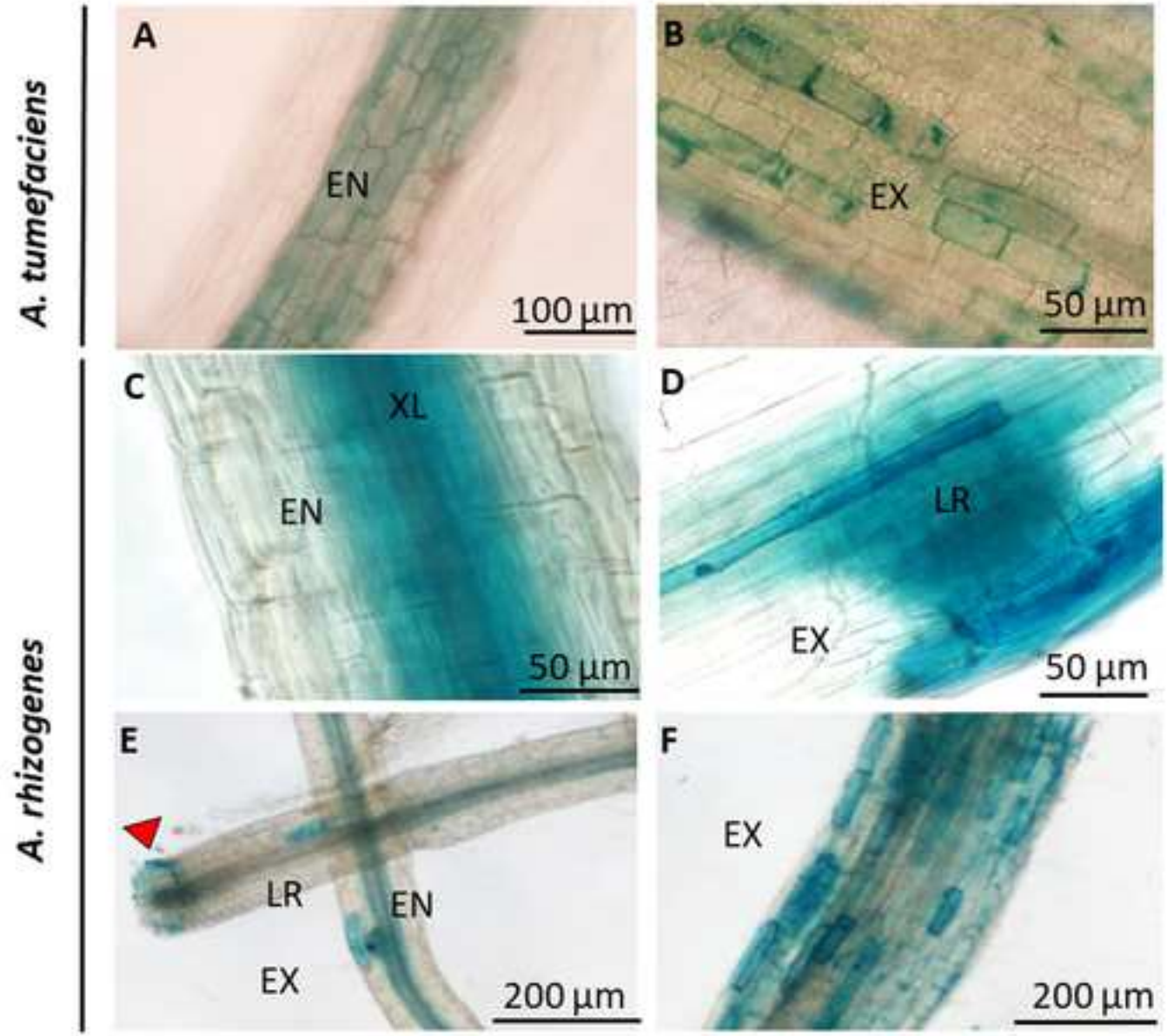


Table 1

PLANT MEDIA

MS	
Murashige and Skoog media including vitamins	4.4 g
MES hydrate	0.5 g
milliQ water	up to 1 L
Adjust the pH to 5.8 with KOH	
Add Gellan gum directly to the bottle for its insolubility at room temperature	2.3 g
Autoclave for 15 min at 121 ºC and 1 atm	

2MS (MS supplemented with 2 % sucrose)	
Murashige and Skoog media including vitamins	4.4 g
MES hydrate	0.5 g
Sucrose	20 g
milliQ water	up to 1 L
Adjust the pH to 5.8 with KOH	
When solid media is required, add Gellan gum directly to the bottle for its insolubility at room temperature	2.3 g
Autoclave for 15 min at 121 ºC and 1 atm	

MG	
Murashige and Skoog media including vitamins	4.4 g
MES hydrate	0.5 g
Glucose	16 g
milliQ Water	up to 1 L
Adjust the pH to 5.8 with KOH	
Add Plant Agar directly to the bottle for its insolubility at room temperature	5.5 g
Autoclave for 15 min at 121 ºC and 1 atm	

Callus Induction Media (CIM)	
Autoclaved and cooled at 60 ºC MG media	1 L
Suplement with:	
Naphthalene acetic acid (NAA) at 1 mg / mL in DMSO	5 mL [5 mg / L]
6-Benzylaminopurine (BAP) at 1 mg / mL in ethanol	100 µl [0.1 mg / L]
Cefotaxime sodium (to kill Agrobacterium) at 250 mg / mL in H_2O	1 ml [250 mg / L]
Kanamycin (or other antibiotic of the selective marker to select transgenic cells) at 50 MG / Ml in H_2O	1 ml [50 mg / L]
Use fresh plates when possible. Alternatively, they can be stored up to 10 days at 4 ºC in a sealed plastic box with the lid side up	

Shoot Induction Media (SIM)	
Autoclaved and cooled at 60 ºC MG media	1 L
Suplement with:	
Zeatin riboside at 2 mg / mL in DMSO	1 mL [2 mg / L]
Naphthalene acetic acid (NAA) at 1 mg / mL in DMSO	20 µL [0.02 mg / L]
Gibberellic acid (GA_3) at 1 mg / mL in ethanol	20 µL [0.02 mg / L]
Cefotaxime sodium (to kill Agrobacterium) at 250 mg / mL in H_2O	1 ml [250 mg / L]
Kanamycin (or other antibiotic of the selective marker to select transgenic cells) at 50 mg / mL in H_2O	1 ml [50 mg / L]
Use fresh plates when possible. Alternatively, they can be stored up to 10 days at 4 ºC in a sealed plastic box with the lid side up	

BACTERIOLOGICAL MEDIUM

YEB medium composition	
Yeast extract	10 g
Beef extract	5 g
Peptone	5 g
Sucrose	5 g
$MgSO_4$	0.49 g
milliQ water	up to 1 L
Adjust pH to 7.2 with NaOH	
Add Bacteriological agar directly to the bottle for its insolubility at room temperature	15 g
Autoclave for 15 min at 121 ºC and 1 atm	
To select all *Agrobacterium* strains add:	1 L
Rifampicin at 50 mg / mL in DMSO	2 mL [100 mg/ L]
Additionally, to select *A. rhizogenes* C58C1:Pri1583 strain add:	
Kanamycin at 50 mg / mL in H_2O	1 mL [50 mg/ L]
Additionally, to select *A. tumefaciens* GV2260 strain add:	
Carbenicillin at 25 mg / mL in H_2O	1 mL [25 mg/ L]
To select the transformed *Agrobacterium* with PK7GWIWG2_II-RedRoot plasmid or pKGWFS7 plasmid add:	
Streptomycin at 150 mg / mL in H_2O	2 mL [300 mg / L]
Spectinomycin at 50 mg / mL in H_2O	2 mL [100 mg/ L]

Table 2

Components	Stock Solution [g / L]	Final concentration [µM]	mL Stock Solution / 10 L
KNO_3	202	2500	12.5
$Ca(NO_3)_2 \cdot 4H_2O$	472	2500	12.5
Iron (Sprint 138 iron chelate)	15	12	7.5
$MgSO_4 \cdot 7H_2O$	493	1000	5
NH_4NO_3	80	500	5
KH_2PO_4 (pH 6.0 with KOH)	136	250	2.5
H_3BO_3	2.86	23	5
$MnCl_2 \cdot 4H_2O$	1.81	4.57	5
$ZnSO_4 \cdot 7H_2O$	0.22	0.38	5
$CuSO_4 \cdot 5H_2O$	0.05	0.1	5
$Na_2MoO_4 \cdot 2H_2O$	0.12	0.25	5
Distilled H_2O			up to 10 L

Table 3

Components	Stock Concentration	Final Concentration	Volume of Stock Solution / 20 mL
X-GlcA (5-Bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylammonium salt)[1]	-	1 mM	1 mL of methanol with 10 mg of X-GlcA dissolved
Sodium phosphate buffer 2X pH 7	1000 mM	50 mM	10 mL
Potassium ferricyanide[1]	100 mM	1 mM	200 µL
Potassium ferrocyanide[1]	100 mM	1 mM	200 µL
EDTA	500 mM	10 mM	400 µL
Triton X-100	10%	0.05%	100 µL
milliQ H_2O			up to 20 mL

[1] Photosensitive reagents, so protect GUS solution from light.

Table 3

Name of Material/ Equipment	Company	Catalog Number
Acetone	Panreac	1.310.071.21
Acetosyringone	Acros	115540050
Aquarium pump	Prodac	MP350
Autoclave	Ragpa Strelimatic	
Bacteriological agar	Lab Conda	1800
BAP	Duchefa	B0904
Beef extract	Lab Conda	1700
Plant growing cabinet	Nuaire	
Carbenicillin	Duchefa	C0109
Cefotaxime sodium	Duchefa	C0111
DMSO	Merck	1029310161
Ecotron infors	HT	29378
Ethanol	Merck	1,009,831,011
Falcon tube	Control tecnica	CFT011500
Ferricyanate	Sigma	101001081
Ferrocyanate	Sigma	100979088
Flask (8.06 cm diameter and 11.3 cm height) and plastic lid for *in vitro* culture	Apiglass	ref16
GA3	Sigma	G7645
Gamborg B5 media	Duchefa	G0210
Gelrite	Duchefa	G1101
Glucosa	Sigma	G5767
Kanamycin	Sigma	K1377
Leukopor tape	BSN Leukopor	BDF47467
Lupe	Wild-Heerbrugg	M420
Magnetic shaker	Agimatic	7000243
MES hydrate	Sigma	M2933-25G
MgSO4	Panreac	131404
Microscope	Olympus	
Minufugue centrifugue 5415R	Eppendorf	

Murashige and Skoog media	Duchefa	M0254.0050
Na2HPO4	Panreac	131679
NAA	Duchefa	N0903
NaCl	Panreac	131659
NaH2PO4	Sigma	58282
NightSea Stereo	SFA Moonting Adapter	
Parafilm	Anorsa	PRFL-001-001
Peptone	Lab Conda	1616
Petri dishes (90 x 14)	Anorsa	200200
pHmetre	Crison	
Phytotron	Inkoa	RFTI-R5485
Plant Agar	Duchefa	P1001
Refrigeratot	Liebherr Medline	
Rifampicin	Duchefa	R0146
Spectinomycin	Sigma	59007
Spectrophotometer	Shimadzu	
Square plates (120 x 120)	Deltalab	200204
Streptomycin	Sigma	S6501
Sucrose	Panreac	131621
Surgical blades	Swann-Morton	201
Surgical needle	NIPRO	015/0204
Triptone	Lab Conda	1612
Triton	Serva	37240
Unimax 1010 shaker	Heidolph	
Vacuum	Dinko	
x-GlcA (5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronic acid, sodium salt anhydrous)	Biosynth	B-7398
Yeast extract	Lab Conda	1702.00
Zeatin riboside	Sigma	1001042850

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Dear Editor,

We are hereby replying the comments raised by the Reviewers (and the Editor) for the manuscript JoVE59119 entitled: "*Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*-mediated transformation of potato and the promoter activity of a suberin gene by GUS staining" by Sandra Fernández-Piñán, Jennifer López, Iker Armendariz, Pau Boher, Mercè Figueras and Olga Serra.

