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Plant Biotechnology

Short communication

Title

***Rhizobium rhizogenes* -mediated Hairy-root Transformation of Daughter Plants from the Model Strawberry *Fragaria vesca*'s Stolons**

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Running Title

Hairy-root transformation of strawberry on stolons

Abbreviations

F. vesca: *Fragaria vesca*

R. rhizogenes: *Rhizobium rhizogenes* (former name, *Agrobacterium rhizogenes*)

R. radiobactor: *Rhizobium radiobactor* (former name is *Agrobacterium tumefaciens*)

CaMV: Califlower Mosaic Virus

cor47: cold-regulated 47 gene 5'-untranslated region

hsp: heat shock protein

LB medium: Luria-Bertani medium

1/2 B5 agar plate: 1/2 Gamborg's B5 medium 0.9% agar plate

Abstract

Strawberry, a member of the *Fragaria* genus within the Rosaceae family, is one of the most cherished fruits worldwide. This perennial herbaceous plant also serves as a model for studying the Rosaceae family. Despite the complex polyploidy of strawberries, extensive efforts in traditional breeding over the years have resulted in improvements in yield, fruit size and shape, berry quality, and various other aspects of strawberry production. However, in addition to these conventional methods, advanced genetic technologies such as genetic modification and gene editing in intricate polyploidy varieties of strawberry are also required. Here, we present the *Rhizobium rhizogenes*-mediated hairy-root transformation of daughter plants from the model strawberry *Fragaria vesca*'s stolons (also called runners), which exhibit diploid genomes. As a case study, new daughter plants were cut from the stolons, infected with *R. rhizogenes* harboring the *mVENUS* gene under Cauliflower Mosaic virus 35S promoter, and then transferred on vermiculite-filled pots. After a couple of months of growth, fluorescence was observed in a few adventitious roots of the daughter plants. The hairy root transformation of daughter plants isolated from its vegetative propagation circumvents the need for seed production or callus formation and subsequent plant regeneration, which are often problematic for maintaining preferred genetic traits in complex ploidy levels. This method, which excludes genetic modification of the above-ground parts, especially the edible fruits, will open new avenues for strawberry breeding, particularly in the areas of plant nutrient absorption and fostering growth through interactions with microorganisms.

(242 words)

Key words

genetic modification, hairy-root transformation, *Rhizobium rhizogenes*, stolon, strawberry

Strawberries are globally cultivated for their adaptability and economic importance (Debnath and Teixeira Da Silva 2007). They belong to the *Fragaria* genus in the Rosaceae family and are rich in valuable metabolites such as essential nutrients and antioxidants beyond their appearance and taste (Dias et al. 2016). Furthermore, the strawberry is one of the model perennial herbaceous plants for studying the Rosaceae family due to its rapid growth, small genome, short reproductive cycle, and facile vegetative and generative propagation (Shulaev et al. 2011). There are three main ways to propagate strawberry plants: division and transplantation of their crowns, germination from seeds, or harnessing stolons, also called runners, for efficient clonal reproduction, which is particularly crucial in managing the intricate ploidy levels, observed in strawberry varieties (e. g. $2n = 8x = 56$ in *Fragaria x ananassa*) (Hirakawa et al. 2014). Conventional genetic breeding via classical fertilization has improved various strawberry traits such as yield, fruit size and shape, and berry quality similar to achievements in other plant species (Isobe et al., 2013; Yamamoto et al., 2021). However, genetic breeding in cultivated strawberry is labor-intensive and time-consuming due to complex ploidy levels, underlining the significance of exploring efficient genetic modification strategies during clonal propagation.

Recent advances in genetic transformation via *Rhizobium radiobactor* (formerly known as *Agrobacterium tumefaciens*) have significantly improved the strawberry genetic traits, enhancing resistance to virus, fungi, insects, herbicides, and stress, as well as achieving better quality (Nehra et al., 1990; Ricardo et al., 2003; Qin et al., 2008; Pantazis et al., 2013). In general, *R. radiobactor*-mediated transformation produces stable and targeted transformants as an efficient tool. However, *R. radiobactor*-mediated transformation, which involves callus formation and regeneration, faces challenges related to the efficiency of regeneration and subsequent organogenesis, as well as the selection and recovery of transformed cells with antibiotic resistance. Despite the occurrence of genome rearrangements and somatic mutations during callus formation and regeneration, addressing these mutations through backcrossing is not realistic in polyploid strawberries, since plants grown from seeds may exhibit different traits from the parental plants that produced the seeds. Moreover, the stable introduction

of foreign genes throughout the entire plant body, including fruits, often faces criticism due to concerns about the presence of transgenes in the edible parts.

