

A method for the production and molecular validation of *Agrobacterium*-mediated marker-free intragenic octoploid strawberry

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Abstract Intragenesis is an all-native engineering technology for crop improvement. Cultivated strawberry (*Fragaria x ananassa*) remains a limited genetic diversity during evolution. Using an intragenic approach to bring genetic diversity of wild strawberry back to cultivated strawberry could expand genetic variability. A robust regeneration protocol was developed for strawberry cv. ‘Shanghai Angel’ by optimizing the dose of TDZ and explant origins, and 100% regeneration was achieved in 5–10 weeks. An intragene expression cassette was assembled with all DNA fragments from *F. vesca*, harboring a sugar transporter gene *FvSTP8* driven by a fruit-preferential promoter of *Knox*. Intragenic strawberry was developed through a routine *agrobacterium* mediated method without any selectable marker. Following the initial direct PCR selection, a probe-based duplex droplet digital PCR (ddPCR) was built to determine the intragene insertion events. A total of 4 independent intragenic shoots were obtained, with a maximum 5.3 % efficiency. Two lines were confirmed as chimeras (0.005 to 0.02 copy insertion / genome), and the rest two were completely intragenic shoots harboring 6 and 11 copy inserts, respectively. The current work optimized the procedures for producing marker-free intragenic strawberry and determining the insertion copies in the novel germplasm. These approaches are promising and suitable for the engineering of ‘Shanghai Angel’ and other strawberry cultivars to improve traits of agronomic importance. Future challenges and possible approaches were discussed.

Keywords: *Fragaria* × *ananassa*; sugar transporter; intragenesis; marker-free transformation; droplet digital PCR

1. Introduction

Cultivated strawberry (*Fragaria* × *ananassa*) is planted worldwide due to its highly nutritional and economic values. Recently, the origin and evolution of cultivated strawberry has been elucidated, confirming its single dominant subgenome provider being the diploid woodland strawberry *F. vesca* [1]. An improved genome and high-quality annotations of *F. vesca* [2,3], together with the updated annotations of the *F. × ananassa* genome [4], built an integrated science context opening a new era of gene functional studies and genetic engineering in cultivated strawberry. The key areas include regulating strawberry flowering, fruit development and quality, as well as disease resistance [5]. Cultivated strawberry has a low genetic diversity, and there is a critical requisite of utilizing engineering and gene editing to change the status for strawberry improvements, as we could learn from *Solanaceae* crops [6].

Molecular breeding technologies have addressed some constraints of the conventional breeding for enhanced resistance, flavor, and nutritional quality of crops [7]. Till mid-2020, although there were only five genetically engineered fruit crops were approved and commercialized worldwide, more than 45 novel fruit germplasms belonging to 20 distinct crops were successfully developed with the accelerated molecular techniques [8]. Many efforts utilizing genetic engineering methods have improved the important agronomic traits in strawberry [9]. Fruit-specific antisense suppression of ADP-glucose pyrophosphorylase catalyzing starch biosynthesis increased soluble sugar content while decreased starch accumulation in ripe fruits of strawberry cv. Anther [10]. Overexpression of the transcriptional activator *FaMYB10* elevated anthocyanins in many organs beyond strawberry fruits [11]. Fruit-specific antisense suppression of the endo- β -1,4-glucanase (EGase) catalyzing the disassemble of hemicellulose molecules in cell wall increased strawberry fruit firmness [12]. CRISPR/Cas9 mediated knockout of the Auxin Response Factor 8 (ARF8) resulted in a considerably faster growth in *F. vesca* seedlings [13]. An efficient transformation protocol was developed for the model cv. Camarosa [14], and *FaTM6* MADS-box gene knockout by genomic editing caused pollen defect and fruit abortion in this variety [15]. CRISPR/Cas9-directed mutagenesis successfully applied in both octoploid *F. × ananassa* and diploid *F. vesca* has offered valuable opportunities for engineering strawberry [16]. Overexpression and CRISPR/Cas9-directed mutagenesis of RAP, a glutathione S—transferase (GST) (a carrier transferring anthocyanins from cytosol into vacuole) changed the coloration of strawberry fruits reversely [17].

Due to the low frequency of plant transformation, markers including selectable type such as hygromycin and kanamycin antibiotic-resistance, and screenable type such as visional GFP and GUS, have long been used to identify the rare transformant [18]. However, the use of markers provokes the public concerns for the biosafety of engineered crops. Many works endeavored to generate marker-free engineered plants via omitting markers in transformation or eliminating markers after transformation. Robust *agrobacterium* transformation of potato [19–21] and wheat [22] enabled production of marker free transformants of these crops without selection. Marker-

removal systems such as using chemically inducible recombinase and bifunctional selectable system (first positive selection for transformants and later negative selection for marker deletion), facilitated the development of marker-free strawberry cv. Calypso [23]. The same vector and marker-removal strategy later were applied in generating marker-free apple [24], and marker-free banana [25]. In model plant tobacco, a double T-DNA vector was changed to a head-to-head pattern for the RB and LB directions, which significantly increased the frequency of marker-free plants in a co-transformation system [26].

To reduce misleading conceptual assumptions on the genetically modified organisms (GMO) and encourage focus on the products rather than the biotechnology and process, intragenic and transgenic categories were proposed [27]. A more precise and explicit nomenclature 'intragenesis' was defined as rearrangements of genomic material from the same sexual compatibility group and lack of any foreign DNA [28]. This biotechnological concept was further explored in genetic engineering as a novel all-native DNA gene technology, using natural DNA fragments in a new combination to produce genetic diversity and novel phenotype [29–36]. Intragenesis was gradually accepted as an innovative tool for crop improvement in precision breeding, since it raised fewer biosafety concerns with higher consumer acceptance than transgenesis. Intragenic approach was useful in bringing lost traits and genetic diversity back to crop from wild germplasms [37]. For fruit crop improvement, intragenesis was recommended as one of the ideal approaches [38]. Many intragenic vectors were developed and tested [39]. Intragenic apple with enhanced resistance to scab was developed [40] and the transformation systems for intragenic apple were further optimized [41]. Intragenic potato exhibiting resistance to bruising and discoloration has been granted commercial approval [7,42]. Intragenic black carrot was proposed [43].

A central task in genetic engineering is to validate the transformation and identify the copy number of DNA insertion. Droplet digital PCR (ddPCR) was developed to detect DNA relying on partitioning individual amplifications into separate droplets, and detecting the endpoint amplification products [44]. This method has been demonstrated to be more sensitive with lower error rate than qPCR, successfully used to determine transgene copy number in banana [25], tobacco [45], and maize [46]. Comparative study using ddPCR and Southern blotting in an array of genetically modified crops established the single duplex ddPCR an accurate method for measuring transgenic insertion events [47]. DdPCR has become a simple and cost-efficient method for analyzing copy number variations, widely used in biology [48].

The unique domestication and evolution history limited the genetic variability of modern cultivated strawberry. This fact hinders its improvements by conventional breeding. So far, the novel intragenesis tool has not been reported in strawberry yet. In addition, although the agrobacterium-mediated transformation of strawberry is easy to operate, it is challenging to use DNA blotting in determining transgene copy number in an octoploid genome. In genetic engineering, plants with single copy insertion of new DNA are desired for the stable transferability to progeny. To meet with these gaps, here we assembled a marker-free vector harboring an intragene expressing cassette with DNA fragments from *F. vesca*. Then, relying on a highly

efficient regeneration method, marker-free intragenic strawberry plantlets were developed using agrobacterium-mediated transformation without selection. Based on direct PCR-based initial selection, intragene copy number in these candidate transformants were determined by a single duplex ddPCR. To our knowledge, **this is a first report of engineering intragenic strawberry** as well as an accurate analysis of copy number by ddPCR in genetically modified strawberry.