We have structured the Reviewers' comments in a question and answer (in blue) format in order to facilitate the identification of the points raised by them.
The original manuscript has been extensively revised and modified according to the reviewers' comments in order to clarify their questions and to increase the general quality and readability of the manuscript. The authors express their gratitude to the Reviewers for their time and for providing helpful comments, which have clearly contributed to the improvement of our original submission. We are confident that, following this revision, the manuscript is clearer and we hope that it will be finally considered acceptable for publication.

Best regards,

Olga Serra
Universitat de Girona.

TITLE:
~~*Agrobacterium tumefaciens* and *AgrobacteriumA. rhizogenes* mediated transformation of potato: the promoter activity of a suberin gene as a proof of concept~~
Agrobacterium tumefaciens and *A*grobacterium *rhizogenes*-mediated transformation of potato and the promoter activity of a suberin gene by GUS staining

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KEYWORDS:
~~*Agrobacterium*~~*Agrobacterium* *rhizogenes*, ~~*Agrobacterium*~~*Agrobacterium* *tumefaciens*, *Solanum tuberosum*, suberin, root, potato

SHORT ABSTRACT:
Here we present two protocols to transform potato plants. The *Agrobacterium tumefaciens* transformation leads to a complete transgenic plant while the ~~*A. rhizogenes*~~*A. rhizogenes* produces transgenic hairy roots in a wild type shoot that can be self-propagated. We then detect the promoter activity by GUS staining in the transformed roots.
~~Here we present two different protocols to obtain *Solanum tuberosum* transgenicmutant lines, using two *Aagrobacterium* strains. As an example, we perform GUS analysis to study tissue specific activity of athe activityfunction of a concrete promoter, nevertheless this protocol allows to perform any analysis or procedure depending on the vector it is used.~~
~~Two systems of *Agrobacterium* mediated plant transformation in potato are elucidated. Both *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens* were transformed with the same binary vector to obtain roots with the *FHT* (a suberin feruloyl transferase) promoter driving the *GUS* reporter gene. GUS expression was detected in the suberized endodermis and exodermis in both cases.~~

LONG ABSTRACT:
Agrobacterium sp. is one of the most widely used methods to obtain transgenic plants as it has the ability to ~~insert~~transfer and integrate its own T-DNA into the plant's genome. Here, we present two transformation systems to genetically modify ~~ied~~ potato (*Solanum tuberosum*) plants. In ~~*A. tumefaciens*~~*A. tumefaciens* transformation, leaves are infected, the transformed cells selected and a new complete transformed plant is regenerated using phytohormones in 18 weeks. In ~~*A. rhizogenes*~~*A. rhizogenes* transformation, stems are infected by injecting the bacteria with a needle, the new emerged transformed hairy roots are detected using a red fluorescent marker and the non-transformed roots are removed. In 5-6 weeks~~Thus~~, the resulting plant ~~-~~is a composite of a wild type shoot with fully developed transformed hairy roots. To increase the biomass, the transformed hairy roots can be excised and self-propagated. We applied both *Agrobacterium* mediated-transformation methods to obtain roots ~~transformed with~~expressing the *GUS* reporter gene driven by a suberin biosynthetic gene promoter. The GUS staining procedure is provided and allows the cell localization of the promoter induction. In both methods the transformed potato roots showed GUS staining in the suberized endodermis and exodermis, and additionally, in ~~*A. rhizogenes*~~*A. rhizogenes* transformed roots the GUS activity was also detected in the emergence of lateral roots. These results suggest that ~~*A. rhizogenes*~~*A. rhizogenes* can be a fast alternative tool to study the genes that have a role in roots.
~~*Agrobacterium* sp. is one of the most widely used methods to obtain transgenic plants as it has the ability to insert its own DNA into the plant's genome. Here, we discuss two transformation systems, one to obtain stable modified potato (*Solanum tuberosum*) plants by *A. tumefaciens* and the other one to obtain transgenic hairy transgenic roots by *A. rhizogenes*. *A. tumefaciens* helps achieve a complete transgenic potato plant. However, when the root is the target organ for the genetic modification, the use of *A. rhizogenes* gains relevance, because it is faster and cheaperless costly. This cost is~~

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~~reduced because hormone application is not necessary to regenerate the whole plant. Transformed potato hairy roots were easily identified in the composite plant using a red fluorescent transformation marker. Moreover, we describe how to increase the root biomass by growing them as composite plants in hydroponic media or autonomously in root growing media. We obtained GUS transcriptional reporter roots for a key suberin biosynthetic gene (*FHT*, a feruloyl transferase). In transformed potato roots using *A. tumefaciens*, GUS expression is detected in the suberized endodermis and exodermis, a comparable pattern obtained for transformed potato hairy roots using *A. rhizogenes* that additionally presented GUS activity in the emergence of lateral roots. Thus, *A. rhizogenes* mediated transformation is useful to study the activation of promoters of suberin related genes, as well as other genes whose expression lies in root tissues.~~

INTRODUCTION:

Aside from economic interest, the generation of transgenic plants has its own relevance in research to demonstrate the ultimate function of genes and to better understand plant physiology and development. The most widely used method for plant DNA insertion is ~~*Agrobacterium*~~*Agrobacterium*-mediated transformation. ~~*Agrobacterium*~~*Agrobacterium tumefaciens* is able to generate crown galls in the infected tissue of many plant species by the action of its tumour-inducing (Ti) plasmid. The plasmid contains a T-DNA region with a set of genes that will be ~~inserted~~ integrated in the plant genome and induce tissue dedifferentiation[1,2]. The exchange of these genes within the T-DNA by the transgene has allowed the generation of specific plant modifications avoiding phenotypic effects[3]. To promote the transgene cloning into the T-DNA, the T-DNA region has been excised in an independent plasmid called a binary plasmid, while the rest of the genes of the Ti plasmid (the virulence genes that allow the T-DNA transfer and insertion mechanisms) have been placed in a helper plasmid. For plant biotechnology ~~and~~ research, transformation by ~~*Agrobacterium A. tumefaciens*~~*A. tumefaciens* has several advantages: it does not need expensive devices, is able to generate both stable and transient plant transformation, and integration of low numbers of gene copies into the chromosome[4]. However, for most plants, but not A~~a~~arabidopsis, the generation of stable transformants requires plant regeneration from a single or a few cells using exogenous phytohormones, making this process ~~expensive~~ laborious and time consuming. ~~*Agrobacterium A. rhizogenes*~~*A. rhizogenes* is also able to modify the plant genome, but in this case, it yields hairy roots or adventitious roots due to the expression of *rol* (root *loci*) genes encoded in the root-inducing (Ri) plasmid[5]. Although less studied than ~~*A. tumefaciens*~~*A. tumefaciens*, ~~*A. rhizogenes*~~*A. rhizogenes* is also used for obtaining transgenic roots.~~biotechnological and research approaches. However, i~~ In this case, the ~~*A. rhizogenes*~~*A. rhizogenes*~~*grobacterium*~~ ~~used still~~ contains the original T-DNA in the Ri plasmid and a ~~, but incorporates a~~ binary plasmid with a second T-DNA ~~that produces~~ carrying the transgene. When the infection site is in stems or hypocotyls, a composite plant can be obtained, with new hairy transgenic roots emerging from wild type shoots. Alternatively, hairy transformed roots can grow autonomously ~~*in vitro*~~*in vitro* in media with carbon source inputs. The use of ~~*A. rhizogenes*~~*A. rhizogenes* instead of ~~*A. tumefaciens*~~*A. tumefaciens* to produce transgenic tissue is gaining relevance when the root is the target organ, because plant regeneration ~~hormone application~~ is not required ~~for plant regeneration~~ and hence it is faster and less costly. Previous~~Many~~ studies~~works~~ have demonstrated this methodology appropriated for the phenotypic characterization of root specific genes[6–9].

The potato ~~plant~~ (*Solanum tuberosum*) is the fourth most important crop ~~plant~~ in the world according to the Food and Agriculture Organization of the United Nations (FAO)~~,~~

since ~~and~~ the tuber has nutritional relevance ~~in~~for human consumption ~~because it~~for being ~~is~~ a good source of vitamins and minerals. ~~To that end~~For that reason, potato has been placed in the spotlight of agricultural biotechnology and also is considered as a good biological model for genetic and developmental studies[10,11]. Potato transformation significantly contributed to the understanding of molecular mechanisms underlying suberized tissues through the characterization of genes involved in suberin and wax biosynthesis[12–17], suberin monomer transport[18] and transcription regulation[19]. The suberin feruloyl transferase gene, *FHT*, is one of these characterized biosynthetic genes; its downregulation gives rise to a strong impairment of the ~~apoplastic~~ periderm protection~~barrier~~, which is correlated with a strong decrease in ferulate esters of suberin and waxes in potato tubers[14]. Concomitantly, in ~~A~~Aarabidopsis ~~roots~~ and seeds of Arabidopsis, the knockout of its putative orthologue (*ASFT/RWP1*) also demonstrated its role in producing alkyl ferulates in suberin[20,21]. In potato, the *FHT* transcriptional reporter line and the FHT antibody showed that the promoter activity and the protein are located in the exodermis, the endodermis, ~~and~~ the phellogen of the periderm and in wounded tissues[15].

In this work, we ~~will~~ detail a protocol using ~~*A. rhizogenes*~~*A. rhizogenes* to produce transgenic hairy roots that ~~will be~~are maintained in a wild type stem, generating composite potato plants, or excised to grow autonomously ~~*in vitro*~~*in vitro*. We ~~will~~ also provide the protocol using ~~*A. tumefaciens*~~*A. tumefaciens* to obtain complete transgenic potato plants. As a case study, ~~*A. rhizogenes*~~*A. rhizogenes* and ~~*A. tumefaciens*~~*A. tumefaciens* transformed with the same binary vector ~~will~~ are~~be~~ used to obtain roots with the *FHT* promoter driving *GUS* reporter gene expression. The results ~~will~~ are~~be~~ reported and compared.