In contrast to *R. radiobactor*, *Rhizobium rhizogenes* (formerly known as *Agrobacterium rhizogenes*) introduces foreign genes only into adventitious roots induced from plant wounds (Tepfer, 1984; Toyoda et al., 1991; Nilsson and Olsson, 1997; Yazaki et al., 1998; Daspute et al., 2019; Tatsumi et al., 2020). *R. rhizogenes*-mediated hairy-root transformation in strawberries has been reported in leaf discs and aboveground sections with cotyledons and hypocotyls from seedlings over the past 20 years (Kyo and Shirai, 1990; Toyoda et al., 1993; Yan et al., 2023). While the hairy-root transformation is suitable for transient assays in roots grown from seedlings, challenges remain in maintaining genetic traits and obtaining stable transformants. Here, we report a new method of *R. rhizogenes*-mediated hairy-root transformation of daughter plants growing on strawberry stolons (Figure 1 and 2). This method does not require callus formation and plant regeneration, transforms only the hairy roots without inserting foreign genes into the above-ground parts, and allows for the expression of foreign genes without seed propagation (Figure 1B).

We chose the diploid woodland strawberry, *Fragaria vesca* Hawaii-4 ($2n = 14$), which is well-characterized with a small genome, short reproductive cycle, and both vegetative and generative propagation (Shulaev et al. 2011), as a model for studying the hairy-root transformation of daughter plants from strawberry stolons. Details about the experimental protocols are described in Supplementary Text. Surface-sterilized *F. vesca* seeds were aseptically grown on 1/2 Gamborg's B5 medium 0.9% agar plates without sugar and vitamin. Four- to six-week-old seedlings were transferred and grown on vermiculite-filled pots in the greenhouse with a heating system and maintained by propagating daughter plants on stolons. For daughter plants transformation, stolons with daughter plants sized 2 to 4 cm were harvested (Figure 2A, B).

To evaluate transgenic efficiencies for the hairy-root transformation, *R. rhizogenes* A13 strain (MAFF-210266) (Chen et al. 1995) harboring a pRI909 vector containing

mVENUS-intron driven by the CaMV 35S promoter with the hsp878 terminator (*35S::mVENUS*) (Matsui et al., 2014; Yamasaki et al., 2018; Otake et al., 2023) was utilized (Figure 1A). The *35S::mVENUS* plasmid was introduced into *R. rhizogenes* by electroporation (Wen-Jun and Forde 1989). The *R. rhizogenes* strain was grown in a 28°C dark growth chamber on LB plates for 3 days.

Daughter plants sized 2-4 cm were cut from the stolons of their parent plants, placed on *R. rhizogenes* colonies harboring the *35S::mVENUS* vector, and infected with the bacteria by puncturing the crown of the daughter plant with a syringe on an LB plate (Figure 2C, Supplementary Text). The infected daughter plants were transplanted into vermiculite-filled pots and wrapped to prevent drying (Figure 2D). After one week, the wrap was removed, and the infected daughter plants were grown in vermiculite for 1 to 2 months till adventitious roots emerged (Figure 2E).

To compare the ratio of transgenic efficiency, we also performed the hairy-root transformation of seedlings as described previously (Díaz et al. 2005) (Figure 1C, Supplementary Text). One- to two-week-old etiolated seedlings, aseptically grown on 1/2 B5 agar plates and cut at the lower part of the hypocotyl, were co-incubated with *R. rhizogenes* harboring the *35S::mVENUS* on 1/2 B5 agar plates without antibiotics for 3 to 4 days. Subsequently, they were transferred and vertically grown on 1/2 B5 agar plates containing 12.5 µg l⁻¹ of meropenem for 2 to 4 weeks to generate hairy roots.