2. Results

2.1 Optimization of shoot regeneration from leaf explants of strawberry cv. 'Shanghai Angel'

Regeneration is the first step of genome modification. Concerning the high heterozygosity of cultivated strawberry, the best-suited explants to be targeted are adult plant materials. Here we first used strawberry leaves from the asepsis shoots of cv. 'Shanghai Angel' from *in vitro* micropropagation of stolon apical tips. Hormonal balance is the dominant factor controlling morphogenesis in cultured explants. Thidiazuron (TDZ) has been reported as a very efficient hormone for shoot regeneration from cultivated strawberry leaf tissues [49]. **To identify the optimal hormonal conditions for regeneration of 'Shanghai Angel', leaf discs from in vitro asepsis shoots were cut and grown on a range of MSB5 media enriched with 0.1 mg.L⁻¹ IBA, as well as varying TDZ from 2.0 to 3.0 mg.L⁻¹. A dark incubation for 2 weeks followed by a photoperiod with a weak light intensity of 40-60 $\mu\text{M m}^{-2} \text{s}^{-1}$ at 25 °C (12h day/12h night) was uniformly used for all treatments. Plant explants were subcultured on the same media every 30 d. The effect of TDZ supplemented in MSB5 media on shoot regeneration was evaluated after 60 d of culture (Figure 1a). The highest rate (54.8%) was obtained by adding 2.5 mg.L⁻¹ TDZ to the regeneration medium.**

Our previous work showed that the surface-sterilized leaf explants derived from strawberry plants in greenhouse possessed a satisfactory regeneration capability and transformability [50]. Thus, we compared the frequency and timing of shoot regeneration between explants from *in vitro* proliferating shoots and surface-sterilized young leaves derived from greenhouse plants of 'Shanghai Angel' (Figure 1b-c). On the same MSB5 medium added with TDZ 2.5/IBA 0.1 mg.L⁻¹, a significant difference was observed between two types of explants. After six weeks of culture, about a regeneration rate of 15-20% was obtained with *in vitro* culture shoot leaves as the **starting** explants, while drastically higher rates of 50-100% were achieved with starting explants from greenhouse young leaves. Clearly, the young leaves from greenhouse plants of 'Shanghai Angel' displayed an exceptional capacity of rapid shoot regeneration.

Indeed, adventitious buds from direct organogenesis were first observed on leaf discs from greenhouse plants after 3 weeks of culture on MSB5 media with TDZ/IBA at 2.5/0.1 mg.L⁻¹, and most explants developed adventitious buds in the subsequent 2 weeks (Figure 1c, lower panel). By contrast, only a few buds were observed on *in vitro* shoot-derived leaf explants after 5 weeks of culture (Figure 1c, upper panel). In addition, both direct leaf organogenesis and caulogenesis were observed on

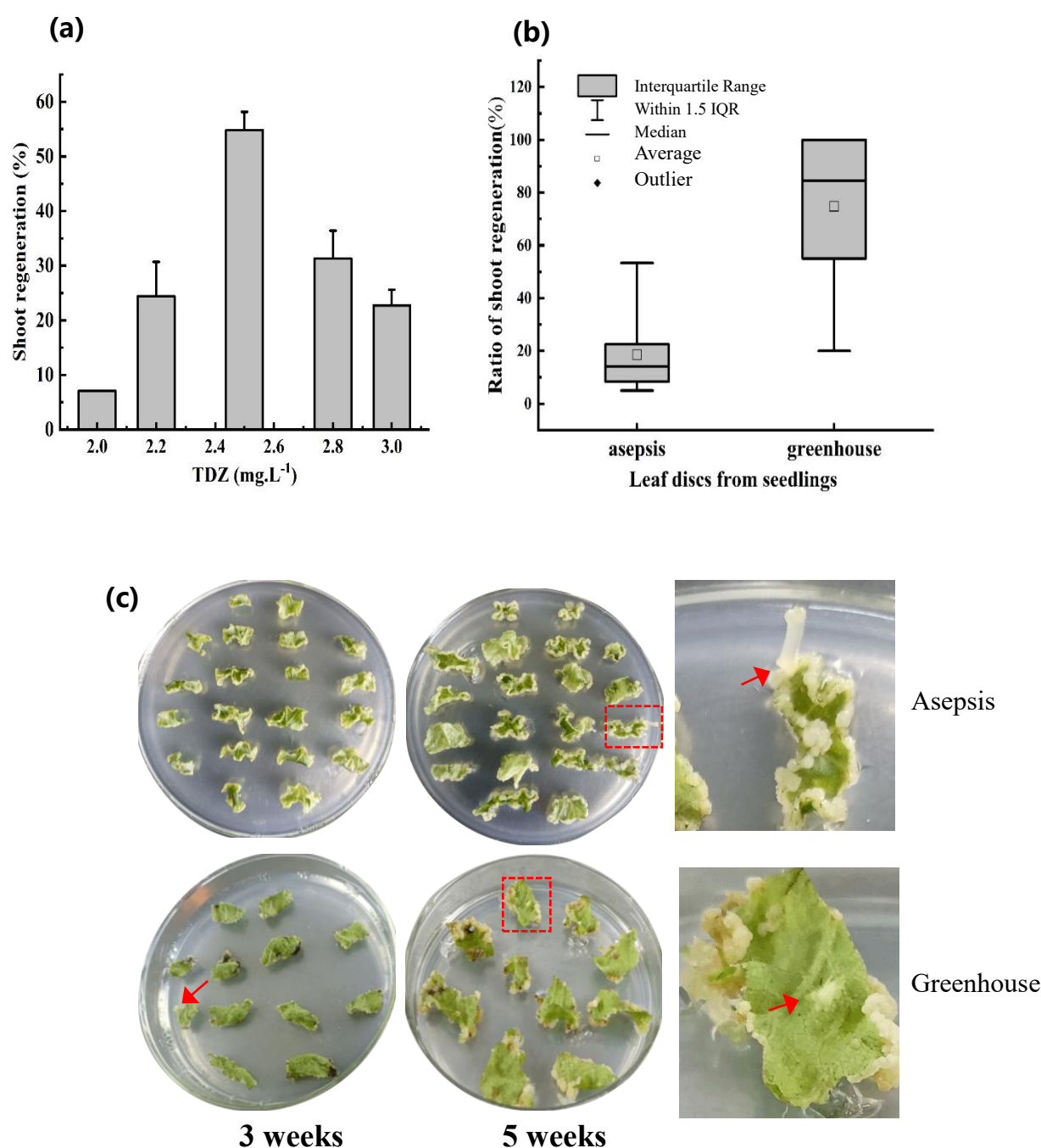


Figure 1. Efficient shoot regeneration of strawberry cv. Shanghai Angel. **(a)** The frequency of regeneration from aseptis leaf discs after 60 days' cultivation on media with thidiazuron (TDZ) at different concentrations. The same MSB5 medium with 0.1 mg.L⁻¹ IBA was used for all treatments. **(b)** The box chart indicates the comparative shoot regeneration of leaf discs from aseptis shoots and greenhouse plants. Data were gained after six weeks' cultivation on MSB5 added with 2.5 mg.L⁻¹ TDZ and 0.1 mg.L⁻¹ IBA. **(c)** Typical morphology of leaf discs after three weeks and five weeks cultivation on the same media. Upper, aseptis shoots-derived leaf discs; lower, greenhouse plants-derived; the right, enlarged leaf discs squared. Arrows indicate the shoot buds differentiated either from callus or direct adventitious organogenesis.

greenhouse leaves derived explants. But on *in vitro* culture shoot leaves-derived explants, the adventitious shoots were largely regenerated after caulogenesis. To achieve 100 % shoot regeneration for cv. 'Shanghai Angel', approximately 6-8 weeks and 10-12 weeks were required for greenhouse-derived and *in vitro* proliferating

explants, respectively.

