Establishment of A Rapid and Stable Infected System by Agrobacterium-Mediated Transformation of Germination Seeds in Diploid Strawberry.

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Methodology

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Title: Establishment of a rapid and stable **infected** system by *Agrobacterium*-mediated transformation of germination seeds in diploid strawberry.

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Highlight: Using the CHLH (the H subunit of magnesium chelatase magnesium chelatase) as a reporter gene, we first have successfully established *Agrobacterium*-mediated transformation of germinating seeds (AMTGS) in diploid strawberry (*Fragaria vesca*), providing a useful tool for identification of gene function.

Abstract

Background

Strawberry (*Fragaria*) is regarded as a model plant for both Rosaceae and non-climacteric fruit ripening. Although much progress has been made in identification of gene function using stable and transient genetic transformation systems in strawberry, the limitation is, more or less, are present. To this end, development of a rapid, efficient, and stable transformation system is required for strawberry research and breeding.

Results

Here, using diploid Hawaii-4 (*Fragaria vesca*) seeds and a reporter gene of *CHLH* (the H subunit of magnesium chelatase magnesium chelatase) key to chlorophyll synthesis, we first develop a rapid, efficient, and stable infected system by the *Agrobacterium*-mediated seed infection to silence the reporter gene, reaching an infection frequency with 28.3% through a series of optimization elements, including seed full imbibition and initial germination, shaking infection for 24 h, dark cultivation on MS medium for 3 d at 24 °C, light culture on MS-Tim medium for 1 week at 24 °C, and vector construction tagged with fluorescence label. Taken together, radicle-emergence germination seeds, appropriate *Agrobacterium* concentration and infection time are critical for successful infection, finally obtaining the infected kanamycin-resistant seedlings of T1 generation by infected wild seeds within 1 month and T2 generation-infected plants within 4 months.

Conclusions

The *Agrobacterium*-mediated infection of germinating seeds (AMTGS) in diploid strawberry (*F. vesca*) is first established, providing a useful tool for gene function identification and improved agronomic traits in strawberry.

Key words: Strawberry; *Agrobacterium*-mediated infection of germinating seeds; reporter gene; CHLH; RNAi

Background

Strawberry (*Fragaria*), as a distinct member of Rosaceae, is not only an important fruit crop worldwide, but is also regarded as a model for studying non-climacteric fruit ripening [1]. In the past years, a large of genes related to fruit ripening and development have been identified via traditional stable and transient infected systems through VIGS (virus induced gene silencing), ihpRNA (intron-containing hairpin RNA), somatic tissue or callus formation in strawberry [2-15], however, some limitations, more or less, is present, including transient injury in the transient infection system and long period, instability of callus induction and low efficiency in the stable infection system [2-4]. To some extents, development of a rapid, efficient, and stable infection system is required for strawberry research and breeding.

In addition to the widely-used leaf-disk infection [14, 19, 20], other tissues are also used for gene infection [21-30]. The mature and immature embryo-generated calli are also used to develop infected plants in rice, maize, wheat and barley [21-23, 27]. Notably, non-tissue culture-based methods, such as the floral dip infection, is widely and efficiently used in Arabidopsis as a model system [24-26], and the *Agrobacterium*-mediated infection of germinating seeds (AMTGS) of Arabidopsis is also early reported [31]. In recent years, the AMTGS of kenaf (*Hibiscus cannabinus*) seeds have been developed within 3 months with 6 % infection efficiency by the efficient, fast, reliable stable, non-tissue culture-infected method [32]. Thereby, to rapidly identify gene function in strawberry, an AMTGS of diploid strawberry 'Hawaii-4' (*Fragaria vesca*) has been established in the present study.

It is previous reported that some factors including suspension *Agrobacterium* concentrations, *Agrobacterium* infection time, kanamycin concentration are considerable in plant infection; and the reporter gene is also a good strategy for early infection selection, such as neomycin phosphotransferase gene (NPT II), green fluorescent protein (GFP), red fluorescent protein (DsRed), and β -glucuronidase (GUS) genes [14-31]. In addition, the H subunit of magnesium chelatase magnesium chelatase (CHLH), a key enzyme involved in chlorophyll biosynthesis, is chosen as a good reporter gene, in that its silencing phenotype was clearly observed through chimeric phenotypes with yellow/white leaves [33-36]. Given that it is tend to usage of the diploid for scientific studies rather than the octaploid, and the results gained from the diploid are appropriate for the octaploid application. Thereby, in the present study, we recruit the diploid and integrate these reported methods, apart from the GFP and GUS genes (Fig. 2), we also used the reporter gene CHLH to construct the recombination pK7GWIWG2 (II) RR-FveCHLH vector carried with the two reporter genes, DsRed and CHLH, which are used as early selection. On the basis of DsRed fluorescence, it is easy to isolate early potential infected seedlings, and further confirming to be positive infected plants by kanamycin selection (Fig. 3 and 4). The first selection by DsRed fluorescence may bypass a large quantity of seeds used for kanamycin selection, not only saving more time and labor, but

also and making it easier to obtain infected plants. One month after the infection, through the reporter gene CHLH, we may observe the leaves of infected plants with a loss-of-green phenotype (Fig. 5). In all, through the two reporter genes, the *Agrobacterium*-mediated infection of strawberry seeds was first established successfully.