In transformation of daughter plants from stolons, we successfully obtained transgenic plants with adventitious roots exhibiting strong *mVENUS* fluorescence 1 to 2 months post-infection (Figure 2F, G, H). The frequencies of the plants exhibiting *mVENUS*-positive roots ranged from 25% to 53.3% of the total plants used in the experiments (Table 1). The number of transformed hairy roots per plant exhibiting fluorescence ranged from one to several (Supplementary Text, Figure S1). The transformation efficiency of daughter plants was comparable to that of seedlings, which were used as a control; the transgenic efficiencies in the cut-off roots of seedlings ranged from 10% to 73.7% of the total inoculated plants (Table 1). *mVENUS* expression was observed in

several emerging roots from the cut end 3 weeks after infection. Thus, *R. rhizogenes* is suitable for the hairy-root transformation of daughter plants grown on the strawberry stolons as well as seedlings cut at their hypocotyls.

Here, we present a novel method for the *R. rhizogenes*-mediated hairy-root transformation of daughter plants grown on strawberry stolons, aiming to address the breeding challenges in the economically significant fruit crop. By harnessing the unique physiology of stolons, we developed a protocol that introduces foreign genes into progeny generated through vegetative reproduction. This approach bypasses the conventional limitations associated with seed propagation and tissue-culture-based transformation methods in higher polyploid plants. Unlike *R. rhizozogenes*-mediated transformation of seedlings (Figure 1C), that of daughter plants from the stolons avoids seed propagation, thus preserving the parental genetic traits.

Our protocol involves cutting daughter plants from the stolons and infecting their crowns, which contain meristems, with the *R. rhizogenes* A13 strain. One to two months post-infection, we observed *mVENUS* expression in some roots emerging from the cut-out daughter plants. Significantly, these daughter plants with transgenic roots continue to grow as clones of their parental plants during the vegetative reproduction stage, thus avoiding seed propagation, callus formation, and plant regeneration. In higher-order polyploid plants, where genetic traits segregate across homologous chromosomes, vegetative propagation without seed is especially important. This suggests that the daughter progenies with transgenic roots maintain the same genetic traits as their parent plants. Extending the *R. rhizogenes*-mediated hairy-root transformation to other strawberry varieties and plant species could allow the expression of foreign genes only in the roots, maintaining the genotype in higher-order ploidy plants and preserving non-genetically modified edible parts.

R. rhizogenes induces adventitious roots with transgenes without requiring callus formation and regeneration. This hairy-root transformation is suitable for transient expression analysis of foreign genes, allowing the study of promoters and gene

functions directly in roots, as transgenic roots are visible in their T0 generation (Yan et al. 2023). Earlier studies in plant biotechnology have demonstrated that *R. rhizogenes*-mediated hairy-root transformation can produce secondary metabolites in medicinal plants (Motomori et al., 1995; Yazaki et al., 1998). While *R. radiobacter* is commonly used in planta assays in mostly dicots and some monocots (Pantazis et al. 2013), it is often inefficient and time-consuming due to the necessity for callus formation and regeneration. In contrast, *R. rhizogenes* can infect a broader range of hosts, making its transformation applicable to more plant species. The *R. rhizogenes*-mediated hairy-root transformation, where genetic modification occurs only in the roots, may improve nutrient absorption, abiotic stress tolerance, and disease resistance in plant roots. Furthermore, combining this transformation system with RNA or protein transport mechanisms from roots to shoots might enable the modification of above-ground phenotypes (Zhang et al. 2016).

Regenerating stable transformants after the *R. rhizogenes*-mediated hairy-root transformation remains challenging. However, transformed roots offer an opportunity to generate transgenic plants by inducing callus formation and subsequent plant regeneration through the application of phytohormones (Yan et al. 2023). Recent advances in plant development have identified several candidates that induce shoots from roots (Ikeuchi et al., 2013; Kyo et al., 2018; Hanano et al., 2020; Debernardi et al., 2020; Yarra and Krysan, 2023). These advancements may enable the conversion of *R. rhizogenes*-mediated transgenic roots into shoots or other organs, ultimately leading to the development of intact transgenic plants. Combining *R. rhizogenes*-mediated transformation with shoot-inducible genes holds significant potential for advancing plant science and biotechnology.

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Author contribution

SH performed all experiments, except for the plasmid construction. KO constructed the 35S::mVENUS plasmid. SH and SS wrote the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

Description of Supplementary Files

Supplementary text is available at Plant Biotechnology online.