PROTOCOL:

Note: The ~~*A. rhizogenes*~~*A. rhizogenes* transformation protocol was adapted and modified from Horn *et al.*[7] and the genotype tested was *S. tuberosum* ssp. *tuberosum* (cv. Désirée).~~and t~~ The ~~*A. tumefaciens*~~*A. tumefaciens* transformation protocol was adapted and modified from Banerjee *et al.*[22] and the genotypes tested were *S. tuberosum* ssp. *tuberosum* (cv. Désirée) *and S. tuberosum* ssp. *andigena*. The main steps of both procedures are summarised in ~~Figure~~Figures 1 and 2, respectively.

Note: In all the steps of the procedure performing ~~*in vitro*~~*in vitro* transfers, do so rapidly, and when possible, maintain the plates or pots closed, thus minimizing plant exposure to the air to avoid wilting and contamination.

Note: Otherwise stated, all the plant incubations were done in cabinets under long day conditions of 12 h 24 ºC light/ 12 h 20 ºC dark and 67 µmol m^{-1} sec^{-1}.

Note: Otherwise stated, perform all the bacteria manipulation and *in vitro* plant transfers in aseptic conditions in a laminar flow hood.

Note: All the media recipes for *Agrobacterium* and *in vitro* plant cultures are provided in Table 1.

Caution: Deposit all material contaminated with genetic modified plants to the appropriated waste container.

[Place Table ~~2~~1 here]

~~1.~~

***Agrobacterium*, cultures used for transformation**

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Note: The strain used for *A. rhizogenes* was the C58C1:Pri1583[7] (kindly provided by Dr. Inge Broer) and that for the *A. tumefaciens* was the GV2260. Both bacteria were transformed with the binary vector carrying the transgene. *A. rhizogenes* was transformed with pK7GWIWG2 II-RedRoot (VIB-Department of Plant Systems Biology at Universiteit Gent) that contains a T-DNA carrying a selectable marker to monitor the hairy root formation. To compare the transformed roots generated by both *Agrobacterium*, *A. rhizogenes* and *A. tumefaciens* were transformed with the binary vector pKGWFS7 which contains a T-DNA carrying the *FHT* promoter driving the *β-glucoronidase* (*GUS*) reporter gene and the Kanamycin resistance as a selective marker[15].

1. Pick a colony of *Agrobacterium* and grow it overnight (O/N) in 5 mL of YEB medium supplemented with antibiotics (Table 1) in a 50 mL centrifuge tube at 28 °C with shaking at 200 rpm.
2. For *A. tumefaciens* transformation, measure the optical density, which must be OD_{600} = 0.6-1.
   2.1. If the optical density is higher, make a subculture lowering it to OD_{600} = 0.3 with fresh media and wait until the culture reaches OD_{600} = 0.6-1.
3. Centrifuge 1 mL of *Agrobacterium* culture at 3,000 x g in a bench-top centrifuge for 10 min at room temperature.
4. Remove the supernatant by pipetting, and resuspend cells in 1 mL of fresh YEB medium without antibiotics. Repeat this step twice to ensure the complete removal of antibiotics.
   4.1. For *A. tumefaciens* transformation, in the last resuspension add the appropriate YEB volume to obtain a final optical density of OD_{600} = 0.8.
5. Keep cells on ice while preparing the plants to be infected.

~~2.~~

~~3. Note: Before starting, 3-to-4-week old potato plants (*S. tuberosum* cv. Désirée or *S. tuberosum* ssp. andigena), produced by stem cuttings and grown in solid 2MS medium, are required (Figure 1A). The *in vitro* pots are grown in cabinets under a light/dark cycle of 12/12 h at 24/20 °C, respectively, and 67 µmol m^{-2} sec^{-1}.~~

~~4. Note: When doing *in vitro* transfers, do so rapidly, and when possible, maintain the plates or pots closed, thus minimizing plant exposure to the air to avoid wilting.~~

Plant material ~~preparation~~for transformation

~~1.~~6. ~~Produce~~ Make one or two node stem cuttings either containing the apical or the auxiliary buds from sterile ~~*in vitro*~~*in vitro* potato plants (donor plants); ~~by stem cuttings and~~ grow~~n~~ them in solid 2MS medium in pots ~~(Figure 1A)~~ for 3 to 4 weeks (Figure 1A and Figure 2A) ~~in cabinets under long day conditions of 12 h 24 ºC light/ 12 h 20 ºC dark, and 67 µmol m^{-1} sec^{-1}.~~

~~2. Grow *in vitro* plants in cabinets under long day conditions of 12 h 24 ºCa light/ 12 h 20 ºC dark cycle of 12/12 h at 24/20 °C, respectively, and 67 µmol m^{-1} sec^{-1} for 3-4 weeks.~~

~~Note: When performing in vitro transfers, do so rapidly, and when possible, maintain the plates or pots closed, thus minimizing plant exposure to the air to avoid wilting.~~

Plant transformation using ~~*A. rhizogenes*~~*A. rhizogenes* (~~*Figure*~~*Figure 1)*

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~~5.~~ Note: ~~Always try to u~~Use fresh media plates, though alternatively, the plates can be kept at 4 °C with the lid side up, tightly sealed with transparent film to avoid media dehydration.

Note: To prepare the square media plates, incline them ~15°, fill with 40 mL of MS, and let solidify. This will help the aerial part of the plant to minimize the contact with the medium.

Note: This procedure allows the obtaining of transformed hairy roots. To evaluate the transgene expression, a negative control is needed. To prepare the negative control, follow the procedure using an *A. rhizogenes* strain either untransformed or transformed with the empty vector that includes the transformation marker.

~~6.~~

~~7.~~ ~~Pick a colony of Grow transformed *A. rhizogenes* C58C1:Pri1583[6] strain transformed with the binary vector and grow it(single colony) overnight (O/N) in 5 mL of YEB medium supplemented with antibitotics (see Table 2) rifampicin [50 µg/mL] (genomic resistance), kanamycin [50 µg/mL] (Ri plasmid selection), streptomycin [150 µg/mL] and spectinomycin [50 µg/mL] (Prom*FHT::GUS* binary vector selection) in a 50 mL centrifuge Falcon tube at 28 °C with shaking at 200 rpm. Repeat this step to obtain a negative transformation control using the *A. rhizogenes* strain untransformed or transformed with the empty vector that includes the transformation marker.~~

~~8. rifampicin [50 µg/mL] (genomic resistance), kanamycin [50 µg/mL] (Ri plasmid selection), streptomycin [150 µg/mL] and spectinomycin [50 µg/mL] (PromFHT::GUS binary vector selection)~~

~~9. Centrifuge 2 mL of *A. rhizogenes* culture at 3,000 x g in a bench top centrifuge for 10 min at room temperature (RT).~~

~~rRemove the supernatant by pipetting, and re-suspend cells in 1 mL of fresh YEB medium without antibiotics. Repeat this step twiceagain to ensure the be sure to completeely removale of the antibiotics.~~

7. Transfer~~Remove~~ very carefully a donor ~~whole~~ plant ~~very carefully~~ from the 2MS~~solid~~ medium to~~and place it on~~ a 120 mm x 120 mm square plate. ~~in sterile conditions.~~

8. ~~Inoculate~~Inject to one stem internode 5 µL of the ~~*A. rhizogenes*~~*A. rhizogenes* culture ~~by means of a puncture with a~~using a surgical needle ~~into the a stem internodes using a 120 mm x 120 mm square plate as a support~~ and r. ~~R~~epeat it twice per plant in different internodes.

Note: -Consider each ~~puncture~~injection as an independent transformation event (~~Figure~~Figure 1B).

~~10. Repeat this step to obtain a negative transformation control using the A. rhizogenes strain untransformed or transformed with the empty vector that includes the transformation marker.~~

~~11. In sterile conditions and using a 120 mm x 120 mm square plate as a support, remove a whole potato plant carefully from the medium and inoculate with 5 µL of A. rhizogenes culture by means of a puncture with a surgical needle into the stem internode of the 3-week-old potato plant (Figure 1B). Repeat twice2 times per plant andin different internodes. Note: Each puncture is considered a different independent transformation event.~~

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9. Transfer immediately the entire plant to a square plate with solid MS medium supplemented with 0.1 mM acetosyringone ~~for two weeks~~. Accommodate 2 plants per plate.

~~12.~~ Note: The 1 M stock of acetosyringone is prepared in DMSO and can be stored at -20 °C.

~~13. Repeat step 3 with a new plant and place the second punctuated plant in the plate used in step 4.~~

~~14.~~10. Seal the plate using surgical ~~Leukopor~~ tape and arrange ~~the petri dishit~~ vertically inside a growth cabinet for 2 weeks ~~under long day conditions of 12 h 24 °C light/ 12 h 20 °C dark~~ ~~a light/dark cycle of 12/12 h at 2~~4~~2~~/20 °C, respectively, and 67 µmol m^{-2} sec^{-1}~~.~~.

~~After 2 weeks, e~~Excise the native roots of the plant, and transfer the plant to a new square plate with MS medium supplemented with cefotaxime sodium [~~(~~500 µg/mL]~~)~~ to kill ~~*A. rhizogenes*~~*A. rhizogenes*.

11. ~~Note:~~ The new hairy roots will start to appear at this time (~~Figure~~Figure 1C, 1D). They can be checked ~~visualized~~ by red fluorescence when using a ~~DsRed2~~DsRed transformation marker (~~Figure~~Figure ~~1~~ 3 D).

~~15.~~

12. To obtain a composite plant (Figure 1E), let the transgenic hairy roots grow for 3-4 weeks in MS medium supplemented with cefotaxime sodium [500 mg/mL] (change the medium every week).

~~16.~~13. Depending on the purpose, ~~p~~Propagate the transgenic hairy roots and the negative controls, ~~Ddepending on the purpose, follow the different options~~ as follow:

~~a.~~13.1. ~~To obtain a composite plant, let the transgenic hairy roots grow for 3-4 weeks in MS medium (change the medium every week). After that, t~~Transfer the whole composite plant to a hydroponic (Table ~~1~~2) or soil medium to allow for massive development. ~~Cau~~~~Critical steption: EnIt is very important to be sure to completely remove that the native roots have been completely excised to reduce the amount of non-transgenic material.~~

13.2. To individually propagate the transformed hairy roots, using a scalpel ~~select and~~ cut~~, using a scalpel under sterile conditions,~~ the roots expressing the red fluorescent transformation marker (~~DsRed2~~DsRed protein) when they are 4-8 cm long (Figure 1E) and~~.~~ ~~Transfer~~ transfer them into a petri dish with Gamborg B5 solid medium supplemented with 2% sucrose and cefotaxime sodium ~~(~~[500 mg/mL]~~)~~. S~~, s~~eal the plates with laboratory film~~Parafilm~~ and grow them in the dark at 20 °C.