In sum, a valid adventitious shoot regeneration system was set up from 'Shanghai Angel' leaf tissues with a frequency up to 100% when explants were cultured on MSB5 medium enriched with 2.5 mg.L⁻¹ TDZ and 0.1 mg.L⁻¹ IBA. The surface-sterilized young leaf explants from greenhouse plants were most appropriate for a rapid and efficient shoot regeneration of cv. 'Shanghai Angel'. The establishment of an efficient regeneration protocol has enabled us to transform strawberry without antibiotic selection.

2.2 Generation of a marker-free binary vector with an intragenic expression cassette

Strawberry cv. 'Shanghai Angel' released by Shanghai Academy of Agricultural Sciences, is a progeny of the cross Benihoppe x Akihime, representing an elite cultivar for Shanghai and eastern China. It is characterized by a superior resistance to *Colletotrichum* spp., the causal agents of anthracnose and a high yield with satisfactory fruit quality under most conditions [51–52]. However, in a climate full of rainy and sunless days, which was frequently occurring at early spring in Shanghai, fruit quality of this variety declined sharply. With an aim to increase its fruit quality under unfavorable weather, sugar transporter gene was selected to explore the possible effect via enhancing fruit-specific expression of a transporter gene, which could mediate the influx of extracellular sugar into fruits.

The fruit-specific activity of the upstream regulatory sequence (2471 bp) of a receptacle-specific expressed KNOX family transcription factor gene03606 (FvH4_4g26090) has been demonstrated via β -glucuronidase (GUS) staining in *F. vesca* [53]. Gene 05814 (FvH4_4g15150) encodes a sugar transporter protein (STP) [54] belonging to the group of monosaccharide transporters (MSTs) which function as proton/sugar symporters for a wide range of monosaccharides [55]. The subcellular locations of FvSTP8 were predicted at plasma membrane and vacuolar membrane, potentially being involved in the influx of extracellular fructose and glucose in strawberry. And in cultivated strawberry, this gene was preferentially expressed in functional leaves while weakly expressed in fruits [54], which is fit for an enhanced expression via genetic modification.

The generation of a marker-free binary vector for fruit-specific expression of *FvSTP8* was mainly achieved through two steps (Figure 2). We started with pMDC162 harboring the promoter of strawberry KNOX gene03606 at the upstream of GUS gene [53]. First, the 1590 bp coding sequence (CDS) of *FvSTP8* was amplified from Hawaii4 cDNAs and used to replace the GUS gene downstream KNOX gene03606 promoter. Simultaneously, the backbone of pMDC162 beyond GUS gene (backbone 1) was generated via a reverse PCR. The recombination events of *FvSTP8* CDS and backbone 1 were screened out by PCR (*FvSTP8* specific primers) integrated with SpeI digestion analysis, followed by sequencing confirmation with multiple T-DNA specific primers.

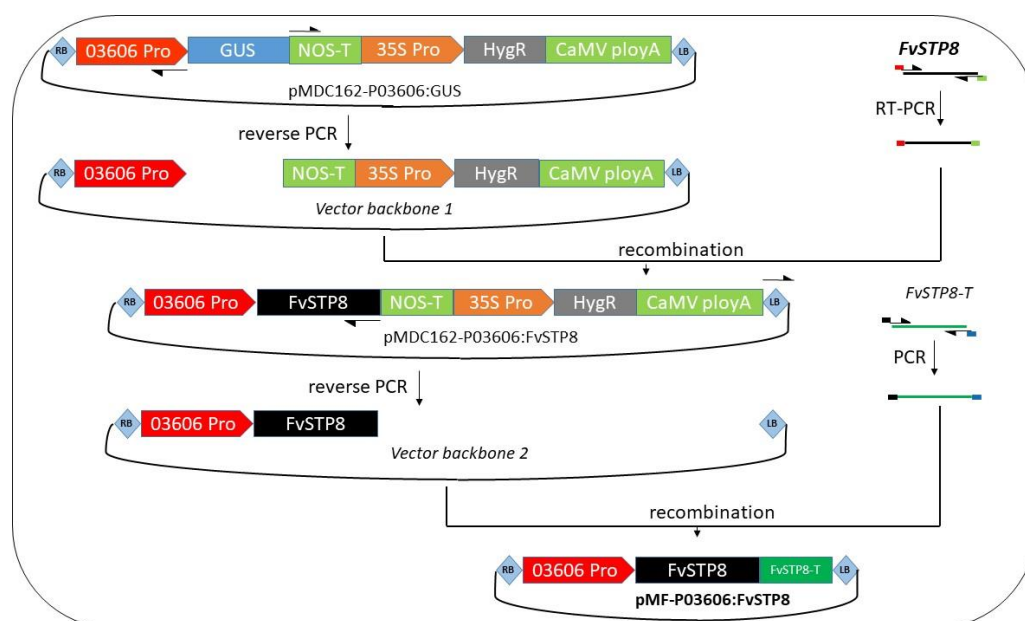


Figure 2. Diagram depicts the T-DNA regions of the 12.5 kb binary vector pMDC162-P03606:GUS and its derivative pMF-P03606:FvSTP8 (11.1 kb). Two recombination reactions sequentially performed first we substituted *FvSTP8* coding sequence (CDS) from Hawaii4 for *GUS* gene, and then replaced the NOS terminator behind *FvSTP8* CDS with its original terminator from Hawaii4 genome and simultaneously deleting the whole expression cassette for hygromycin selection marker. LB/RB, left/right border; STP8, coding sequence of *FvSTP8* from Hawaii4; STP8-T, terminator of *FvSTP8* from Hawaii4; NOS-T, terminator of *A. tumefaciens* NOS gene; 35S, CaMV 35S promoter; HygR, hygromycin resistance gene with a CaMV 35S promoter and CaMV polyA signal terminator. Arrows indicate the primers for PCR or reverse PCR; color columns added to the primers for the overlapping sequences used in recombination.

A second recombination reaction was performed to substitute the natural terminator of *FvSTP8* gene for the antibiotics marker expression cassette as well as NOS terminator downstream *FvSTP8* CDS. A 401bp fragment downstream the stop code of *FvSTP8* was cloned from the genomic DNA of Hawaii4. At the same time, the backbone 2 harboring only gene03606 promoter and *FvSTP8* CDS on T-DNA region was amplified and recombined with the aforementioned *FvSTP8* natural terminator. The resultant clones were first screened out by PCR with *FvSTP8* terminator specific primers and then validated by sequencing with primers specific to three components of the novel intragene *FvSTP8* driven by KNOX promoter (FvKNOX-pro: *FvSTP8*) on T-DNA.

2.3 Developing *maker*-free intragenic strawberry plants via *Agrobacterium*-mediated transformation without selection

Transformation of ‘Shanghai Angel’ began with the pre-cultured leaf explants (Figure 3a). Occasional shoot buds on explants were carefully discarded before *agrobacteria* infection. After 15–20 min infection in a freshly activated *agrobacteria* solution, surface-dried strawberry explants were co-cultured at 22 °C no longer than 45 hours in dark, which was crucial for suppressing the overgrowth of *agrobacteria* on strawberry materials. After co-culture, explants were carefully washed and rinsed with antibiotics (Carbenicillin and Timentin) for the removal of *Agrobacteria*.

Subsequently, explants were maintained on MSB5 medium with 200 mg.L⁻¹ carbenicillin and sub-cultured every 2-4 weeks (Figure 3b). Those explants with visible resurgent agrobacteria were directly discarded during subculture, since the overgrowth of agrobacteria was uncontrollable at later stages.