Results

Determination of seed imbibition extent and optimum kanamycin screening concentration

To establish rapid and stable infected system in strawberry, the *Agrobacterium*-mediated infection of germinating seeds in diploid strawberry was first studied based on the previous reports [31, 32]. We first find that there was no significant difference in the germination rate of strawberry seeds under different light culture conditions, including light culture, dark culture, and light culture after dark culture; also the three treatments had a few, half, and 90 % of seed germination respectively with 4-d, 5- d and 6-d imbibition (Fig. 1a).

To confirm optimal kanamycin concentration for screening, the sterilized strawberry seeds were cultured in MS selection medium with kanamycin concentration at 0, 25, 50, 75, 100 mg/L for 15 days. The results showed that as the concentration of kanamycin was 0 mg/L, 95 % of the seeds germinated, the plants grew normally and the leaf color was dark green. Compared with no selection pressure, when the concentration of kanamycin increased by 25 mg/L, 91.7 % of the seeds germinated, except for the roots with slightly-slow growth, the germinated seedlings continued to growth as the same as the wild-type; when the concentration of kanamycin was 50 mg/L, 41.7 % of the seeds germinated, but the first leaf of the germinated seedlings could not fully unfold; when the selection pressure was increased to 75 mg/L, only 20 % of the seeds could germinate and the color of the two cotyledons of the seedlings were yellow and white; when the selection pressure increased to 100 mg/L, 5 % of the plants germinated and died of browning (Fig. 1b, c). Taken together, 75 mg/L of kanamycin in MS-selection medium was selected as an antibiotic selection concentration.

Optimization of infection parameters

After 3-d culture in the selection medium, the GFP could be observed in the successfully-infected plants (Fig. 2a, b). Similarly, the unsuccessfully-infected seedlings had no GUS expression (Fig. 2c, e), while the successfully-infected seedlings showed blue in the roots and true leaves with GUS expression (Fig. 2d, f). Base on the observation of both GFP and GUS expression in infected seedlings, a 1.5-OD₆₀₀ value of the *Agrobacterium* infection solution was optimum infection concentration (Fig. 2g). Seeds infected at the stage of radicle emergence had highest infection efficiency (Fig. 2h) during 24-h infection (Fig. 2i). Taken together, the optimum parameters of *Agrobacterium*-mediated infection of

germinating seed in diploid strawberry are as follows: 75 mg/L of kanamycin in MS-selection medium, seeds infected at the stage of radicle emergence, 24-h infection in 1.5-OD₆₀₀ value of the *Agrobacterium* infection solution, and then it was dark cultured at 24 °C for 3 d.

Establishment of a rapid and stable infected system by the Agrobacterium-mediated infection of germinating seeds by a reporter gene of FveCHLH in strawberry

The *CHLH* encoding the H subunit of magnesium chelatase magnesium chelatase involved in chlorophyll biosynthesis is chosen as a reporter gene, which is used to early assess effectiveness in the seed infection system, due to its silencing phenotypes with yellow/white leaves easy to be observed [33-36].

Based on the optimal infection conditions obtained above, we infected germinating seeds at the stage of radicle emergence of diploid strawberry 'Hawaii-4' using *Agrobacterium* strain GV3101 strain carrying the pK7GWIWG2 (II) RR-*FveCHLH* vector. After 3-day infection under dark culture, the seeds were transferred to MS-selection medium. After 3 days again, the seeds were screened by DsRed fluorescence. Then after 2 weeks, the fluorescent seedlings were transferred to quartz sand and watered using nutrient solution containing 75 % kanamycin. The whole processes for infection of germination strawberry seeds are shown in Fig. 3. We found that the DsRed⁺ fluorescence observed in 3-day-old plants (Fig. 4a, b) persisted in 7-day-old plants (Fig. 4c, d). DsRed fluorescence was also expressed in the roots (Fig. 4e, f) and stolons (Fig. 4i, j) of the plants after three months, but not in the wild-type plants of the roots (Fig. 4g, h) and stolons (Fig. 4k, l).