~~b.~~ Note: The roots can be manipulated under a stereomicroscope equipped to detect the fluorescence ~~with an adapter~~ (see table of materials) ~~to detect the fluorescence~~. ~~Alternatively, the NightSea Stereo Microscope Fluorescence Adapter (Model SFA) can be used.~~ ~~To better propagate the roots, cut and transfer them when they are 4-8 cm long.~~ ~~Critical step Caution: The Gamborg B5 media is prone to contamination, so cut and transfer the roots to fresh media every 4-6 weeks and have at least two replicates.~~

~~c.~~13.2.1. For biomass production (i.e. gene expression analysis), cut a 5 cm long hairy root and propagate it in a 150 mL Erlenmeyer flask with 20 mL of Gamborg B5 liquid medium supplemented with

2% sucrose and cefotaxime sodium [(500 µg/mL]). Grow it for 6 weeks in the dark at 20 °C and 60 rpm.

[Place ~~Figure~~Figure 1 here]

Plant transformation using ~~*A. tumefaciens*~~*A. tumefaciens* (~~*Figure*~~*Figure* 2)

Note: This procedure allows the obtaining of transformed plants. To evaluate the transgene's effect, a negative control is needed. One option is to follow the procedure using an ~~*A. tumefaciens*~~*A. tumefaciens* transformed with the empty vector. Alternatively, wild type plants can be used.

~~17. Note: The MG medium is MS medium supplemented with 1.6% glucose and solidified with 0.6% of plant agar (Duchefa). 2MS medium is MS medium supplemented with 2% sucrose and, when solidified, with 0.23% of gellan gum Gelrite (Duchefa), as stated before. Try to uUse fresh plates with media containing phytohormones; alternatively, you can keep them with the lid side up at 4 °C, tightly closed with a film or a bag for a maximum of 10 days.~~

~~18.~~

~~1. Pick a colony of Grow transformed *A. tumefaciens* GV2260 strain (single colony) O/Novernight and grow it O/N in 510 mL of YEB medium supplemented with antibiotics (see Table 2) rifampicin [50 µg/mL] (genomic resistance), carbenicillin [50 µg/mL] (helper plasmid selection), streptomycin [150 µg/mL] and spectinomycin [50 µg/mL]) (PromFHT::GUS binary plasmid selection) into a 50 mL centrifuge Falcon tube at 28 °C with shaking at 200 rpm O/Novernight.~~

~~2. Measure the optical density, which must be OD_{600} = 0.6 1lower than 0.6 1 UA. Note: If higher, make a subculture lowering it to OD_{600} = 0.3 0.3 UA with freshnew media and wait until the culture reaches OD_{600} = 0.6 1.0.6 1 UA.~~

~~Take 1 mL of culture and centrifuge it for 10 min at 3,000 x g in a bench top centrifuge at RT.room temperature.~~

~~RrRemove the supernatant by pipetting, and re-suspend cells in 1 mL of mL of fresh YEB medium without antibiotics. Repeat this step twice to ensure complete removal of antibiotics.Repeat this step twiceagain to be sure to completely remove the antibiotics from the medium. In the last step, add the appropriate YEB volume to obtain a final optical density of OD600 = 0.88 UA.~~

~~3. Note: Keep cells on ice and infect the potato explants immediately.~~

~~In sterile conditions, use cut one~~Place a leaf from the 3-4-week old ~~potato~~ plants (Figure 2A), ~~place it in~~in a petri dish. ~~Using a scalpel to proceed with the cut.~~

14. ~~and using Use a scalpel, cut and to~~ exclude the petiole and make transverse ~~sections~~ cuts (1-3 depending on the leaf size) ~~onin~~from the c~~ the c~~entre of the leaf ~~and extend it~~ to the edges avoiding cutting them off (Figure 2B). ~~(Figure 2B). Caution: From the cuttings to the leaf, transfer to 2 MS medium, proceeding immediately to avoid wound healing that may prevent the A. tumefaciens infection.~~

~~4.~~

15. Imme~~e~~diately place the leaf floating on 10 mL of fresh ~~and sterile~~ 2 MS liquid media in a petri dish with the abaxial side up and close the plate. Repeat the step ~~with as many leaves as can be~~ accommodati~~n~~ng~~ed~~ up to ~~in the petri dish (8~~105

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leaves for cv. Désirée and 2~~0~~5 leaves for ssp. *andigena* (depending on the leaf size) ~~maximum~~).

~~5.~~ ~~Immediately a~~

16. ~~Inoculate~~Add~~dd~~ 80 µL of ~~*0.8 UA A. tumefaciens*~~*A. tumefaciens* culture at OD_{600} = 0.8 in the liquid media and ~~to the plate with the leaves, and h~~H~~h~~omogenise the plate manually for 1 min to distribute the bacterial solution.

~~6.~~17. Carefully seal with ~~laboratory~~ sealing film, cover with aluminium foil and incubate for 2 days in a dark chamber at 24 °C to let the ~~transfection~~~~infection~~transformation occur. ~~Caution: Do not leave the wounded leaves for a long time without A. tumefaciens since it can heal before the infection and then the transformation of the plants will not take place.~~

18. Transfer the leaves keeping abaxial side up~~(again with the abaxial side up)~~ to CIM medium ~~supplemented with antibiotics (see Table 2) (~~Figure~~~~Figure 2B~~C~~)~~ and~~, Callus Induction Medium (CIM: MG medium with naphthalene acetic acid (NAA) [5 mg/L], 6-benzylaminopurine (BAP) [0.1 mg/L]), supplemented with cefotaxime sodium [250 mg/L] to kill the a~~Agrobacteriumia) and kanamycin [50 mg/L] or the corresponding binary vector selective marker (Figure 2C)~~, incubate them for one week in a growth cabinet.

Note: Scrape the CIM medium with the tweezers so that the leaves can be better accommodated on the media.

~~7. Callus Induction Medium (CIM: MG medium with naphthalene acetic acid (NAA) [5 mg/L], 6-benzylaminopurine (BAP) [0.1 mg/L]), supplemented with cefotaxime sodium [250 mg/L] to kill agrobacterium) and kanamycin [50 mg/L] or the corresponding binary vector selective marker (Figure 2C). (pa la tabla)~~

~~8. Incubate for one week in growth cabinets under long day conditions of 12 h 24 ºC light/ 12 h 20 ºC dark cycle a light/dark cycle of 12/12 h at 24/20 °C, respectively, and 67 µmol m-2 sec-1. Note: Appreciate the callus formation after a week incubation.~~

19. Transfer the leaves keeping abaxial side up~~(again with the abaxial side up)~~ to SIM medium ~~supplemented with antibiotics (see Table 2) (~~Figure~~~~Figure 2C~~D~~)~~. and incubate them in a growth cabinet, refreshing the medium every 7-10 days, until the shoots are about 2 cm tall (Figure 2E).

~~Shoot Induction Medium (SIM: MG media with zeatin riboside [2 mg/L], NAA [0.02 mg/L] and gibberellic acid (GA3) [0.02 mg/L]), supplemented with cefotaxime sodium [250 mg/L] to kill the Agrobacteria) and kanamycin [50 mg/L] or the corresponding binary vector selective marker (Figure 2D).~~

Note: Scrape the SIM medium with the tweezers so that the leaves can be fully surrounded by the media. When the emerged shoots reach the lid, work with tall petri dishes.

~~Note: Appreciate the callus formation after a week incubation.~~

~~9. Shoot Induction Medium (SIM: MG media with zeatin riboside [2 mg/L], NAA [0.02 mg/L] and gibberellic acid (GA3) [0.02 mg/L]), supplemented with cefotaxime sodium [250 mg/L] to kill agrobacterium) and kanamycin [50 mg/L] or the corresponding binary vector selective marker (Figure 2D).~~

~~Incubate for 3-8 weeks, refreshing the medium every 7-10 days, until the shoots are about 2 cm long. Transfer the leaves to fresh SIM media every 7-10 days.~~

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Note: The callus will form after 2-3 weeks in SIM medium (Figure 2D) and the shoots after 6-7 weeks. The shoots will be considered as independent transformation events when they emerge from callus formed from independent wounds. ~~every callus that came out from a single wound is considered as a single transformation event.~~

~~10.~~ Note: The shoots will form after 6-7 weeks in SIM medium (Figure 2E).

20. ~~Take~~ Cut three shoots ~~of~~ emerged from each ~~callus~~ callus (considered the same transformation event) (Figure 2E).~~(same transformation event) and~~ transfer them to culture flasks with MG medium supplemented with cefotaxime sodium [250 mg/L] ~~(Figure 2D)~~to allow rooting, label the subset with a number and incubate in a growth cabinet for 3-4 weeks or until the shoots are vigorous (Figure 2F).

~~supplemented with cefotaxime sodium [500 mg/L]. Number each independent transformation event separately.~~ Critical step: When cutting the shoots, remove well the callus otherwise the root will not form.

20.1. Repeat the step as many times as independent lines are needed.

20.2. Up to 5 different transformation events can be placed in a culture flask with a diameter of 8 cm to work in full confidence that the plants from different events are not mixed.

~~Incubate the shoots for 3-4 weeks or until the shoots plants are vigorous, approximately 3-4 weeks.~~

~~Note: Up to 5 different transformation events can be placed in a culture flask with a diameter of 8.2 cm.~~

~~11. Critical stepaution: When cutting the shoots, be aware of not taking part of the callus, in the case that itthat occurs, roots will not grow.~~

21. Select the most vigorous plant of each event, cut ~~it~~the apical segment of the shoot with 3-4 internodes ~~,~~and place it in a new culture ~~dish~~flask ~~flask~~ with 2 MS medium supplemented with cefotaxime sodium [250 mg/L] ~~(Figure 2E)~~ .

~~Note~~Note: ~~In this medium, i~~In 3-~~4~~ 6 weeks~~,~~ the plant will grow efficiently, developing ~~the~~ a vigorous~~complete~~ shoot and roots ~~organs (it could take up to 6 weeks)~~.

~~12.~~ ~~Critical step~~Note~~aution~~~~Note~~: ~~Save~~ Bring back to the chamber the ~~remaining~~ non-selected shoots until ~~the one selected~~the plant selected ~~has developed roots~~fully developed.

22. Make stem ~~e cuts~~segments from the ~~fully developed~~ plant with at least one internode or with the apical bud and transfer ~~it~~ them to new ~~2~~ 2-MS medium ~~(Figure 2E)~~ supplemented with cefotaxime [250 mg/L]. Incubate them in the growth cabinet.

22.1. Replicate every 3-4 weeks to establish the ~~*in vitro*~~*in vitro* transformed lines ~~and grow in parallel the negative control plants~~.

~~13. Let it grow for 3-4~~5 weeks and replicate it again. Repeat this step ~~every 3-4~~5 weeks to maintain ~~in vitro~~ lines. Note: The ~~medium has to be supplemented with~~ cefotaxime sodium is needed in at least three subsequent transfers to 2 MS medium to be sure to kill the ~~*A. tumefaciens*~~*A. tumefaciens*; afterwards, if ~~*A. tumefaciens*~~*A. tumefaciens* overgrowth is observed, transfer plant lines again to ~~fresh~~ 2 MS media supplemented with cefotaxime sodium ~~[500 mg/L]~~.