In current system, once the shoot buds were apparently regenerated, plant materials were immediately transferred to a new MS medium without the plant growth regulators TDZ and IBA. Depending on the status of differentiated buds, zeatin was added (buds in health) or not (buds in vitrification) to overcome the inhibition of TDZ on shoot elongation [56]. The regenerated adventitious shoot buds were then transferred to MS media with BA and IBA at low doses to facilitate shoot growth (Figure 3c). Putative transformed shoots through molecular validation were rooted and hardened before transferring to pots (Figure 3d). The pipeline steps of *Agrobacterium*-mediated transformation of ‘Shanghai Angel’ leaf discs for marker-free strawberry are shown in Figure 3e. This method permits the development of intragenic strawberry without antibiotics selection in 3 to 6 months.

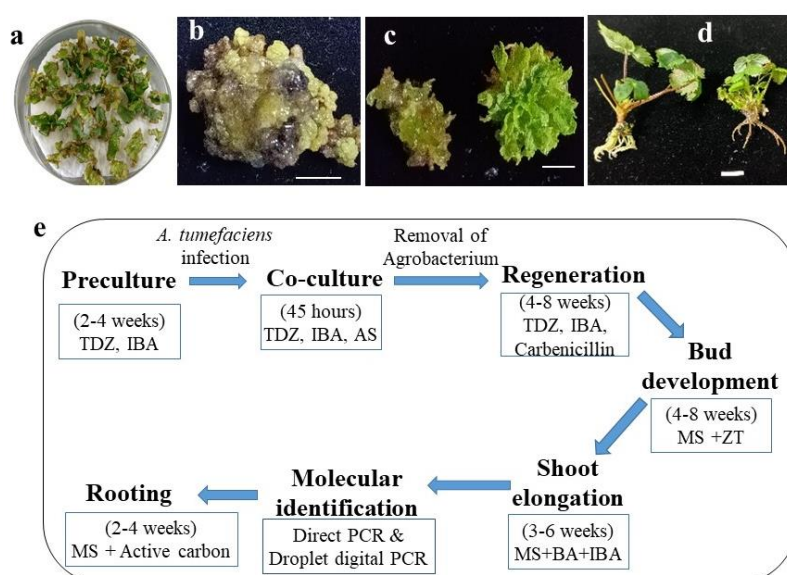


Figure 3. Agrobacterium-mediated transformation of strawberry cv. ‘Shanghai Angel’ without selection. (a) Explants were rinsed with sterile water containing 200 mg.L⁻¹ Carbenicillin and 200 mg.L⁻¹ Timentin after 45 hours’ co-culture at 22°C in darkness. (b) Transformed calli undergoing differentiation on MS medium with B5 vitamins (MSB5) media with 2.5 mg.L⁻¹ TDZ, 0.1 mg.L⁻¹ IBA and 200 mg.L⁻¹ Carbenicillin. (c) The bushy buds and shoots after regeneration. (d) Two transformed plantlets validated by PCRs were washed before transferring into soil substrate. Scale bars in (b) and (c) 0.5 cm, in (d) 1 cm. (e) Brief scheme of the methodology followed for strawberry transformation with the marker-free vector pMF-P03606: *FvSTP8*. MSB5 was used in the earlier three steps; ordinary MS medium was used after shoot regeneration. AS for acetosyringone at 100 µM to facilitate agrobacteria (strain GV3101) infection.; ZT for zeatin at 0.22 mg.L⁻¹ to enhance bud development; BA 0.1 mg.L⁻¹ and IBA 0.01 mg.L⁻¹ for shoot elongation; active carbon at 0.4 mg.L⁻¹ for rooting.

2.4 Initial selection of marker-free intragenic transformants using direct PCR

In the absence of antibiotics selection, the total number of adventitious shoots regenerated from each survived explant was very high, which was consistent with previous observation in other plant species [57]. Since the novel intragene we constructed would not express and display any visible phenotype in young shoots, it

was indeed difficult to identify transformants. Here we developed a two-step method for screening intragenic plantlets. The initial step was to use a direct PCR selection of potential transformants at early stage. This could be performed once the regenerated shoots higher than 2 cm with 3 to 4 compound leaves in an *in vitro* culture container. At most 4 shoots oriented at distinct directions of one callus were reserved and sampled for direct PCR identification.

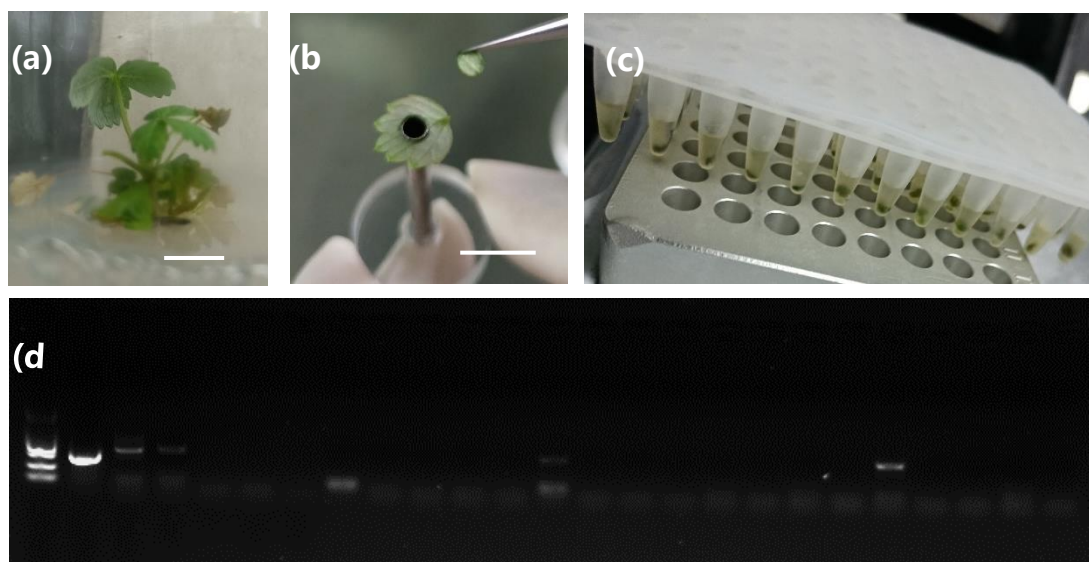


Figure 4. Direct PCR identification of the sterile shoots potentially transformed with pMF-P03606:*FvSTP8*. **(a)** One regenerated young plantlet. **(b)** A piece of leaf in a diameter 3 mm was sampled for direct PCR analysis. Scale bars in **(a)** and **(b)**, 1 cm. **(c)** Coarse lysis solution served as DNA template for direct PCR analysis. **(d)** Gel photo showing the amplification of intragenic construct in one direct PCR analysis of 22 plantlets. 1.0 % agarose gel, 120 V for 30 min. M for DL2000 DNA marker; + for positive control with the diluted plasmid pMF-P03606:*FvSTP8* as PCR template; - for negative control against untransformed cv. ‘Shanghai Angel’ strawberry DNA template.

Each adventitious shoot reserved was sampled on a clean bench, and a coarse DNA lysis solution was prepared at 95 °C for 10 min (Figure 4a-c). This coarse DNA solution was used as PCR template to amplify a 528 bp fragment that spanning the 3’ end of KNOX promoter and the middle of *FvSTP8* CDS with primer pairs CM2140 and CM2340 (supplementary Table S1). PCR products were visualized on 1% agarose gel. The false positive amplification was observed, probably resulted from unspecific amplification or *Agrobacterium* contamination. Only those samples with amplicon bands identical to positive control (plasmid template) were accepted as PCR positive (Lane 19 for sample #9-d, Figure 4d).