One month after transplanting, in comparison to the control, a proportion of the infected seedlings showed yellow spots (Fig. 5 a, phenotype-1), or half green and half white in a leaf (Fig. 5a, phenotype-2). Genome-based PCR was performed to detect both kanamycin and *DsRed* gene fragments in the T-DNA inserted infection plants. The expected 400-bp Kana and 964-bp DsRed products were detected in five *FveCHLH*-RNAi plants but not in the wild-type plants (Fig. 5b, c). Compared with the wild-type plants, the expression of *FveCHLH* gene in the *FveCHLH*-RNAi plants was significantly down regulated (Fig. 5d). In the process of infection, phenotype-1 plants accounted for 9.43 % of the total infected plants, and phenotype-2 plants accounted for about 18.87 % of the total infected plants. It may be concluded that the transformation efficiency (The number of positive seedlings detected by DsRed and PCR divided by the total number of infected seedlings) reached by 28.3 % (Fig. 5e). These results showed that based on the *Agrobacterium*-mediated infection of germinating seed, the pK7GWIWG2 (II) RR-*FveCHLH* vector was successfully transformed, and the *FveCHLH* expression was inhibited, also the *FveCHLH*-RNAi infected plants have chlorosis phenotypes.

T2 seedlings of FveCHLH-RNAi plants retains kanamycin-resistance

T1 seeds of *FveCHLH*-RNAi infected plants were spread on the quartz sand soaked in the nutrient solution containing 75 mg/L kanamycin. In the presence of kanamycin, the seeds sensitive to kanamycin cannot germinate or the cotyledons turn yellow 10 days after germination, and the true leaves could not develop. T2-resistant seedlings could germinate on quartz sand with kanamycin and continued to grow normally (Fig. 6a). The Kanamycin resistant bands and DsRed bands were detected in T2 generations (Fig. 6b). These results demonstrate that the infected plants can obtain by seed infection and the relative genetic characteristics may be integrated into the T1 progeny. **To this end, the rapid and stable infected system is successfully established by the *Agrobacterium*-mediated infection of germinating seed.**

Discussion

Strawberry is a model plant for both Rosaceae and non-climacteric fruit ripening. **In the past years, considerable progress has been made in understanding the underlying molecular mechanisms of strawberry fruit ripening by using transient expression and stable transformation systems. However, limitations of these systems include transient-infection with injury and stable-infection long infection with period, instability of callus induction, and low efficiency [1-14, 16-18, 37].** Notably, it is previously reported that the *Agrobacterium*-mediated seed infection may facilitate the entry of *Agrobacterium tumefaciens* into the seeds to integrate a target gene into the embryo cells [31-32], similar to the pollen-tube mediated genetic infection method widely used in *Arabidopsis thaliana* [24,30]. Reference of the seed infection method [31-32], **in the present study**, we first establish the *Agrobacterium*-mediated infection of the germination seeds in diploid strawberry by a series of optimization conditions.

First, some factors key to infection efficiency were confirmed, including seed imbibition time, suspension *Agrobacterium* concentrations, *Agrobacterium* infection time, kanamycin concentration in selection of infected plants. First, full imbibition is requisite for seed infection **and thus should be determined**. It is early reported that in *Arabidopsis* seed infection, 12-h imbibition has the highest infection efficiency, less than 9 h showing no transformants [31]. In the strawberry, we demonstrate that seed imbibition to reach both testa rupture and, radicle emerging is vital for infection efficiency (Fig. 2). Second, *Agrobacterium* concentrations and infection time are also critical to infection efficiency [38], and thereby we further confirm them, finding that the 24-h infection time has maximum infection efficiency, in consistent with the previous reports in seed infection [31, 32]. Generally, in the conventional leaf disk method, *Agrobacterium* with concentration at 0.3-0.5 of OD₆₀₀ has high vigor and is used as an optimal concentration for infection [39], in that a higher *Agrobacterium* concentration causes harm to explant differentiation including browning even death, and a lower

concentration is not enough *Agrobacterium* for T-DNA integration of explants with lower infection efficiency. In the present study, we find that the germination seeds were more tolerant to *Agrobacterium tumefaciens*, and OD₆₀₀ of 1.5 is an optimal concentration for highest infection efficiency (Fig. 2). Finally, the appropriate concentration of kanamycin is also pivotal for selection of infected positive plants. In Arabidopsis seed infection, T1 and T2 generation positive seedlings could be selected on medium containing 100 mg/L kanamycin [31]. In Kenaf seed infection, F0 and F1 plants were successfully obtained on medium containing 50 mg/L kanamycin [32]. Here, we find that the strawberry seedlings were selected on medium containing 75 mg/L kanamycin, in which the positive infected plants could developed true leaves, whereas not in the control (Fig. 2). Taken together, we **determine** high efficiency infection parameters in the *Agrobacterium*-mediated infection in the germination seeds of diploid strawberry, including radicle initially-emerging seeds, 1.5-OD₆₀₀ *Agrobacterium*, 24-h infection, and 75 mg/L kanamycin in infected plant selection.