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23. To characterize the plant phenotype, ~~If the objective is to massively develop plants, then~~ transfer ~~the plants~~plants to ~~an hydroponic culture or to~~ soil for their full characterization or to hydroponics for root inspection~~ media~~.

~~14.~~23.1. Keep the tubers produced in soil to propagate and maintain the established lines.~~The plants can be transferred to a hydroponic medium or soil to allow for massive development.~~

[Place ~~Figure~~Figure 2 here]

Hydroponic culture

24. Prepare the Hoagland's solution to a half strength (0.5X) (Table ~~1~~2) in a 10 L bucket.

~~Place the components of Table 1 to get 0.5X Hoagland's solution (Table 1) in a bucket and fill it up to 10 L of distilled water.~~

25. ~~.~~ ~~i~~Immerse an aquarium pump to maintain homogeneity and proper oxygen conditions~~. and homogeneity in the medium. and c~~

~~1.~~26. Cover the walls of the ~~-~~bucket with aluminium foil to ~~allow the roots to~~ grow roots in ~~the~~ dark conditions.

27. Avoiding root damage,~~Take~~ transfer ~~*in vitro*~~*in vitro* plants ~~and transfer them~~ to hydroponic culture.

Note: R~~r~~emove any remaining *in vitro*~~*MS*~~ medium from roots to avoid microorganism proliferation during incubation by ~~immersing~~shaking carefully the ~~roots~~roots ~~in~~immersed in water.

~~and transfer them to the hydroponic culture.~~

28. ~~Remove the plants from the solid in vitro medium and carefully transfer them (avoiding root damage) to the hydroponic culture.~~ Cover the plants with transparent film like a glasshouse to allow adequate acclimatization and incubate in the ~~to allow for proper acclimation.~~ growing chamber.

29. Make holes in the film after 3 days and remove it completely ~~remove it~~ one week after~~. remove it a week after~~ after a week~~.~~

30. Replace with fresh media every 10 days.

~~2. Maintain the culture in the growing chamber to keep the photoperiod (dark/light 8/16 h), temperature (20 °C) and optimal humidity optimal (63 %) for growing.~~

~~3. Let the plants grow 2-3 weeks to allow the full development of the root system. Note: At this step, different analyses can be performed, as in this same example, GUS assay. . In our case, we proceed with the GUS assay.~~

~~19.~~

[Place Table ~~1~~ 2 here]

~~[Place Table 2 here]~~

GUS histochemical reporter gene assay

Note: In our case the GUS analysis was performed with roots of 2-3 weeks grown in hydroponics.

31. Fix the roots ~~tissue~~ with 90 % chilled acetone (v/v) and incubate it for 20 minutes on~~in~~ ice.

32. Perform two washings with distilled water.

33. ~~and a~~Add fresh GUS staining solution~~,~~ (Table 3) and a~~(1 mM X-GlcA (5-Bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylammonium salt) from a~~

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stock of 1 mg / 100 µL of methanol, 50 mM sodium phosphate buffer 2X pH7, 1 mM potassium ferricyanide, 1 mMMm potassium ferrocyanide, 10 mM EDTA and 0.05% (v/v) triton X-100). Apply vacuum (-70 Pa) for 20 min.

34. and iIncubate at 37 °C in the dark to protect the photosensitive GUS (X-Gle photosensitivity) for 4 hours or until a blue colour is visible.

Note:

Note: The presence of ferri- and ferrocyanide in GUS solutionthe incubation media is a critical point for the visualization reaction; they minimize the diffusion of primary reaction products and provide more precise localization. Caution: Protect GUS solutionThe GUS solution needs to be protected from light because it contains photosensitive X-GlcA, ferri- and ferrocyanide reagents. It can be stored in the dark at 4 °C.

Caution: Caution: Use a fume hood and wWear glovesprotective clothing when handling the toxic cyanide derivatives in GUS solution (, as the potassium ferricyanide and potassium ferrocyanide). contain toxic cyanide that should be handled in an extraction cabinet. The GUS substrate and the disposal material should be disposed safely.

1. Dispose tThe disposable material that has been in contact with the solution must be disposed of in appropriate containers. The GUS substrate should be disposed safely. a temporary container in an extraction cabinet to facilitate its subsequent treatment.

35. Remove the GUS staining solution and discard it in appropriate containers.

36. Perform two washings with ethanol 70% (v/v).

2. Observe under a and keep the sample in this solvent at 4 °C until observation under a bright field microscope.

37.

3. Note: GUS staining is stable for a few weeks; however, during the first week, the GUS signal is clear and diffuses less into neighbouring cells. For longer storage, seal the tube and store it at 4 °C. CautionCaution: Discard the GUS solution in an appropriate container. Collect tTransgenic plants must be collected and disposed inside them into biological containers to be duly processed.

[Place Table 3 here]

REPRESENTATIVE RESULTS:

***Agrobacterium*Agrobacterium *rhizogenes*-mediated potato transformation**

The first protocol described iIn this manuscript the step-by-step procedure is set up to obtain, step-by-step, transformed root transformation wwith *A. rhizogenes*A. *rhizogenes* from native wild-type plants is presented.. FigureFigure 1 presents an overview of the assay, which altogether takes around 21 days5-6 weeks (from injection of *A. rhizogenes*A. *rhizogenes* to selection of fluorescent roots). Once the hairy roots areobtain obtainedfully developed hairy roots). Then, the plant can be studied as a composite (wild type shoot, transgenic root) or the transgenic hairy root clones can be excised andto let them grown autonomously in solid Gamborg B5 medium supplemented with 2% sucrose. Alternatively, the hairy roots can be massively propagated using the liquid Gamborg B5 media. The procedure presented has been carried out with *S. tuberosum* spp. *tuberosum* (cv. Désirée).

The method to monitor the procedure and to obtain potato transgenic hairy roots has been validated using the DsRed2 transformation marker using a binary vector with the

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~~DsRed2~~DsRed as a transformation marker (~~i.e. pKGW-RedRoot or~~ PK7GWIWG2_II-RedRoo~~o~~t from VIB-Department of Plant Systems Biology at Universiteit Gent). This~~that~~ allowed~~s~~ the easy distinction ~~identification~~ of ~~the~~ transgenic hairy roots from non-~~those that are not~~ transgenic by the~~using~~ red fluorescence. According to that, i~~I~~n ~~Figure~~Figure 3~~,~~ the transformed hairy roots ~~that contains the T-DNA from the binary vector~~ exhibited red fluorescence when ~~they were~~ illuminated ~~them~~ with green light. ~~A~~The negative control using the untransformed ~~A~~*A~~*Agrobacterium* showed no red fluorescence (~~Figure~~Figure 3C), overall indicating the suitability of the ~~DsRed2~~DsRed transformation marker to ~~show~~ identify the transgenic hairy roots (~~Figure~~Figure 3D). Other transformation markers such as antibiotic resistance can be used as described by other authors[23, 24], however, the antibiotics in the media can produce a growth delay in transgenic roots containing the marker.

[Place ~~Figure~~Figure 3 here]

***~~Agrobacterium~~Agrobacterium tumefaciens*-mediated potato transformation**

The second protocol described in this manuscript is set up to obtain, step-by-step, a complete potato plant ~~transformation~~ transformed with ~~*A. tumefaciens*~~*A. tumefaciens*. ~~Figure~~Figure 2 presents an overview of the procedure, which altogether takes between 1~~5~~5-1~~8~~8 weeks (from leaf infection with ~~*A. tumefaciens*~~*A. tumefaciens* to obtaining fully regenerated plants). The most time-consuming part of the procedure is the plant regeneration by organogenesis~~, which takes around 5-6 weeks~~. This particular step ~~is the one that~~ makes this method more laborious than ~~that~~ using ~~*A. rhizogenes*~~*A. rhizogenes*. The procedure has been carried out with *S. tuberosum* spp. *tuberosum* (cv. Désirée) and *S. tuberosum* ssp. *andigena*, the former being less dependent on short-day conditions to induce tuberization.

In the ~~*A. tumefaciens*~~*A. tumefaciens*-mediated transformation, in contrast to the ~~*A. rhizogenes*~~*A. rhizogenes*-mediated transformation, the regenerated plants are completely transgenic organisms. However, though the transgenic plants are regenerated in a kanamycin selective media, not all the lines efficiently express the transgene. Hence, validation of the transgene expression is needed.

Comparison of the promoter *FHT* activity in roots obtained using ~~*A. tumefaciens*~~*A. tumefaciens* and ~~*A. rhizogenes*~~*A. rhizogenes*

The aforementioned procedures were applied to produce roots expressing the *GUS* gene under the promoter of *FHT* gene. The complete transformed plants with ~~*A. tumefaciens*~~*A. tumefaciens* were previously reported[15], using the binary vector pKGWFS7 containing the *FHT* promoter. Now, this binary vector has been used to produce new transformed hairy roots to compare the tissues where the promoter is active and therefore to test the hairy root system as a tool to ~~test~~study promoter activation. ~~Potato transformed hairy roots to express the GUS gene under a suberin biosynthetic promoter (*CYP86A33*) were previously reported (Bjelica et al.,2016), however the GUS expression in roots was bulk quantified using a fluorometric assay, hence the expression in suberized tissues was only presumed.~~

~~Figure~~Figure 4 shows GUS ~~expression~~ staining in roots of transgenic plants obtained by *A. tumefaciens* (Figure 4A-B) and transgenic hairy roots obtained by ~~*A. tumefaciens* and roots of transgenic plants obtained by *A. rhizogenes*~~*A. rhizogenes* (Figure 4C-F), respectively. As can be seen, roots transformed with ~~*A. tumefaciens*~~*A. tumefaciens* and grown ~~*in vitro*~~*in vitro* show blue staining in the endodermis (~~Figure~~Figure 4A), a cell layer between the cortex and the stele. In more developed roots, the blue labelling is patchy in the external layer corresponding to the exodermis (~~Figure~~Figure 4B). ~~This~~

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binary vector was used to produce new transgenic hairy roots to determine the tissues where the *FHT* promoter was active and compare it with roots obtained using *A. tumefaciens*. In transformed hairy roots grown in hydroponics, the GUS marker was specifically located in the endodermis (FigureFigure 4C, 4E), in the emergence of lateral roots (FigureFigure 4D, 4E), in the wounded areas where the root presented wounded areaswas probably wound healinging the damaged tissue andbroke, hence in the wound healing areas (FigureFigure 4E), and in the exodermis (FigureFigure 4F). The roots showed no GUS stain in negative controls that were either hairy roots without the *PromFHT:GUS* T-DNA cassette or wild type roots.