‘Shanghai Angel’ leaf explants were infected with agrobacterium in 4 independent experiments using greenhouse-derived young leaves after 2-4 weeks preculture (Table 1). Through agrobacterium co-culture less than 2 days followed by subculture without selection, a total of 241 shoots regenerated in five to six months after initial infection. Indeed, more than half explants were discarded during subculture for the resurgent growth of agrobacterium. The survived materials often developed bushy shoots on explant. Till now, direct PCR identification had performed for all shoots from 4 transformation trials. A total of 4 independent shoots were identified as PCR positive. Therefore, when transformation efficiency was expressed

as the percentage of PCR positive shoots over the number of initial leaf disc explants, the maximum transformation efficiency achieved in this work was 5.26%. If a transformation frequency was expressed as the percentage of PCR positive shoots over the number of tested shoots, it was about 0 - 2.38%.

Table 1. The efficiency for the marker-free transformation of pMF-P03606:*FvSTP8* to the strawberry variety ‘Shanghai Angel’ revealed by direct PCR.

Experiment	No. of initial explants	^a No. of Shoots	No. of PCR positive shoots	^b Transformation efficiency (%)
I	30	53	0	0
II	51	58	1	1.96%
III	40	46	1	2.50%
IV	38	84	2	5.26%

^aNumber of regenerated shoots was calculated at five to six months after transformation.

^bTransformation efficiency was expressed as the percentage of PCR positive shoots over the number of initial explants.

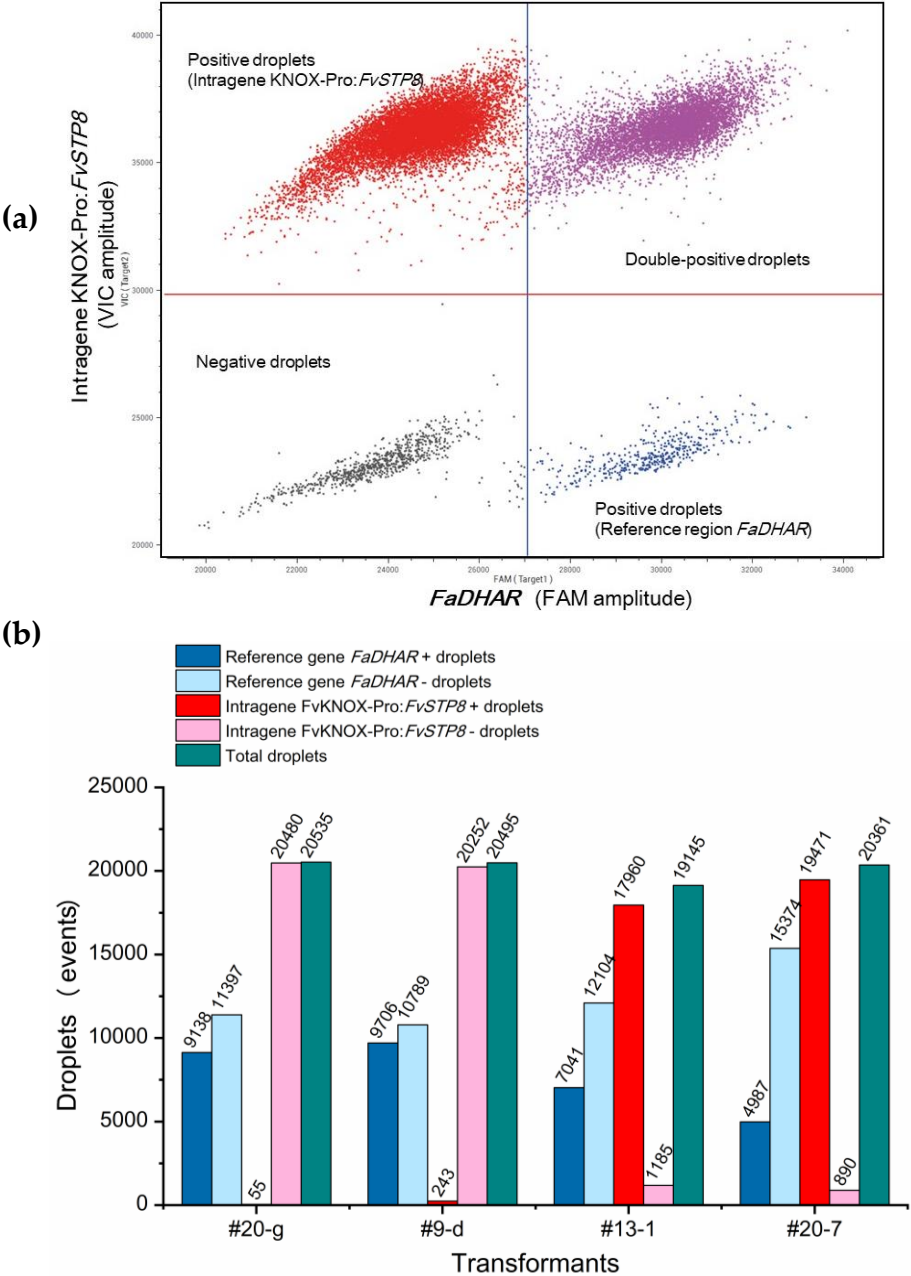
2.5 Fine identification and determination of the intragene copy number in marker-free transformants using ddPCR

To determine whether the direct PCR accurately identified transformants, a probe-based duplex ddPCR was further used to estimate the intragene integrations in the four independent T0 transformed events of ‘Shanghai Angel’. Primers and probes were designed to detect the reference gene *FaDHAR* (DeHydro Ascorbate Reductase) and the intragene *FvSTP8* driven by KNOX promoter (Figure 2). *FaDHAR* is a low-copy nuclear gene [58] with four copies per octoploid strawberry genome [4]. Using the updated DNA sequence information in *Fragaria × ananassa* Reference Genome v1.0 (FANhybrid_r1.2) at GDR (www.rosaceae.org) [59], reference primers and probe (supplementary Table S2) were designed to detect the single copy FxC_26g17540 (FANhyb_rscf00000137.1.g00005.1) in octoploid strawberry genome.

DdPCR was independently performed twice with untransformed ‘Shanghai Angel’ as negative control (Figure 5). Among different ddPCR assays, 20072 – 21444 droplets (mean, 20739) were generated per reaction. Nearly 7386–10772 (mean, 9291) positive droplets for the reference amplicon as well as varying positive droplets for the intragene amplicon were quantified per 20 µl reaction harboring 5 ng fractioned DNA template. The original one-dimensional plots of droplets for all four transformed lines and the blank sample (untransformed ‘Shanghai Angel’) indicated that the droplet patterns were largely regular for an accurate quantification in this study (supplementary Figure S1). The typical two-dimensional (2-D) plot of droplets measured in line #13-1 for fluorescence signals emitted from both reference gene (blue, FAM-labeled) and target intragene (red, VIC-labeled) regions clearly showed the general pattern of ddPCR (Figure 5a). The 2-D droplet patterns for other lines and the blank control were displayed in supplementary Figure S2. The number of VIC-labeled positive droplets of intragene in transformants dramatically ranged from 55 to 19471 (Figure 5b), while the blank sample of un-transformed ‘Shanghai Angel’ within each

run displayed a background VIC amplitude including 4 positive droplets across this study (data omitted).

The ddPCR results for four lines selected from direct PCR were shown in Figure 5c. The intragene copy number in #9-d and #20-g were very low, estimated to be significantly lower than 1, suggesting the chimeric status in these two lines. Chimerism was occurring at a very high frequency (about 50%) in this study, and the two chimeras were rejected for further experiments. By contrast, the DNA insertion values in #13-1 and #20-7 were particularly high, close to integers 6 and 11 copies, respectively. The latter two lines were recovered and maintained. It's a pity that the plantlets harboring an ideal low copy number (averaging 1-2 copies) of the novel intragene were not obtained yet and awaited to be identified in the near future.