In addition, how to early select positive infected seedlings is more important in genetic infection. It is previously demonstrated that reporter genes are good strategy for early infection selection, such as neomycin phosphotransferase gene (NPT II), green fluorescent protein (GFP), red fluorescent protein (DsRed), and β -glucuronidase (GUS) genes [31,40]. In the present study, apart from the GFP and GUS genes (Fig. 2), we also used the reporter gene encoding magnesium chelatase H subunit (CHLH) functioned in chlorophyll biosynthesis, in that when its expression is downregulated, resulting in yellow or white leaf phenotype and thus using as a good report gene [33-36]. The recombination pK7GWIWG2 (II) RR-FveCHLH vector with two reporter genes, DsRed and CHLH, which used as early selection, were constructed. On the basis of DsRed fluorescence, it is easy to screen early potential infected seedlings, which is further confirmed to be positive infected plants by kanamycin selection (Fig. 3 and 4). The first selection by DsRed fluorescence may bypass a large quantity of seeds used for kanamycin selection, not only saving more time and labor, but also making it easier to obtain infected plants. One month after the infection, through the reporter gene CHLH, we may observe the leaves of infected plants with a loss-of-green phenotype (Fig. 5).

To further confirm the *Agrobacterium*-mediated infection of the germination seeds, we also carried out kanamycin resistance and PCR analysis in T2 seedlings (Fig. 6): (a) when strawberry seedlings were selected on medium containing 75 mg/L kanamycin, the true leaves of kanamycin-resistant T2 seedlings can develop normally, whereas the growth of wild-type seedlings and kanamycin-sensitive T2 seedlings was partially inhibited and true leaves failed to develop; (b) through PCR test, the integration of DsRed gene and Kanamycin resistance gene into the genome of kanamycin-resistant T2 seedlings have been confirmed, whereas in wild-type seedlings and kanamycin-sensitive T2 seedlings, corresponding gene integration has not been detected. By analysis of the infected characteristics of transformed progeny (T1 and T2 generation), we provide a line of evidence to demonstrate that the *Agrobacterium*-mediated infection of strawberry seeds

was first established successfully. Based on the early report [41], we also speculate that transgenic epidermal cell division (L1 layer) is likely as a result of phenotype 1, and the transgenic anticlinal cell divisions (L2 layer) is likely as a result of phenotype 2, to some extents, the phenotype 1 is easy to generate T2, the chimeric phenotypes are generated.

Conclusions

On the basis of the CHLH (the H subunit of magnesium chelatase magnesium chelatase) as a reporter gene and the germinating seeds with radicle appearance, the *Agrobacterium*-mediated infection of germinating seeds (AMTGS) in diploid strawberry (*Fragaria vesca*) has first been established, especially finding that appropriate *Agrobacterium* concentration and infection time are critical for the infection. The successful infected kanamycin-resistant seedlings (T1) are gained within 1 month and T2 infected plants within 4 months.

In summary, we have first established a fast and efficient protocol for *Agrobacterium*-mediated infection of strawberry seeds: (1) seed imbibition time (just to the point of radicle emergence) is a key step; (2) *Agrobacterium* concentration and the infection time are also critical for successful infection; (3) the selection of kanamycin-resistant seedling takes 1 month, T2 generation infected plants are obtained within 4 months; (4) the transformation efficiency is 28.3 %. Thus, this method can greatly shorten the experimental cycle and simplify the operation processes. Give that strawberry is a model plant for both *Rosaceae* and non-climacteric fruit ripening, the *Agrobacterium*-mediated infection of strawberry seeds is to be widely used and will facilitate fruit development research and breeding.

Methods

Plant materials and seeds germination

The diploid strawberry ‘Hawaii-4’ (*Fragaria vesca*) plants were grown in green house at 25±1 °C. The seeds were taken from ripe fruits and residual receptacle was removed, then the seeds were put at room temperature to dry for 1-2 d. The dried seeds are put into 2-mL tube and stored at 4 °C for use. Seeds were sterilized by 1 % sodium hypochlorite for 8 min and washed with sterile water three times. The sterilized seeds were transferred onto MS-germination medium [4.43 g/L MS (Phyto Technology Laboratories M519), 2 % sucrose (Sangon, China), 0.8 % agar (Sangon, China), pH 5.8], and left at 4 °C for 24 h to break dormancy. The dormancy-broken seeds were treated with three test treatments: light culture in a light incubator (PRX-350C, Sdfu, China), dark culture wrapped with tin foil, and light culture after 2 days of dark culture. The seed culture condition in light incubator is as a 16 h photoperiod with a light intensity of 30,000 LX (250μmol·m⁻²·s⁻¹) at 24 °C. Each treatment was inoculated with 20 seeds (three repeats) for germination.