[Place FigureFigure 4 here]

FIGUREFIGURE AND TABLE LEGEND:

FigureFigure 1: Timeline to obtain potato transgenic hairy roots using *A. rhizogenesA. rhizogenes*. The lower part of the figure shows tThe cumulative weeks to reach each stage of the processtransformation process and the subsequent steps to grow the hairy roots are shows and its brief description. Representative iThe upper part depicts images of different stages are depicted: . First, the initiation of the process using 3-week-old *in vitroin vitro* plants (A), then infection of the plants by puncture injectingwith *A. rhizogenesA. rhizogenes* (B), the formation of the callus proliferative tissue (C,(arrows) with emerging hairy roots (DC), and the transgenic developed hairy roots expressing the red fluorescent transformation marker DsRed2DsRed (D)(E).

FigureFigure 2: Timeline to obtain potato transformed plants using *A. tumefaciensA. tumefaciens*. The cumulative weeks to reach each stage of the transformation process and the subsequent steps to grow the plants are shown. The lower part of the figure shows the cumulative weeks to reach each stage of the process and its brief description. Representative images of different stages are depicted: The upper part depicts images of different stages. First, the initiation of the process using leaves from 3-week -old *in vitroin vitro* plants (A), then potato leaf cutting patterns to infect with *A. tumefaciens* (B), the transfer of the wounded and infected leaves to the effect of callus generation by CIM media (BC), the leaves when transferred to SIM media (C), the visualization of the callus around the wounded areas after 2-3 weeks in SIM media (D), the shoot formation after 9-11 weeks in SIM media (E), and the shoots after being transferred to MG media (F). effect of shoot generation by SIM (CD),shoot propagation in MG medium (D), and plant propagation in 2MS medium (D) once they are self-sufficient enough to maintain the generated lines and perform different analyses, (DE).

FigureFigure 3: Fluorescent transgenic hairy roots of potato (cv. Désirée) transformed by *A. rhizogenesA. rhizogenes*. The hairy roots were obtained using a non-transformed *A. rhizogenesA. rhizogenes* (strain C58C1: pRI1583) (A and C) and with *A. rhizogenesA. rhizogenes* (strain C58C1: pRI1583) transformed with the empty binaryvector pK7GWIWG2- (II) Red-Root carrying a DsRed2DsRed transformation marker (B and D). TThe hairy roots are observed formed under a stereomicroscope in both infections (A, B) but only red fluorescence is only observed in hairy roots transformed with the *A. rhizogenes* containing the binary vector. The images were taken with a stereomicroscope equipped with a lamp and a specific filter to visualize the red fluorescence. roots are observed in the epifluorescence microscope when using *A. rhizogenes* transformed with the binary vector (C, D). Scale bar: A µm

FigureFigure 4: Histochemical observation of transgenic potato roots expressing the *GUS* expression reporter gene driven by the promoter of *FHT* in potato. (cv. Désirée) transgenic plants and transgenic hairy roots obtained with *A. tumefaciens* (*S. tuberosum*

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~~ssp. *andigena*) and *A. Rhizogenes* (*S. tuberosum* ssp. *tuberosum* cv. Désirée) respectively.~~ The roots from complete transgenic plants obtained by *A. tumefaciens* (*S. tuberosum* ssp. *andigena*) transformation (A-B) ~~The roots transformed with *A. tumefaciens* (A and B)~~ show blue staining in the endodermis (A) and exodermis (B). The transgenic hairy roots obtained by *A. rhizogenes* (*S. tuberosum* ssp. *tuberosum* cv. Désirée) transformation (C-F) ~~The roots transformed with *A. rhizogenes* (C-F)~~ display GUS ~~labelling~~ staining in the ~~endodermal cells~~endodermis (C and E), in the lateral root emergence (D and E), in the ~~the~~ wound-healing zone (E) and in the exodermis (F). Endodermis (EN); Exodermis (EX); Xylem (XL); ~~(LR)~~ Primordia of a lateral root (LR). The red arrow indicates the wounded area ~~zone, while black arrows indicate only cells~~.

Table 1: Media recipes used for growing bacteria and *in vitro* plants.

Table ~~1~~2: Half strength ~~Nutrient for Solutions in Components for H~~Hoagland's s~~S~~olution for growing potato plants in hydroponics.~~components~~preparation, ~~specifying their stock solution and and the required volume of each one in 0.5X Hoagland's solution (10 L) stock concentrations and mL needed for 1 L and 10 L.~~

~~Tabe 2: Components for the preparation of p~~Table 3: GUS staining solution recipe.~~lant media and bacteria media. The hormones, vitamins and antibiotics are specified for each one of them~~

DISCUSSION:

In potato, the most common system to obtain stable complete transgenic plants uses the transformation by ~~Agrobacterium~~*Agrobacterium tumefaciens* strains that require organogenesis using exogenous phytohormones. Although the *Agrobacterium* based protocols has the potential to integrate non-T-DNA vector sequence[25], this methodology is still the easiest and less expensive available to transform potato plants. During last years, the interest in *A. rhizogenes*-mediated transformation has got the attention of researchers for allowing to obtain transgenic roots in shorter periods than using *A. tumefaciens*. The *A. rhizogenes* still preserves the root-inducing (Ri) plasmid that carries a set of genes encoding enzymes for the phytohormone auxin control and cytokinin biosynthesis, and encoding for opines[26]. Once the Ri T-DNA is inserted into the host genomic DNA, the new hormonal balance deregulates the infected cells inducing the formation of proliferating roots, called hairy roots, emerging at the points of infection[27]. When an additional binary vector is used for integrating a foreign DNA, the preservation of the Ri plasmid confers the possibility to obtain transformed hairy roots with no need of organogenesis using exogenously applied phytohormones[28]. The hairy roots can be maintained attached to the wild type shoot generating a composite plant or can be self-propagated. This ability of hairy roots to self-propagate is being exploited to produce in several plants hairy roots as a biological system for mass-producing valuable metabolites or foreign proteins, generating interests in pharmaceutics and even phytoremediation areas (see for review[29,30]). In potato (var. Kufri Bahar), transgenic complete plants were infected with wild type *A. rhizogenes* strain to produce hairy roots expressing the Hepatitis B surface antigens (HBsAg)[23]. Alternatively, a complete transgenic plant regenerated from hairy roots can be obtained, but potato plant and tubers showed distinct development compared to the untransformed controls. These differences in the phenotype are due to the original Ri T-DNA integrated within the genome[31, 32].

~~However, though potato plants developed from hairy roots have been proven to develop normally[28], this strategy may present limitations, especially when studying tightly-controlled developmental processes, because the Ri plasmid still contains the T-DNA~~

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genes responsible for the induction of hairy roots (rol A, B, C and D) and the synthesis of opines and auxin genes (necessary for the maintenance of these roots). Once the T-DNA is inserted into the host genomic DNA, it will hormonally deregulate the infected cells, leading to root-like proliferation and cellular organization, generating hairy roots at the points of infection29. The *A. tumefaciens* system does not present this limitation since the original T-DNA, responsible for inducing the tumour, is completely removed. Another limitation of generating composite plants using *A. rhizogenes* with a native scion is that the system is not stable between generations, as the tuber or floral organs are still wild type. However, some examples of functional genetic studies have been successful using *A. rhizogenes* in potato, tomato and eucalyptus to demonstrate gene function[5-8], and to study the hormonal response[30,31] and promoter activity[32,33].

, but this system is costly in terms of time and money, since it is necessary to regenerate a whole plant using phytohormones. This has contributed to the development of alternative methods that can overcome these limitations. The use of *Agrobacterium rhizogenes* to achieve transformed hairy roots is an example. In this work, the detailed procedures to obtain transgenic stable hairy roots using *A. rhizogenesA. rhizogenes* and transgenic stable plants using *A. tumefaciensA. tumefaciens* are presented (Figure 1 and 2). The fully developed transgenic hairy roots were obtained in 3-45-6 weeks, while transgenic roots using *A. tumefaciensA. tumefaciens* needed 15-18 weeks due to the organogenesis requirement from transformed cells, and the selection and propagation of transformed plants. In our hands, the *A. tumefaciensA. tumefaciens* transformation procedure works efficiently in *S. tuberosum* ssp. *andigena* and ssp. *tuberosum* (cv. Désirée), with a reported transformation efficiency around 35 %[22] and 48 %[12], respectively. The described *A. rhizogenesA. rhizogenes* transformation procedure is based on that reported by Horn[7], which showed a high (80–100 %) transformation efficiency in cv. Deésirée and other three potato cultivars (Albatros, Deésirée, Sabina and Saturna).

To present *A. rhizogenes* as an alternative transformation system for potato functional studies and to indicate whether the initial presence of Ri T-DNA was matter of concern, we used both systems to transform potato roots with the same binary vector that contained a T-DNA with a promoter of

Moreover, tThe roots transformed using both Agrobacterium systems were compared to determine the activity of a promoter of a suberin biosynthetic gene (*FHT*) driving the expression of the *GUS* reporter gene. The histochemical analysis revealed that in *A. tumefaciens* transformed plants, *have proven to be useful for the study of the promoter's activity*. In detail, the activity of the *FHT* promoter was in the inner and outer suberized layers of the roots (endodermis and exodermis, respectively) (Figure 4A-B) and also in the wounded areas of the leaf, stem and tuber[15]. In *A. rhizogenes* transformed hairy roots, the activity was also detected in endodermis and exodermis and in the wounded root areas (Figure 4E). The co-occurrence of the suberin promoter activity in both types of transformed roots indicates that the Ri integrated T-DNA is not affecting the developmental processes related to suberization at least in these root tissues. In transformed hairy roots we also detected and in the wounded areas of the leaf, stem and tuber[14], and the results indicated that both systems have proven to be useful for the study of the promoter's activity. In detail, the activity of the *FHT* promoter was in the inner and outer suberized layers of the roots (endodermis and exodermis, respectively) and in the wounded areas of the leaf, stem and tuber[14]. The transformation with *A. rhizogenes* confirmed the tissue-specific activity of the *FHT* promoter found with *A. tumefaciens* transformation, but also showed induction of the *FHT* promoter in the wounded root and in in the areas surrounding the emergence of lateral roots (Figure 4D, 4E). The

~~induction of the *FHT* promoter closer to the lateral root emergence.~~ This agrees with the promoter activity reported by other genes involved in the transport of suberin monomers such as *ABCG11 / WBC11*[33–36] or the regulator *StNAC103*[19]. ~~The co-occurrence of the expression of *FHT* in roots of potato plants transformed with *A. tumefaciens* and *A. rhizogenes* indicates that the appearance of adventitious roots does not affect plant development, at least those development processes related to suberization.~~ The potato transformation by ~~*A. rhizogenes*~~*A. rhizogenes* to study a suberin gene~~,~~ has already been ~~yet~~ reported ~~in the past~~recently[37] and also in that case hairy roots have allowed to show the promoter activation of *CYP86A33*, a fatty acid ω~~w~~-hydroxylase. However, ~~Potato transformed hairy roots to express the GUS gene under a suberin biosynthetic promoter (*CYP86A33*) were previously reported (Bjelica et al.,2016), however the GUS expression~~staining in roots was bulk quantified using a fluorometric assay, hence the specific expression in suberized tissues was only presumed.