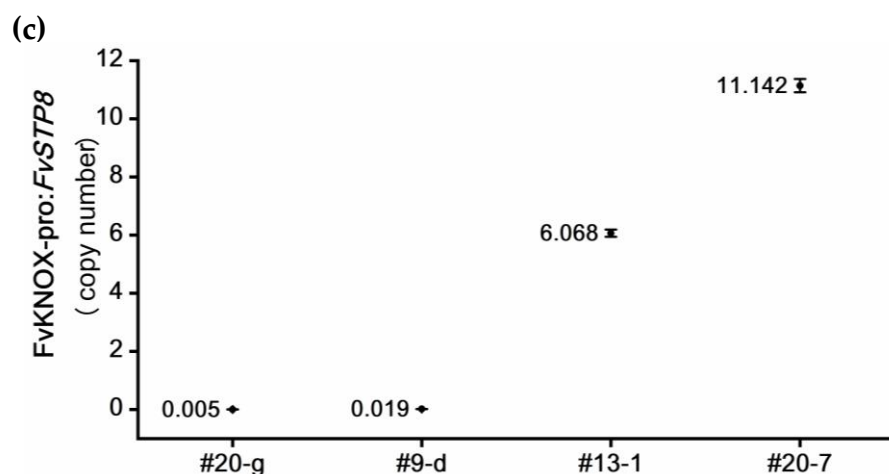


Figure 5. Intragene copy number measurement in strawberry cv. ‘Shanghai Angel’ via ddPCR. **(a)** Two-dimensional visions for fluorescence signal emitted from the endogenous reference gene *FaDHAR* (FAMTM labeled, blue positive droplets), and the intragene KNOX Pro:*FvSTP8* (VICTM labeled, red positive droplets) for line #13-1. Negative droplets are black. The droplets with both fluorescent probes are purple. **(b)** Bar graph highlighting the relative abundance of positive and negative droplets relative to the total number of droplets obtained in four independent lines. **(c)** Copy number of intragene in T0 strawberry lines after variation processing in Sniper SightPro software where the reference gene copy was set to 1 in octoploid genome based on BLASTN analysis. The error bars represent the maximum and minimum Poisson distribution for the 95% confidence interval generated by the Sniper SightPro software.

3. Discussion

Current work aimed to develop intragenic octoploid strawberry with enhanced capability of fruit sugar accumulation under unfavorable climate. We constructed a marker-free vector using only DNA fragments of woodland strawberry origin to produce strawberry with fruit-preferential expression of a sugar transporter. Based on an efficient regeneration protocol, agrobacterium-mediated transformation of cv. ‘Shanghai Angel’ without antibiotic selection successfully introduced a novel intragenic expression cassette FvKNOX-Pro: *FvSTP8* into this cultivar. The described transformation system and intragenic vector construction approach could be generally applied to create novel strawberry germplasms with improved agronomic traits, especially useful in bringing wild type genetic diversity back to cultivated strawberry. In addition, a ddPCR assay was developed for octoploid strawberry, thereby enabling an accurate monitoring of DNA integration in the engineered strawberry or detecting mutations in a germplasm population at an affordable cost.

Results of Southern blot analysis sometime are complex and not allow an accurate determination of genomic integration pattern, especially for multiple insertions at a single locus and the hybridized signal being influenced by other factors [47,60]. The genome size of cultivated strawberry is 813 Mb [1], and one copy genome is ca. 0.89 pg (<https://cvalues.science.kew.org/>). According to previous suggestion for appropriate amount of template using BioRad ddPCR system [47], we started with 25 ng strawberry genomic DNA template in ddPCR using Sniper DQ24. However, there occurred too many positive droplets to allow accurate counting. When we reduced the template amount to 4 ~ 5 ng, a satisfactory determination of positive and negative

droplets was achieved. This inconsistency might be caused by distinct effectiveness in sample amplification and/or fluorescent signal acquisition in two different systems.

Direct PCR combined with ddPCR revealed a maximum 5.3% transformation efficiency of cv. 'Shanghai Angel' without selection in this study, although the four independent transformants obtained were composed of two chimeras and two with multiple-copy integration. Transformation frequency of strawberry varied greatly and was affected by the cultivar and methods. Efficiency in early reports before 2005 was largely lower than 10%. The cultivar Rapella [61] and Redcoat [62] were transformed at a frequency of 0.95% and 3%, respectively. A transformation of strawberry cv. Firework using *Agrobacterium* strain CBE21 for an overexpression of traumatin II gene achieved an efficiency of about 11% [63]. Later, a work using LBA4404 *agrobacterium* mediated transformation of cv. Chandler reached an extraordinarily high efficiency ranging 40% to 90% [64]. It was reported that an octoploid strawberry genotype Laboratory Festival #9 (LF9) displayed a near 100% transformability under 5 mg/L Kan selection [49].

Here, a striking difference was found in the regeneration frequency of leaf disks from green-house derived young leaves with those from *in vitro* sterile shoots of cv. 'Shanghai Angel'. Apparently, green-house derived young leaf materials exhibited a faster and greater capability in regeneration than *in vitro* materials. Consistently, transformation with leaf pieces from 'Chandler' seedlings was successful at a 4.16% frequency with plantlets differentiated, while only transformed callus not differentiated was obtained at a 1.11% frequency with leaf disks from shoots of micropropagation [65]. The reduced regeneration capability in sub-cultured strawberry might result from the changed levels and balance of plant internal hormones during shoot micropropagation. In current transformation without antibiotic selection, the use of bactericidal antibiotics would also affect explant growth and development. Previous report showed that carbenicillin did not affect the regeneration of sugar beet even at high concentrations ca. 1000 mg/L, but this antibiotic inhibited the growth of callus at low concentrations at 250 mg/L in the same plant [66]. Carbenicillin added at 250-500 mg/L stimulated callus growth and shoot formation of *Antirrhinum majus* which was potentially related to the generation of the auxin phenylacetic acid from its break-down [67]. Future efforts on adjusting plant growth regulators application in the presence of antibiotics could optimize cv. 'Shanghai Angel' transformation. Additionally, an increased transformation efficiency might also be obtained by optimizing the use of antibiotics for a satisfactory suppression of *agrobacteria* resurgence, since in current case more than half explants with *agrobacteria* contamination were discarded during subculture.

Reducing the rate of chimerism in transformed shoots is of the similar bottlenecks in genetic engineering for strawberry and other *Rosaceous* plants [68]. To dissociate chimeras and recover completely transformed shoots in a transformation without selection is a great challenge. It's readily comprehensible that a high rate of chimerism in this study, since when there is no selection or even the selection applied is low, non-transformed cells would escape and chimeras will survive. Besides, the chimerism frequency is associated with the regeneration pathways of explants, and there is a high risk of obtaining chimeric plants from organogenesis [69]. Clearly, the leaf explants of

cv. 'Shanghai Angel' directly harvested from greenhouse showed a higher organogenic capacity than those from *in vitro* culture shoots. The independent transformed lines 9-d and 20-g obtained in this study were chimeras, and the possibility could not be excluded that these seedlings might be regenerated from transformed cells via organogenesis. Most leaf explants of cv. 'Shanghai Angel' from micropropagation regenerated via caulogenesis, which would reduce the frequency of chimerism during transformation. Thus, we suggest the use of *in vitro* strawberry of micropropagation as donor plants for genetic transformation. The explant materials should not be sub-cultured more than five times, since a complete loss of shoot regeneration capacity of cv. 'Shanghai Angel' has been observed after six-months' culture for leaf discs from shoots sub-cultured more than eight times (data omitted).