Vector construction and *Agrobacterium tumefaciens* culture

Two *Agrobacterium* strains were used to optimize infection parameters, namely GV3101 strain carrying pCAMBIA 1300 vector with a Green fluorescent protein (GFP) reporter gene and carrying pBI121 vector with a β -glucuronidase (GUS) reporter gene. The RNAi recombinant plasmid was constructed using the Gateway system and target genes were silenced in strawberry fruit by intron splicing RNA (ihpRNA). The 465-bp *FveCHLH* were amplified and cloned into the pDONRTM 221 vector (Invitrogen, Beijing China) then into the pK7GWIWG2 (II) RR vector using Gateway LR Clonase II enzyme (Invitrogen 11791-020, USA) combination technology. The primers for constructing *FveCHLH*-RNAi were: forward, 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCTGCCAACAATCCATCT-3' and reverse, 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCTTCCAAGTCTCAGCCA-3'. The pK7GWIWG2 (II) RR vector contains the red fluorescence protein (DsRed) reporter gene and kanamycin resistance gene.

The recombinant plasmid was transformed into *A. tumefaciens* GV3101, and a single colony was picked and placed in Luria broth (LB) medium containing antibiotic 20 μ g/mL rifampicin (Sangon, A600812, China), 50 μ g/mL gentamicin (Sangon, A100304, China), and 100 μ g/mL spectinomycin (Sangon, B541016, China) at 28 °C, and 180 rpm overnight. The single colony of GV3101 strains (GV3101 strain of pCAMBIA 1300 and GV3101 strain of pBI121) were put into an LB medium containing antibiotics 20 μ g/mL rifampicin, 50 μ g/mL gentamicin, 100 μ g/mL kanamycin (Sangon, A506636, China) at 28 °C, and 180 rpm overnight. Above three kinds of bacteria liquid were collected with a 50 ml tube (Sangon, NO. F602788, China) by centrifugation at 6,000 rpm for 5 min, and suspended using MS-infection [4.43 g/L MS, 2 % sucrose, 200 μ mol/L Acetosyringone (As, Sigma-Aldrich, D134406, USA)], pH 5.8 to prepare an *Agrobacterium* infection solution. The volume of *Agrobacterium* infection solution was 7 ml.

Determination of appropriate Kanamycin concentration for screening

The sterilized seeds were cultured on the MS-selection medium containing different concentrations of kanamycin with 0, 25, 50, 75 and 100 mg/L in order to determine most suitable antibiotic screening concentration. Twenty seeds were placed in each petri dish and the experiment were done with three repetitions.

Optimization of parameters related to infection efficiency

The pre-imbibed seeds were placed in *Agrobacterium* infection solution and co-cultured for 24 h in the dark at 120 rpm, 28 °C, then the seeds were placed in MS-germination medium and wrapped in tin foil at 25 °C for 3 d. Subsequently, the seeds were further placed in MS-selection medium [4.43 g/L MS, 2 % sucrose, 75 mg / L kanamycin, 200 mg /L

1 Timentin (Phyto Technology Laboratories T869), 0.8 % agar, pH 5.8]. After 3 days, GFP/DsRed fluorescent tag and GUS
2 reporter gene were used for early screening and statistics. After 2 weeks, the seedlings with fluorescence were
3 transplanted into quartz sand and covered with cling film for three days to prevent water loss, and watered with the
4 kanamycin-added nutrient solution to allow the seedlings to grow under appropriate selection pressure at condition with a
5 greenhouse at 24 °C, light/dark 16/8 h.

6 To determine factors related to infection efficiency, we performed a series of experiments, including OD values
7 (optical density at 600 nm, OD₆₀₀=0.5, 1, 1.5, and 2), infection time (12 h, 24 h, and 36 h), and seed germination status (1
8 represents the seed that has not yet exposed the radicle; 2 represents the seed that has just sprouted the radicle; 3
9 represents that the seed has two cotyledons.). The optimal infection parameters were determined by investigation of GFP
10 using stereo fluorescence microscope (ZEISS-Axiocam 506 color) and GUS Histochemical Stain Kit (Solarbio, G3060).
11 Infection efficiency was calculated by the proportion of GFP fluorescent protein expression plants to the total infected
12 plants or Gus expression plants to the total infected plants. During the infection process, 20 seeds were placed in each
13 petri dish, and three replicates were performed.

14 15 *Identification of infected plants by PCR*

16 In order to confirm infection of T-DNA in diploid strawberry genome, leaves of one-month-old infected strawberry
17 plants with red light were collected and genomic DNA was extracted by Plant genomic DNA Extraction Kit (Aidlab
18 Biotech, DN15, China). The leaves of wild type plants were used as control. The fragments of Kanamycin resistance
19 genes and DsRed genes were amplified by PCR. Primers for PCR identification were: Kan forward,
20 5'-CGATAGAAGGCGAT GCGCTG-3' and reverse, 5'-CGCTTGATCCGGCTACCTG-3'; Red forward,
21 5'-CGCCCTTGGTACCTTCAGCTTCAC-3' and reverse, 5'-CAATGCAGTGGGACCCACGGTTC-3'.