Altogether these results ~~demonstrated~~ evidence that ~~*A. rhizogenes*~~*A. rhizogenes* transformation is a faster alternative tool to ~~a suitable and faster method to study~~explore the cell type-specific promoter activation of ~~suberin~~suberin-related genes in roots, ~~-related promoters an~~which may be extended to~~d~~ studies based on other processes that occur in roots. In agreement, some other functional genetic studies have been successful using *A. rhizogenes* in potato, tomato and eucalyptus to demonstrate the gene function[6–9], to study the hormonal response[38,39] or the promoter activity[40,41]. However, this strategy still may present limitations, especially when studying tightly-controlled developmental processes that may be deregulated by the Ri T-DNA or when the whole plant or the tubers or other organs different from roots want to be studied. In these situations, the *A. tumefaciens* transformation system is still preferred.

~~However, though potato plants developed from hairy roots have been proven to develop normally[28], this strategy may present limitations, especially when studying tightly-controlled developmental processes, because the Ri plasmid still contains the T-DNA genes responsible for the induction of hairy roots (rol *A*, *B*, *C* and *D*) and the synthesis of opines and auxin genes (necessary for the maintenance of these roots). Once the T-DNA is inserted into the host genomic DNA, it will hormonally deregulate the infected cells, leading to root-like proliferation and cellular organization, generating hairy roots at the points of infection[29]. The *A. tumefaciens* system does not present this limitation since the original T-DNA, responsible for inducing the tumour, is completely removed. Another limitation of generating composite plants using *A. rhizogenes* with a native scion is that the system is not stable between generations, as the tuber or floral organs are still wild-type. However, some examples of functional genetic studies have been successful using *A. rhizogenes* in potato, tomato and eucalyptus to demonstrate gene function[5–8], and to study the hormonal response[30,31] and promoter activity[32,33].~~

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DISCLOSURES:
The authors have no conflicts of interest to disclose.

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Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have revised it.

2. Title: Please revise the title to avoid punctuation.

The new title: “Agrobacterium tumefaciens and Agrobacterium rhizogenes-mediated transformation of potato and the promoter activity of a suberin gene by GUS staining”.

3. Please provide an email address for each author.
We have added it.

4. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …” We have modified the abstract according to JoVE Instructions for Authors.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Gelrite, Duchefa, Falcon, Leukopor, etc.
We have modified it according to JoVE Instructions for Authors.

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
We have modified it accordingly.

7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
We have modified it according to JoVE Instructions for Authors.

8. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
We have modified it accordingly.

9. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.
We have modified it accordingly.

10. Line 126: What volume of fresh YEB medium is used to resuspend cells? We have specified the volume as 1 mL of fresh YEB.

11. Line 183: Please specify at what wavelength the optical density is measured. We have specified it.

12. Line 247: What components? We referred to the Hoagland's media nutrient components. We have rephrased it.

13. Please include single-line spaces between all paragraphs, headings, steps, etc.
We have included it.

14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
We have modified it accordingly.

15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
We have modified it accordingly.

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
We have modified it accordingly.

17. Figure 3: Please include a scale bar to provide context to the magnification used. Define the scale in the appropriate figure Legend.
We have modified it accordingly.

18. Table 1: Please include a space between all numbers and their corresponding units (i.e., 2 M, 1 M, etc.). Please change "ml" to "mL". Please make the number 2 in H2O a subscript.
We have modified it accordingly.

19. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

We have modified it accordingly.

Reviewer #1:
Dear Authors,
I have reviewed the manuscript entitled, "Agrobacterium tumefaciens and A. rhizogenes-mediated transformation of potato: the promoter activity of a suberin gene as a proof of concept." Although the manuscript provides detailed methods for potato transformation, many of the main elements required by the journal are not met and I therefore am not recommending this manuscript for publication. The following are the questions I considered when making this decision and my responses to them:

*Are the title and abstract appropriate for this methods article?
The title seems appropriate but the abstract lends little information towards what is actually provided in the methods and is more of a general overview of the advantages of either transformation system (i.e. A. tumefaciens vs A. rhizogenes).
The long abstract was modified according the suggestions of the reviewer focusing more on the methods, their purpose and a brief summary of the advantages.

*Are there any other potential applications for the method/protocol the authors could discuss?
One application is discussed where A. rhizogenes transformation can be used to study root phenotypes and materials generated in less time than using A. tumefaciens transformation. However, this potential application is limited and other applications, such as ones related to propagation of transgenic hairy root clones using the propagation system included are not discussed.
We have included the potential of hairy roots to act as a biological system for mass-producing valuable metabolites or foreign proteins, generating interests in pharmaceutics and even phytoremediation areas. We also have provided a potato example in which hairy roots were used to produce the Hepatitis B surface antigens (HBsAg).

*Are all the materials and equipment needed listed in the table? (Please note that any basic lab materials or equipment do not need to be listed, e.g. pipettes.)
Yes, these seem to be in order.

*Do you think the steps listed in the procedure would lead to the described outcome?
Yes, possibly. Depending on the potato genotype used which is not mentioned or discussed.
The genotypes tested with these protocols were already mentioned in the plant material and the results sections.

*Are the steps listed in the procedure clearly explained?
No. The methods are overly complicated where they could be simplified to ease application to other labs (e.g. "Plant transformation using A. rhizogenes" could be accomplished using methods similar to A. tumefaciens). Furthermore, steps critical to the success of the protocol are not detailed enough (e.g. step 4 of "Plant

transformation using A. tumefaciens") and figures do not aid in understanding or carrying out critical steps.
We have changed the protocol steps with the aim to make it clearer using shorter sentences and including Tables for the recipes that makes the reading less distorting. We have also grouped some overlaying parts of the procedures in Agrobacterium cultures used for transformation and Plant material for transformation. We have made an accurate review to highlight the critical steps, which will be included in the video format. We have modified the figures to better represent the steps that are more relevant for the whole procedure.

*Are any important steps missing from the procedure?
Yes, the way in which the plant materials are grown that are used for transformation is not described.
The plant material used for transformation was already described in the first version of the manuscript, however, to make it clearer to the reader, we added a section entitled "Plant material for transformation".

*Are appropriate controls suggested?
No, no controls are suggested.
In each transformation procedure we specified that negative controls are needed. Regarding the protocol of *A. rhizogenes* we proposed to use the *A. rhizogenes* strain untransformed or transformed with the empty vector that includes the transformation marker. In the *A.tumefaciens* transformation we proposed to use the wild type or plants transformed with the empty vector.

*Are all the critical steps highlighted?
No, no critical steps are highlighted.
There are some critical steps that were warning by word "Caution" in the previous manuscript version. Now in the present manuscript, these steps have been highlighted as Critical steps. The word "Caution" is only used when special measures must be taken with the chemicals or with wasted plant material. We have highlighted all the critical steps for the video production.

*Is there any additional information that would be useful to include?
Yes, please see previous comments.
We already amended previous comments.

*Are the anticipated results reasonable, and if so, are they useful to readers?
No quantifiable results are provided towards efficiency of the methods and the visual data comparing the two methods (i.e. GUS staining) is inconclusive.
The goal of this manuscript is to compare the localization of promoter activity using both transformation systems. To get this aim we used a chromogenic substrate to perform a histochemical analysis, but it is not appropriated for quantification. In the discussion section now we are providing information about the efficiency of both methodologies.

*Are any important references missing and are the included references useful?
These seem to be in order.
Sincerely,
Reviewer

We thank to the Reviewer her/his comments that help to improve the new version of the manuscript. We hope that this revised version fulfils the expectations of the Reviewer.

Reviewer #2:
Manuscript Summary:
The manuscript "Agrobacterium tumefaciens and A. rhizogenes-mediated transformation of potato: the promoter activity of a suberin gene as a proof of concept" (JoVE59119) submitted by Fernández-Piñán et al, describes the Agrobacterium-mediated transformation of potato, using both A. tumefaciens and A. rhizogenes. As proof of concept, the authors demonstrate the promoter-driven expression of GUS in root tissue, based on a suberin-associated gene, FHT.

Major Concerns:
None; this MS is timely, as potato transformation is becoming more important in the analysis of potato genes. With the potato genome sequence being published ~8 years ago, there has been a much greater interest in this plant species as an experimental model (primarily because of its importance as a food crop). Having a routine and robust transformation protocol for potato is important for advancing studies on the crop and for implementing biotechnological solutions to its production and storage. There have been other protocols for potato transformation published (indeed the author's protocols are based on previously published protocols), but the JoVE environment allows for a more detailed description of the procedure.
We thank the Reviewer for her/his positive comments and also for all the suggestions raised below. We think that her/his help has contributed to improve the revised manuscript.

Minor Concerns:
There are a few minor typos (ln 62, roo.ts; ln 72, "works" should be "studies" and ln 268, "Mm should be "mM").

These mistakes have been amended.

Also, it would be good if the authors provided some discussion about cultivar choice. Are all potato cultivars amenable to this protocol? There are older references that compare cultivars and show differences. The utility of the protocol would be greatly enhanced if it were universally applicable to any potato cultivar.

In our laboratory we are working with *S. tuberosum* ssp. *tuberosum* cv. Désirée because it is the model of choice of many laboratories working on plant molecular genetics. We also are working with ssp. andigena because its tuberization is

photoperiod-sensitive, a feature that is very valuable in some physiological studies. However different potato cultivars were successfully transformed by *A. tumefaciens* in the past (Block 1988) including economically important potato cultivars (Beaujean et al., 1998). Regarding *A. rhizogenes*, when setting up the procedure in our laboratory, we tested different Agrobacterium strains and explants, and very clearly the procedure described by Horn et al., 2014 was the most robust and successful. These authors also reported the efficient transformation by *A. rhizogenes* of four different cultivars. Hence both systems of transformation are efficient for different potato cultivars and probably can be applicable to any potato cultivar.

We added in the discussion the information about the transformation efficiencies of both procedures to highlight that they are acceptable (35-48 %) using *A. tumefaciens*, and superior (80-100%) using *A. rhizogenes*, thus encouraging the scientists to apply the procedures to their working potato cultivars.

This issue should at least be addressed. Also, a recent paper by Bjelica et al (2016) Plant Cell Reports 35, 2435-2448 described hairy root transformation as a methods to study root-specific processes, and used promoter:GUS construct based on a suberin associated gene (CYP86A33); this should be acknowledged since the gist of the JoVE article is that *A. rhizogenes* is a viable, cheaper and faster alternative to A. tumefaciens, but this is not novel.

We acknowledge the Reviewer to make us notice that the Bjelica et al (2016) work was by mistake not included in the manuscript. In their work, they used a promoter:GUS construct of a CYP86A33 gene and in our case we used the promoter of FHT gene, both suberin biosynthetic genes. The main difference relies in that they quantified the GUS using a fluorometric assay from bulk root material and we used a histological GUS staining to localize in which tissue the suberization occurs. This allowed us to compare the tissues where the promoter was active in both the transformed hairy roots generated with *A. rhizogenes* and the complete transformed plants generated with *A. tumefaciens.* The similar localizations demonstrated that the hairy root transformation system is at least as good as the *A. tumefaciens* to study the suberization process and maybe other processes occurring in roots.