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transport in plants. *Plant Cell* 28:1237–1249.

Table 1. The ratio of survival and transgenic efficiencies

	Type of plants	total	survive	<i>mVENUS</i>	no signal	transgenic rate per total (%)
Exp. 1	Daughter plants	12	8	3	5	25.0
Exp. 2	Daughter plants	15	13	8	5	53.3
Exp. 3	Daughter plants	15	9	5	4	33.3
Exp. 4	Daughter plants	16	14	7	7	43.8
Exp. 5	Daughter plants	16	15	6	9	37.5
Exp. S1	Seedlings	19	19	14	5	73.7
Exp. S2	Seedlings	37	35	10	25	27.0
Exp. S3	Seedlings	22	20	6	14	27.3
Exp. S4	Seedlings	20	10	2	8	10.0
Exp. S5	Seedlings	45	41	10	31	22.2
Exp. S6	Seedlings	17	17	4	13	23.5

Survival and transgenic efficiencies in a series of experiments on daughter plants from stolons (Exp. 1 to 5) and seedlings cut at the lower part of hypocotyl (Exp. S1 to S6). Several plants did not survive due to drought or bacterial and fungal infections. The transgenic rate (%) is calculated as the number of plants expressing *mVENUS* fluorescence divided by the total number of plants used in the experiments.

Figures Legends

Figure 1. Schematic representation of the 35S::mVENUS construct and

transformation of daughter plant grown on stolon. (A) The 35S::mVENUS construct used in this study. LB, left border; RB, right border; NPT II, neomycin phosphotransferase Type II gene; NOS, nopaline synthase; 35S, Cauliflower mosaic virus 35S; P, promoter; Ter, terminator; UTR_{cor47-2mod}, 5'-untranslated region for a highly efficient translation; hsp878, heat shock protein 878. (B) Schematic representation of the transformation process for daughter plants grown on stolons. Daughter plants harvested from stolons elongated from parent plants were infected and co-cultivated with *Rhizobium rhizogenes* harboring foreign genes. Some of the emerging roots from daughter plants could contain foreign genes. (C) Schematic representation of seedling transformation. Ex-seedlings with cut roots were co-cultivated with *R. rhizogenes*.

Figure 2. Daughter plant transformation reveals transgenic roots. (A) Daughter

plants growing on strawberry *Fragaria vesca* stolons. The dimensions of the pot – 7 cm × 7 cm at base, 9 cm × 9 cm at the top, and 8 cm in depth – were represented to estimate the size of plants. (B) Daughter plants were cut from the stolons of the parental plants, (C) placed on *R. rhizogenes* colonies harboring the 35S::mVENUS plasmid and infected with bacteria by puncturing their crowns with a syringe on a LB plates. The mark on the Petri dish indicated was 5 mm. (D) Infected daughter plants were planted in vermiculite-filled pots with a wrap. (E) The wrap was removed after 1 weeks, and daughter plants were grown on vermiculites for 1 to 2 months. A top edge length of each pot was 9 cm. (F) Variation in mVENUS expression in the transgenic roots of the strawberry *F. vesca*. mVENUS Fluorescence photographs were captured with a PowerShot G16 digital camera (Canon, Tokyo) with a PA-V300f filter under 475 to 515 nm LED (Bio Craft, Tokyo). Microscopy images were observed under a SZX12 fluorescence microscope system (G) without and (H) with a GFP filter (Olympus, Tokyo). The difference in fluorescence colors, (F) yellow and (H) green, depends on the filter used.