The plant transformation is actually a war of cell survival, where the plant defense system interacts with the intruding pathogen *Agrobacterium* [68]. To obtain a desirable transgenic or intragenic plant with a single copy of novel DNA is a matter of two organisms. Although the disadvantage of multiple inserts during plant transformation is well known, multiple inserts are often occurred in *agrobacterium*-mediated transformation. In the only three independent lines of marker-free transgenic apple developed, copy number of target gene ranged 2 to 5 [24]. In a transformation of wheat cv. Fielder, only 24% lines contained a single copy of transgene, while the remains harbored two or more copies of T-DNA insertions, with a maximum 7 copies [22]. Concerning the frequency of T-DNA insertion in *Agrobacterium*-transformed strawberry, various observations have been reported. According to Southern blot assay, a high frequency (four of six) of single-copy T-DNA integration was suggested in transgenic strawberry cv. Calypso [23]. However, Southern blot just provided evidences for transformation or potential low-copy T-DNAs, but did not resolve the copy number of DNA insertions in strawberry of other two cases [10,70]. In LBA4404 transformed cv. Kaster, the transgene was presumed to present in single copy in all four strawberry lines [71], although the results provided in that work were not explicitly convincing. In transgenic cv. Chandler, Southern blot assay revealed copy number ranging 1 to 3 [64]. In transgenic cv. Pajaro from LBA4404 inoculation, the copy numbers of inserts ranged from 1 to 5, and only one line contained a single copy of T-DNA [72]. In AGL0 mediated transformation of cv. Calypso, average 3 copies of T-DNA insertions were detected, and only 1 of 6 lines was confirmed to be a single copy transformant [73]. In EHA105 transformed cv. Toyonoka, the exogenous gene was present in 1 to 5 copies, and half (3 of 6) lines had single copy insert [74-75]. Apparently, *agrobacterium* strains have distinct capacity to deliver T-DNA into different hosts, even plants of the same species. In developing marker-free banana, LBA4404 mediated transformation resulted in averaging 2 copies of transgene in two cultivars, while AGL1 generated very high copy numbers of averaging 5 and 13 in these varieties, respectively [25]. Whether the *Agrobacterium* strain LBA4404 or EHA105 is less virulent than GV3101 to strawberry cv. 'Shanghai Angel' needs to be revealed in future.

Future work will focus on testing different *Agrobacterium* strains and adjusting the use of antibiotics and hormones, to increase the competence of both strawberry and *Agrobacterium* for transformation and create an orchestration of their survival. The

final goal is to improve transformation efficiency meanwhile reducing the frequency of chimerism and multiple inserts in strawberry transformants.

4. Materials and Methods

4.1 Plant materials and growth conditions

For strawberry transformation, leaves from cv. 'Shanghai Angel' plants in both greenhouse and tissue culture were utilized. Strawberry cv. 'Shanghai Angel' were grown in a greenhouse under a 10 h photoperiod with a light intensity of $200 \mu\text{M m}^{-2} \text{s}^{-1}$ at 25°C (day)/ 22°C (night) in soil-less medium. Its tissue-culture shoots were developed from the apical tips of stolons. Stolon tips were cut into around 10 cm in length from greenhouse, thoroughly cleaned with a brush and a droplet of dishwashing liquid, and then washed under running tap water for 1 h. Later, the stolon tips in around 5 cm length were surface-sterilized via twice soaking in 4% (V/V) commercial sodium hypochlorite solution (Cat. No. S1944-500ml, 5.68% active chlorine, Sangon, Shanghai) and gently agitated for 5 min each time, followed by eight times rinsing with sterile water. The apical tips around 0.5 cm were cut from the sterilized stolon on sterile filter paper and placed on MS medium with 0.1 mg L^{-1} GA and 0.3 mg L^{-1} BA till germination. The micro-propagated shoots were sub-cultured every 6 to 8 weeks on MS medium with BA 0.3 mg L^{-1} and IBA 0.01 mg L^{-1} . Subculture times for asepsis shoots were limited to less than 5 times to ensure an admirable capability of regeneration in leaf discs.

4.2 Explant preparation and strawberry transformation

Newly unfolded trifoliate leaves of strawberry cv. 'Shanghai Angel' were obtained from greenhouse and similarly disinfected as stolon tips. Leaves were cut into around $1 \times 1 \text{ cm}$ (greenhouse leaves) or $0.3 \times 0.5 \text{ cm}$ (*in vitro* cultured) discs and oriented with adaxial side of the explant on the regeneration medium. Pre-culture and shoot regeneration from leaf disks were achieved on MSB5 (MS basal salts with B5 vitamins, M404, PhytoTech, USA) (pH 5.7-5.8) containing 20 g L^{-1} sucrose, 7.5 g L^{-1} plant agar (A800728, Macklin, Shanghai, China), and 2.5 mg L^{-1} thidiazuron (TDZ) (A600746-0025, Sangon, Shanghai) together with 0.1 mg L^{-1} 3-indole butyric acid (IBA) (A600725-0025, Sangon, Shanghai) [76]. Cultivation for two weeks in darkness and then three to four weeks with a low light intensity (about $40\text{--}60 \mu\text{M m}^{-2} \text{s}^{-1}$) at $25 \pm 1^\circ\text{C}$ was carried out during pre-culture.

Agrobacterium strain GV3101 harboring target marker-free construct was over-night cultured in 4 ml Luria Broth (LB) liquid containing Kanamycin 50 mg L^{-1} , rifampicin 20 mg L^{-1} and gentamycin sulfate 25 mg L^{-1} at 28°C in a shaker 250 rpm. Transformation was largely performed as previously reported [77]. Over-night culture was centrifuged at 5000 rpm for 8 min and the pellet was suspended in liquid MSB5 medium (M404, PhytoTech, USA) pH 5.5, 2% sucrose, $100 \mu\text{M}$ AS (acetosyringone, Cat. No. A601111, Sangon, Shanghai) ($\text{OD}_{600\text{nm}} \approx 0.1$) in a flask and gently activated for 2-3 hr. at 25°C under darkness in a shaker at 50 rpm. The pre-cultured explants were immersed into the *Agrobacterium* suspension for 15-20 min on the clean bench. After infection, the explants were dried on sterile filter paper and cultivated on MSB5 medium plate with 2% sucrose, $100 \mu\text{M}$ AS, 2.5 mg L^{-1} TDZ, 0.1 mg L^{-1} IBA, pH 5.5 in

darkness at 22°C for about 45 hrs. After co-cultivation, the explants were soaked in sterile water containing 200 mg L carbenicillin and 200 Timentin for 15-30 min, and then rinsed three times before dried on filter paper. After washing, the explants were transferred to shoot induction medium MSB5 with 2% sucrose, 200 mg L⁻¹ carb, 2.5 mg L⁻¹ TDZ, 0.1 mg L⁻¹ IBA, pH 5.8. Subculture was carried out every 2-4 weeks on the same media until shoot generated. The bushy shoot buds were transferred to MS medium (M519, PhytoTech, USA) without any hormones (if vitrification happened) or with 0.22 mg.L⁻¹ Zeatin (no vitrification) to facilitate shoot development [56]. After cultivation on MS without plant hormones or with ZT only for once, the bushy shoots were then transferred to MS medium with BA 0.1 mg L⁻¹ and IBA 0.01 mg L⁻¹ to enhance shoot elongation.

4.3 *Agrobacterium* strain and binary vector

The binary plasmid pMDC162 harbors the promoter of strawberry KNOX gene03606 at the upstream of GUS gene which displayed a receptacle-specific activity [53]. The original pMDC162 vector (pMDC162-P03606:GUS) was modified mainly through two recombination reactions. First, *FvSTP8* CDS was cloned from leaf cDNAs of *F. vesca* Hawaii4 into pClone007B blunt vector (TSV-007B, Tsingke, Beijing) through a RT-PCR and validated by sequencing. *FvSTP8* CDS was re-amplified with primers CM2268 and CM2269, to replace the GUS gene downstream KNOX gene03606 promoter. The 11.77kb backbone of pMDC162 without GUS gene (for short, backbone 1) was generated via a reverse PCR using Phanta EVO Super-Fidelity DNA Polymerase (P503-d1, Vazyme, Nanjing, China) with primers ZT067 and ZT068. Then *FvSTP8* CDS fragment and the pMDC162-P03606 backbone1 were recombined using the Exnase II from the ClonExpress II One Step Cloning Kit (C112, Vazyme, Nanjing, China). The resultant clones (for short, pMDC162-P03606:FvSTP8) were first identified via PCR with *FvSTP8* specific primers combined with restriction enzyme SpeI digest analysis, since the original pMDC162 plasmid harbors two recognition sites but the resultant novel construct has three sites. Furthermore, one of the resultant plasmids was selected via sequencing with 8 specific primers spanning the whole T-DNA region.