22 23 *Real-time PCR (qPCR)*

24 Total RNA from leaves of infected and wild-type plants was extracted using an OMEGA RNA extraction kit (OMEGA
25 biotek, USA) according to manufacturer's protocols. To generate first-strand cDNA, 400 ng of total RNA was reverse
26 transcribed using the Trans kit (Transgen, China) according to manufacturer's protocols. The first-strand cDNA was used
27 as a template for PCR amplification for real-time PCR on a Light Cycler 96 Real Time PCR System (Roche Diagnostics
28 GmbH, Mannheim City, Germany) using TransStart Top Green qPCR SuperMix (Transgen, China). The reactions of 10
29 µL contained 5 µL qPCR SuperMix, 0.25 µL forward specific primer (10 µM; Sangon, China), 0.25 µL reverse specific
30 primer (10 µM; Sangon, China), 2 µL cDNA template and 2.5 µL ddH₂O. *Actin* was used as a reference gene. Relative

gene expression was analyzed by LightCycler® 96 SW 1.1. The primers used for real-time PCR were: forward, 5'-TGGGTCCCCTGATAAC-3' and reverse, 5'-CCAAATCCCCTGTCC-3'.

Kanamycin resistance test of T1 seeds

A number of seeds were obtained from a single inbred T1 generation, sprinkled on quartz sand, and irrigated with kanamycin (75 mg/L). After 10 days of culture, the sensitivity of the plants to kanamycin resistance was observed, and DNA molecules were analyzed for the resistant seedlings.

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Availability of data and materials

The datasets supporting the conclusions and a description of the complete protocols are included within the article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

PH and XJ performed the experiments. YY designed the research and wrote the article.

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References

1. Li CL, Jia HF, Chai YM, Shen YY. Absciscic acid perception and signaling transduction in strawberry: A model for non-climacteric fruit ripening. *Plant Signaling and Behavior*. 2011; 6:1950-1953.
2. Guidarelli, M, Baraldi, E. Transient transformation meets gene function discovery: the strawberry fruit case. *Frontiers in Plant Science*. 2015;12(6):444.
3. Haddadi F, Aziz MA, Abdullah SN, Tan SG, Kamaladini H. An efficient Agrobacterium-mediated transformation of strawberry cv. Camarosa by a dual plasmid system. *Molecules*. 2015; 20:3647-66.
4. Zhao Y, Mao W, Chen Y, Wang W, Dai Z, Dou Z, Zhang K, Wei L, Li T, Zeng B, Liu T, Fan Y, Yan J, Li B, Jia W. Optimization and standardization of transient expression assays for gene functional analyses in strawberry fruits. *Horticulture Research*. 2019; 6:53.
5. Nehra NS, Chibbar RN, Kartha KK, Datla RSS, Crosby WL, Stushnoff C. Genetic transformation of strawberry by Agrobacterium tumefaciens using a leaf disk regeneration system. *Plant Cell Reports*. 1990; 9:293-8.
6. El Mansouri L, Mercadi JA, Valpuesta V, Lopez-Aranda JM, Pliegi-Alfaro F, Quesada MA. Shoot regeneration and Agrobacterium -mediated transformation of *Fragaria vesca* L. *Plant Cell Reports*. 1996; 15:642-646.
7. Haymes KM, Davis TM. Agrobacterium -mediated transformation of 'Alpine' *Fragaria vesca* and transmission of transgenes to R1 progeny. *Plant Cell Reports*. 1997; 17:279-283.
8. Balokhina NV, Kaliayeva MA, BuryanovYa I. The elaboration of a shoot regeneration system for the genetic