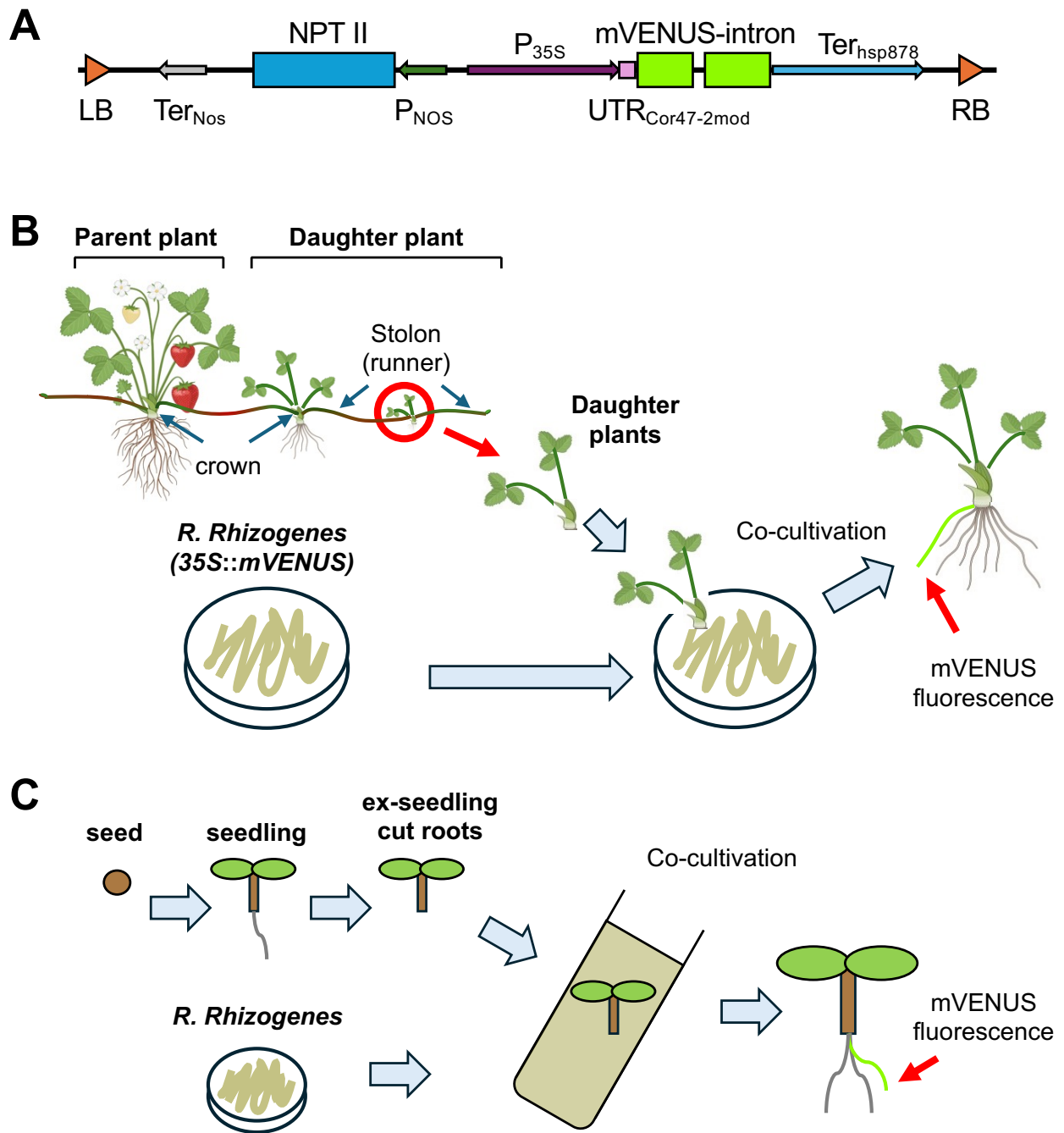


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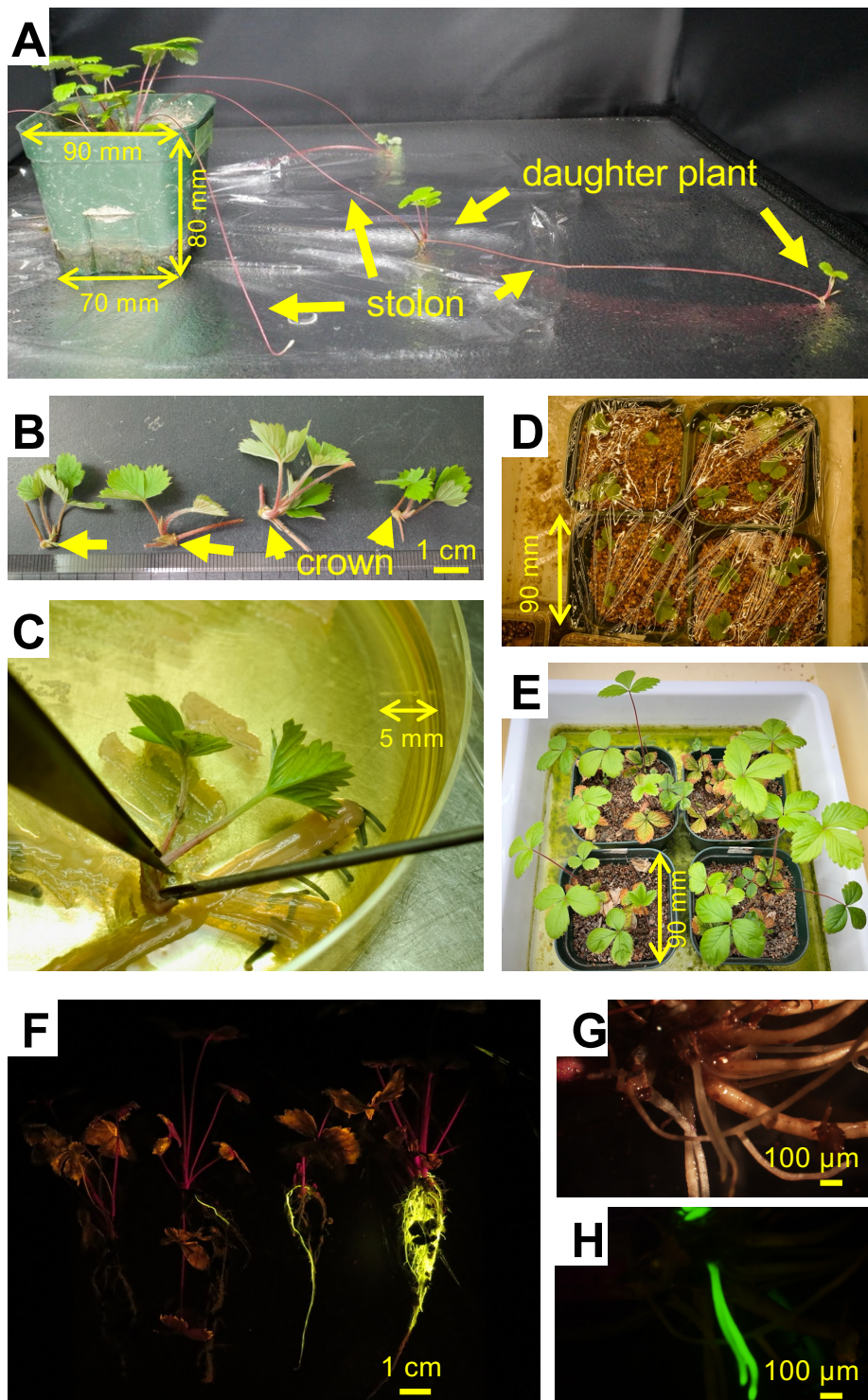


Figure 2. Daughter plant transformation reveals transgenic roots. (A) Daughter plants growing on strawberry *Fragaria vesca* stolons. The dimensions of the pot – 7 cm × 7 cm at base, 9 cm × 9 cm at the top, and 8 cm in depth – were represented to estimate the size of plants. (B) Daughter plants were cut from the stolons of the parental plants, (C) placed on *R. rhizogenes* colonies harboring the 35S::mVENUS plasmid and infected with bacteria by puncturing their crowns with a syringe on a LB plates. The mark on the Petri dish indicated was 5 mm. (D) Infected daughter plants were planted in vermiculite-filled pots with a wrap. (E) The wrap was removed after 1 weeks, and daughter plants were grown on vermiculites for 1 to 2 months. A top edge length of each pot was 9 cm. (F) Variation in mVENUS expression in the transgenic roots of the strawberry *F. vesca*. mVENUS Fluorescence photographs were captured with a PowerShot G16 digital camera (Canon, Tokyo) with a PA-V300f filter under 475 to 515 nm LED (Bio Craft, Tokyo). Microscopy images were observed under a SZX12 fluorescence microscope system (G) without and (H) with a GFP filter (Olympus, Tokyo). The difference in fluorescence colors, (F) yellow and (H) green, depends on the filter used.