To obtain a marker-free plasmid, a second recombination was designed to discard the antibiotics marker gene expression cassette in pMDC162-P03606:*FvSTP8*. A 401bp natural terminator of *FvSTP8* gene (directly downstream the stop code of *FvSTP8*) was cloned from the genomic DNA of Hawaii4 into pClone007B vector. After sequencing for validation, this terminator was reamplified with primers CM2328 and CM2329. Simultaneously, the backbone of pMDC162-P03606:FvSTP8 without the NOS terminator behind *FvSTP8* CDS as well as the whole HygR expression cassette (CaMV35S: HygR gene-CaMV polyA signal) (for short, backbone 2) was amplified via a reverse PCR using primers CM2269 and ZT069. The *FvSTP8* terminator and the above backbone 2 were recombined using the Exnase II of C112 kit (Vazyme, Nanjing, China). Clones were first screened by PCR with *FvSTP8* terminator specific primers and then validated by sequencing with three specific primers. Finally, the resultant plasmid was named as pMF-P03606:*FvSTP8*, which

was introduced into *Agrobacterium* strain GV3101 by the freeze thaw method [78]. Primers used to generate pMF-P03606:*FvSTP8* were listed in supplementary Table S1.

4.4 Direct PCR analysis of regenerated shoots

Before rooting and acclimation, independent transformants were first genotyped using direct PCR. A piece of round leaf disc in a diameter of 3 mm was sampled from each candidate transformant in a clean bench and immersed into 50 µl buffer A (100 mM NaOH, 2% Tween-20) where it was lysed at 95 °C for 10 min. This coarse lysate was then mixed with 150 µl buffer B (Tris-HCl 100 mM, EDTA-2Na 2mM, pH 2.0) for dilution and the resultant solution was directly used as template for PCR.

Specific primers matching to a junction region of the T-DNA in pMF-P03606:*FvSTP8* construct were designed to screen transformed plantlets. Amplification with strawberry *Actin* (gene 18570-v1.0 hybrid, *FvH4_1g23490*) specific primers 5'-CGACCTTAATCTTCATGCTGCTTGG-3' and 5'-TCATTGGAATGGAAGCTGCTGGCATT-3' [79] was performed to ensure the quality of templates meeting for direct PCR. Primers used for direct PCR screening were displayed in supplementary Table S1. PCR reaction cocktail contained 1 µl the above coarse sample lysate, 0.3 µl each primer (stock- 20 µM), 0.5 µl dNTP (stock-2.5 mM), 2 µl 10x PCR buffer with Mg²⁺, 0.3 µl Taq DNA polymerase (5 U/µl, Biocolor, Shanghai, China). Reactions were carried out in a program consisting of an initial denaturation at 94 °C for 3 min, followed by 32 cycles of 94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 40 sec, then a final extension at 72 °C for 5 min. PCR products were separated on 1.5 % agarose gel electrophoresis, visualized with GelRed dye under UV light and photographed with Tanon 1600 Gel Photographer (Tanon, Shanghai, China).

4.5 DNA purification and ddPCR analysis

Total strawberry genomic DNA was isolated from leaves of transformed plantlets using a Super Plant Genomic DNA kit for polysaccharides and polyphenolics-rich samples following the manufacture's instruction with minor modification (TianGen, Beijing, Cat. No. DP360). Briefly, strawberry leaves were homogenized at 55Hz for 1 min using Tissue Lyser-24 (Jingxin, Shanghai). Twenty to 40 mg plant powder was used to mix with 400 µl GPA buffer followed by a water bathing at 65°C for 30 min. The purified DNA was visualized on 1% agarose gel electrophoresis and then quantified using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and the dsDNA quantification kit (Magic dsDNA HS Assay Kit, Cat. No. Q32854, Magic Biotech, Hangzhou, China). One hundred ng genomic DNA per sample was fractionated with 8 U of SpeI-HF (NEB, R3133L) at 37°C for 30 min. Four or 5 ng DNA after digestion was used for each dPCR reaction. A negative control (blank) was performed using untransformed 'Shanghai Angel' DNA for each new ddPCR assay.

A low-copy nuclear gene DHAR (DeHydro Ascorbate Reductase) (four copies per octoploid genome) previously used for phylogenetically analysis in genus *Fragaria* [58] was chosen as the reference gene for current ddPCR. For the probe-based duplex ddPCR, primers and probe were designed at Primer3Plus: <http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/> [80]. Candidate primer pairs and probes were further selected through evaluation at

<https://mfeprimer3-0.igenetech.com/> [81] following the criteria suggested previously [47]. The sequence information of primers and probes for ddPCR was given in supplementary Table S2. Reference gene specific primers and probe were designed to match only FxC_26g17540, one copy of DHARs, corresponding to the loci FANhyb_rscf00000137.1.g00005.1 in the database *Fragaria x ananassa* Reference Genome v1.0 (FANhybrid_r1.2) scaffolds at GDR (www.rosaceae.org).

A ddPCR mixture was prepared with 10 µl 2×TIANexact Genotyping qPCR Pre Mix (probe) (no dUTP; Cat. FP211, Beijing, China), 450 nM of each primer pair and 250 nM of each probe for both reference gene and intragenic construct, 5 ng digested genomic DNA in a final volume 20 µl. Droplets generation (Vibro Ject injection technology), PCR amplification, fluorescence detection, droplet reading and quantification were accomplished using DQ24 digital PCR machine (Sniper, Suzhou, China). The program was set as an initial 65°C 5 min, 95°C 5 min, and 40 cycles of 95°C for 20 sec and 63°C for 30 sec. Following droplet counts accomplished, the measurements of intragene copy number were performed using the Sniper SightPro software with default settings for the threshold to separate positive and negative droplets. DdPCR was independently repeated twice and similar results were obtained. Results presented were from one of experiments in which the positive and negative signal intensities were separated most satisfactorily (supplementary Fig. S1). Average frequency (λ) of certain molecule in ddPCR was calculated following the equation: $\lambda = -\ln(1 - \text{positive droplets} / \text{total droplets}) / \text{volume of droplet}$. The copy number (C) of intragene per genome was obtained using the equation $C = 1 * \lambda_{\text{target}} / \lambda_{\text{ref}}$, where the constant 1 represents for the copy number of the detected reference *FaDHAR* region per genome, λ_{target} for average frequency of intragene KNOX-Pro: *FvSTP8*, and λ_{ref} for the average frequency of detected *FaDHAR* region.

Supplementary Materials: The following are available online at <https://www.mdpi.com>. Table S1: List of primers used for vector construction and direct PCR analysis of transformants. Table S2: Sequence information of the oligonucleotides used for droplet digital PCR (ddPCR) in the study. Figure S1: One-dimensional plot of droplets measured for fluorescence signal emitted from the endogenous reference gene *FaDHAR* (FAMTM labeled, positive droplets are blue) or the intragene KNOX Pro:*FvSTP8* (VICTM labeled; positive droplets are red) in four distinct transformants and untransformed cv. ‘Shanghai Angel’. Negative droplets are black. Figure S2: The droplets visualized in two dimensions for ddPCR analysis with the genomic DNA templates of #9-d and #20-7. The colors are as described in Fig.S1 but the droplets with both fluorescent probes are purple.

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