- transformation of the wild strawberry (*Fragaria vesca* L.). Biotekhnologiya. 2000; 16:46–51.
9. Jimenez-Bermudez S, Redondo NJ, Munoz-Blanco J, Caballero JL, Lopez-Aranda JM, Valpuesta V, Pliego-Alfaro F, Quesada MA, Mercado JA. Manipulation of strawberry fruit softening by antisense expression of a pectate lyase gene. Plant Physiology. 2002; 128:751-759.
 10. Agius F, Gonzalez-Lamothe R, Caballero JL, Munoz-Blanco J, Botella MA, Valpuesta V. Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. Nature Biotechnology. 2003; 21:177–181.
 11. Oosumi T, Gruszewski HA, Blischak LA, Baxter AJ, Wadl PA, Shuman JL, Veilleux RE, Shulaev V. High-efficiency transformation of the diploid strawberry (*Fragaria vesca*) for functional genomics. Planta. 2006; 223:1219–1230.
 12. Qin Y, Jaime A, da Silva T, Zhang L, Zhang S. Infected strawberry: state of art for improved traits. Biotechnology Advances. 2008; 26:219–232.
 13. Zhang Q, Folta KM, Davis TM. Somatic embryogenesis, tetraploidy, and variant leaf morphology in trasgenic diploid strawberry (*Fragaria vesca* subspecies vesca ‘Hawaii 4’). BMC Plant Biology. 2014; 14:23.
 14. Song GQ, Prieto H, Orbovic V. *Agrobacterium*-Mediated Transformation of Tree Fruit Crops: Methods, Progress, and Challenges. Frontiers in Plant Science. 2019; 10:226.
 15. Barcelo, M, El-Mansouri, I, Mercado, J.A, Quesada, M.A., Pliego Alfaro, F. Regeneration and transformation via *Agrobacterium tumefaciens* of the strawberry cultivar Chandler. Plant Cell Tissue and Organ Culture. 1998; 54:29–36.
 16. Folta KM, Dhingra A. Invited review: Transformation of strawberry: The basis for translational genomics in *Rosaceae*. In Vitro Cellular and Developmental Biology Plant. 2006; 42:482–490.
 17. Hoffmann T, Kalinowski G, Schwab W. RNAi-induced silencing of gene expression in strawberry fruit (*Fragaria x ananassa*) by agroinfiltration: a rapid assay for gene function analysis. The Plant Journal. 2006; 48:818-26.
 18. Miyawaki K, Fukuoka S, Kadomura Y, Hamaoka H, Mito T, Ohuchi H, Schwab W, Noji S. Establishment of a novel system to elucidate the mechanisms underlying light-induced ripening of strawberry fruit with an *Agrobacterium*-mediated RNAi technique. Plant Biotechnology. 2012;29:271–277
 19. Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G., and Fraley, R. T. A simple and general-method for transferring genes into plants. Science. 1985; 227:1229–1231.
 20. Cappelletti R, Sabbadini S, Mezzetti B. Strawberry (*Fragaria* × *ananassa*). Methods in Molecular Biology. 2015; 1224:217-27.
 21. Potrykus I. Gene transfer to cereals: an assessment. Trends in Biotechnology. 1990.8:535–542.

22. Ishida Y., Saito H., Ohta S., Hiei Y., Komari T., Kumashiro T. High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nature Biotechnology*. 1996; 14:745–750.
23. Ishida Y., Tsunashima M., Hiei Y., Komari T. Wheat (*Triticum aestivum* L.) transformation using immature embryos. *Methods in Molecular Biology*. 2014; 1223:189–198.
24. Clough SJ, Bent AF. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal*. 1998; 16:735–743.
25. Bent A. *Arabidopsis thaliana* floral dip transformation method. *Methods in Molecular Biology*. 2006; 343:87-103.
26. Zhang X., Henriques R., Lin S. S., Niu Q. W., Chua N. H. *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nature Protocol*. 2006; 1:641–646.
27. Hiei Y, Ohta S, Komari T, Kumashiro T. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant Journal for Cell and Molecular Biology*. 1994; 6:271-282.
28. Harwood W. A. A protocol for high-throughput *Agrobacterium*-mediated barley transformation. *Methods in Molecular Biology*. 2014; 1099:251–260.
29. Ratanasut K, Rod-In W, Sujipuli K. In planta *Agrobacterium*-mediated transformation of rice. *Rice Science*. 2017; 24:181–186.
30. Van Eck J. The Status of *Setaria viridis* Transformation: *Agrobacterium*-Mediated to Floral Dip. *Frontiers in Plant Science*. 2018; 9:652.
31. Feldmann KA, Marks MD. *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana*: a non-tissue culture approach. *Molecular and General Genetics*. 1987; 208:1-9.
32. Hanana M, Ayad R, Mzid R, Khouja ML, Hanachi AS, Hamrouni L. Hanana, Mohsen; Ayadi, Rekaya; Mzid, Rim. Efficient method of seed transformation via *Agrobacterium tumefaciens* for obtaining transgenic plants of *Hibiscus cannabinus* L. *Industrial Crops and Products*. 2018; 113:274-282.
33. Hiriart JB, Aro EM, Lehto K. Dynamics of the VIGS-mediated chimeric silencing of the *Nicotiana benthamiana* ChlH gene and of the tobacco mosaic virus vector. *Molecular Plant Microbe Interactions*. 2003; 16:99-106.
34. Hiriart JB, Lehto K, Tyystjärvi E, Junttila T, Aro EM. Suppression of a key gene involved in chlorophyll biosynthesis by means of virus-inducing gene silencing. *Plant Molecular Biology*. 2002; 50:213-24.
35. Park SH, Choi H, Kim S, Cho WK, Kim KH. Development of Virus-Induced Gene Expression and Silencing Vector Derived from Grapevine Algerian Latent Virus. *Plant Pathology Journal*. 2016; 32:371-6.
36. Pignatta D, Kumar P, Turina M, Dandekar A, Falk BW. Quantitative analysis of efficient endogenous gene silencing

in *Nicotiana benthamiana* plants using tomato bushy stunt virus vectors that retain the capsid protein gene. Molecular Plant Microbe Interactions. 2007; 20:609-18.