We have added the Bjelica et al. work and discussed it accordingly.

Reviewer #3:

Manuscript Summary:

The manuscript describes the method to transform potato cultivar Desiree and Andigena potato using Agrobacterium tumefaciens with helper Ti or A. rhizogenes with Ri plasmid. Binary vector PromFHT::Gus was used to compare the plant transformation systems. Authors state that Agrobacterium spp. are cost effective and less time consuming to generate hairy root phenotype or novel transgenic lines of potato. Gus activity in lateral roots has been demonstrated using A. rhizoegens. Additionally, the application of A rhizogenes to study the activation of promoters of suberin related genes in root tissue has been reported.

Major Concerns:
Line 54: Change DNA to T-DNA transfer and insertion mechanisms....
We have changed it.

Line 55: remove and between biotechnology research.
We have changed it.

Line 57 58: requires a reference to support the statement that MAT gives high transformation.
We have added the reference at the end of the sentence.

Line 64: change Agrobacterium to A. rhizogenes.
We have changed it.

Lines 71-72: Change many works to Previous studies...
We have changed it.

Line 74: remove plant.
We have removed it.

Line 76: add for human consumption between relevance and because.
We have added it.

Line 84: Italicize Arabidopsis.
We have maintained the upright format because in this case Arabidopsis corresponds to the common name, no the scientific name.

Lines 100-104: Media recipes in a tabular form would be much useful to replicate the media preparation in an easier way.
We provide the recipes in a new table (Table 1).

Line 105: change 3-to-4-week to 3 to 4 weeks old sterile potato plants.
We have changed it.

Line 106: stem cuttings does not clearly indicate what authors mean. Please provide an image and specify the stem with apical buds or auxiliary buds were used to generate these plants as donor plants.
We have included the information suggested by the reviewer to clarify the sentence.

Line 107: It is not clear why authors used 12/12 photo period and why there is 24/20 deg Celsius variation.
Our intention was to provide the conditions of our growing chamber that allows a successful transformation.

Lines 109-110: Please specify where this has direct relevance in the protocol.

This is a general good practice when working with *in vitro* plant material, to avoid dehydration and contamination. We have rephrased it and indicated that it is a general recommendation.

Line 113: please remove try to instead recommend using fresh media plates
We have rephrased it.

Line 122: A schematic representation of binary vector to illustrate the T-DNA would be beneficial.
We have described a more general procedure in which we are not mentioning any specific binary vector. To better understand the representative results, in the Results section we have indicated the sources of the binary vectors used.

Lines 124-127: Why there is no optical density measurements taken for A. rhizogenes overnight cultures before transformation?
We always have proceeded with no optical measurements, then we did not indicate any density because we think it is not relevant.

Line 128: rephrase
We have rephrased it to make it clearer.

Lines 133-135: Time??For how long?
We have specified it.

Line 139: Why the incubation temperature changed to 22/20 Deg C?
We acknowledge the reviewer to detect this mistake.

Line 148: MS medium containing any antibiotics or hormones?
MS medium does not contain neither antibiotics nor hormones. We have attached a table with the media recipes for clarification.

Lines 162: If careful aseptic conditions are not maintained, any sucrose or sugar based medium will get contaminated. How authors can prove that Gamborg B5 media is prone to contamination than other media reported in this protocol? Has this been tested during the protocol development. Also, there is no recipe for Gamborg B5 medium is provided.
We specifically indicated this caution for the Gamborg B5 medium because it is for growing roots and when they are contaminated it is not possible to disinfect the material using bleach based methods. But, we agree with the reviewer that all the high sugar containing media are prone to contamination. For this reason, we deleted it.

Line 178: Why A. tumefaciens is grown in 10 ml YEB and A. rhizogenes in 5 ml YEB? Also, the starter cultures are not specified, whether it was a single colony or a -80 deg C glycerol stock???
For *A. tumefaciens*, we have used different volumes of YEB, and depending on the colony biomass we inoculate that culture the day after if it is at the optimal OD_{600}=0.6-1. Since we consider that the starting volume is not critical for the protocol, we

homogenized both procedures to 5 mL as the reviewer suggested. We have also specified that the cultures were inoculated with a colony.

Line 183: OD was measured at what wavelength? Elaborate UA at least once.
UA should not be used. We have specified the wavelengths.

Line 184: change 'new' to fresh.
We have changed it.

Line 196 & 235, 238: remove space between 2MS
We have removed it.

Lines 198-200: Please provide a specific number of leaves that will fit in a specific sized petridish. Overcrowding may result poor transfection.
We have specified it based on the cultivar.

Line 204: IS 24 deg C optimum for transfection?
These are the conditions we use.

Lines 204-206: Please rephrase and also justify why authors think that leaving wounded leaves for long (how long?) can result in no transformation. Was this studied during the protocol development. If so, please provide this data to help readers in replicating this protocol in their laboratory. It will be much appreciated to warn readers with significant figures and data.
A. tumefaciens enters inside the leaf by the wounded areas and is in these areas around the wounds where the cells get transformed (under selective marker pressure the callus are generated). Therefore, we surmise that a longer delay in adding the bacteria after wounding allows higher wound healing and as a consequence less Agrobacteria can enter and poorer transformation efficiency is achieved. However, we agree with the reviewer that we do not have scientific evidence on that and therefore we have deleted the sentence to be more rigorous.

Line 210 & 218: 'the corresponding binary vector selective marker' is this indicating the T-DNA on binary vector? If so, why this was not introduced int he section where authors provide GUS gene and promoter information. This is why a schematic representation of binary vector is essential.
We were referring to the T-DNA selective marker. We rephrased the sentence to avoid misinterpretation. In the results section, we provide more information and references about the binary vectors used together with the representative results obtained for each of them. Also, the selective markers are provided in Table 1.

215: Provide medium SIM recipe in a table of media used in this protocol.
We have provided it in Table 1.

Line 219: Leaves by now would have some calli formed?
Not yet, the callus will be formed after 2-3 weeks in SIM media. Now we have specified it.

Line 222: Provide pictorial evidence for these steps to ease replication of protocol.
We agree with the reviewer that the images always help to replicate the procedure, for this reason we modified the Figure accordingly.

Line 225: What is MG medium?
It is now specified in the Table 1.

Line 228: What kind of culture flask?
In the figure 1A, you can see the type of culture flask, it is a flask used for apiculture (honey). It was obtained from a local provider. We specified the features and the distributor in the Materials table.

Line 244: Figure 2 should include images for 12 and 15 weeks. Also, donor plants (fig 2A) and transgenic plants obtained (fig 2E) are same.
At the end of the procedure the transgenic plants are grown in the same kind of flask as the donor plants. To show it to the reader, we used the same image. However to avoid misunderstanding, we changed the images of figure 2.

Line 261: This table need some amendments such as Components, stock concentration, stock preparation, and Hoagland's solution preparation (10 L). Make four columns.
We have changed the table to make it clearer following some of the recommendations of the reviewer.

Line 264: replace in with on.
We have replaced it.

Line 271: incubation media change to GUS solution.
We have replaced it.

Line 278: of is off. What is temporary container? Instead state: the GUS substrate should be disposed safely.
We have changed it.

Line 281: Why storage at 4 deg C is required?
We usually store the GUS stained tissues at 4 ºC to avoid ethanol evaporation but it could also be stored at room temperature. We have rephrased it including that the tube needs to be sealed.

Lines 288 to 322: There is no mention of transformation frequency which authors have achieved while developing these protocols. Providing such figures will allow a comparative replication of procedures in reader's own laboratory.
In the revised version of the manuscript we provide the transformation efficiencies in the discussion section based on our and other works.

Lines 310-311: Pleas rephrase to read well.
We have rephrased this sentence and also the corresponding for *A. rhizogenes*, which was also not properly builded.

Line 341: Pleas provide a control root image to compare the GUS expression in transgenic roots.
When there is no transgene, the root appeared with no GUS signal. To make it clear, we are stating it specifically in the results section.

Line 350: Figure needs more images and change the 2E.
We do not agree with the Reviewer that this Figure would need more images, since the reported images are those representatives of the different tissues where the GUS stain was positive. In our opinion, the addition of more images introduce redundancy, which we would like to avoid. To us, Figure 2E is relevant since it shows the FHT promoter activity in the wounded areas. We have resized the red triangle that indicates this area to be clearer.

Line 372: Rephrase the table legend.
We have rephrased it
.
Lines 376-378: AMT technology is much cheaper than any other non-Agrobacterium based techniques. How authors are making claim that AMT is time consuming and expensive. Please provide strong citation for such statements.
We wanted to indicate that *A. tumefaciens* is more time-consuming and is expensive compared to *A. rhizogenes*, but we agree with the Reviewer that the message could lead to her/his interpretation. We rephrased it.

Lines 402- 415: There is not specific structure in this part. There is a lot of jumping from A. rhizogenes to A. tumefaciens and back to A. rhizogenes. Moreover, this section does not highlight the used of A. tumefaciens and the benefits of generating stable transformants. Why would someone follow a protocol to generate transgenic roots or full transformed plants? Also, conclusive statements are missing in this section and thus the section ends with no conclusion. Authors should also consider the genotype dependency as major limitation of Agrobacterium based protocols widely reported for potato transformation (Petti et la 2009).
We agree that the structure of the discussion was somewhat confusing and the main conclusions were not visible. We have rewritten the discussion following the recommendations of the Reviewer.

The manuscript is hard to follow to perform a transformation experiments. The language is unclear and confusing at times. Authors have used the same image in Figure 1A and 2A, which still can be accepted, but image in Fig 2E transformed plant is also same as non-transformed plants (Fig 1A & 2A) in food jar. Moreover, a table of media recipe with clear concentrations and addition of stocks before autoclaving and post-sterilization would be very helpful to replicate the experiments. I am afraid that it is hard to follow this protocol, however it is interesting work.

All these points raised by the Reviewer have been used to improve the manuscript, to make it clearer and well-organized. We sincerely acknowledge to the reviewer for her/his exhaustive review of the manuscript, for her/his valuable comments and for the dedicated time to make this manuscript more readable and understandable. We hope that this new version fulfills the Reviewer expectations.

Minor Concerns:
There are some language issues that can be resolved.
We have revised the whole manuscript to solve the language problems.

Reviewer #4:
Manuscript Summary:
The manuscript is appropriate and well written for Jove journal. The information is very detailed, accurate and extensive.

Minor Concerns: The format of the manuscript is not consistent. The authors will have to do major corrections on this issue. Please, avoid using UA for absorbance, since absorbance doesn't have units. The authors should report the wavelength they measured their O.D., i.e., Abs600=0.8. After writing the full names of common acronyms (e.g., room temperature, RT, and overnight, O/N), the authors should use them consistently. It makes it easier to read the protocols. Please, review the English in the tables. There are some Spanish words there.
We thank the Reviewer for her/his comments. The new version of the manuscript is fully revised, including the suggestions raised by this Reviewer.