37. Folta KM, Davis TM. Strawberry genes and genomics. Critical Reviews in Plant Sciences. 2006; 25:399–415.

38. Marutani-Hert M, Bowman KD, McCollum GT, Mirkov E, Evens TJ, Niedz RP. A dark incubation period is important for *Agrobacterium*-mediated transformation of mature internode explants of sweet orange, grapefruit, citron, and a citrange rootstock. PloS One. 2012; 7:10.

39. Zhao S, Ruan Y, Sun H, Wang B. Highly efficient *Agrobacterium*-based transformation system for callus cells of the C3 halophyte *Suaeda salsa*. Acta Physiologiae Plantarum. 2008; 30:729-736.

40. Naylor LH. Reporter gene technology: The future looks bright. Biochemical Pharmacology. 1999; 58:749-757.

41. Stewart RN, Burk LG. Independence of tissues derived from apical layers in ontogeny of tobacco leaf and ovary. American Journal of Botany. 1970; 57:1010-1016

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2 **Figures**

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4 **Fig.1** Determination of culture parameters in germination seeds and the optimal kanamycin screening concentration. **a**5 Seed germination rates in different light culture condition and imbibing time. **b** Seed germination rates in different6 kanamycin concentrations after 15-d cultivation. **c** Phenotypes in different kanamycin concentrations after 15-d

7 cultivation. Each treatment was treated with 20 seeds. The error bars represent the standard error (n=3). Different letters

8 indicate statistically significant differences at $P < 0.05$ as determined by Duncan's test.

Fig. 2 Determination of factors influencing in *Agrobacterium*-mediated transformation of germinating seeds. **Infected seedlings** after 3-d culture in MS-selection medium. **b GFP expression in infected plants** of Fig.2a. **c and e** No GUS blue in the un-infected seedlings. **d and f** GUS blue in successfully-infected seedlings. **c and d** Infection seedlings after 3-d culture in MS-selection medium. **e and f** Infected seedlings after 2 weeks culture. **g** Effect of OD values on infection efficiency. **h** Effect of seed germination status on infection efficiency (1 represents the seed that has not yet exposed the radicle; 2 represents seed infected at the stage of radicle emergence; 3 represents that the seed has two cotyledons). **i** Effect of infection time on infection efficiency. Each treatment was treated with 20 seeds. The error bars represent the standard error (n=3). Different letters indicate statistically significant differences at $P < 0.05$ as determined by Duncan's test. Bars = 0.2 cm.

Fig. 3 Transformation flow chart of *FveCHLH*-RNAi T1 plants.

Fig. 4 Expression of *FveCHLH*-RNAi T1 plants red fluorescent protein. **a** and **b** 3-day-old transgenic plants (left) and 3-day-old wild-type plants (right). **c** and **d** 7-day-old transgenic plants (left) and 7-day-old wild-type plants (right). **e** and **f** 3-day-old infected plants roots. **g** and **h** 3-day-old wild-type plants roots. **i** and **j** 3-day-old transgenic plants stolon. **k** and **l** 3-day-old wild-type plants stolon.

Fig. 5 Phenotype of the *FveCHLH*-RNAi plants in strawberry. **a** Loss of green color in the *FveCHLH*-RNAi plants. **b** The 400-bp Kana products were detected in five *FveCHLH* -RNAi plants (lane 2-6) but not detected in the wild-type plants (lane 1) . **c** The 964-bp DsRed products were detected in five *FveCHLH* -RNAi plants (lane 2-6) but not detected in the wild-type plants (lane 1). **d** *FveCHLH* gene expression of transgenic plants and wild type plants. **e** Proportion of infected seedlings showing phenotype 1 and 2 plants to total infected plants. These data are derived from 20 seedlings per replicate, three replicates. The error bars represent the standard error (n=3). Different letters indicate statistically significant differences at $P < 0.05$ as determined by Duncan's test. Bars = 0.5 cm.

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2 **Fig. 6** Isolation and identification of T2-resistant seedlings. **a** The phenotype of T2-sensitive seedlings for 4 d and 10 d
3 under the irrigation of 75 mg/L kanamycin nutrient solution (left); the phenotype of T2-resistant seedlings for 4 d and 10
4 d under the irrigation of 75 mg/L kanamycin nutrient solution (right). **b** DsRed gene (left) and Kanamycin resistance
5 gene (right) of T2-resistant seedlings of *FveCHLH*-RNAi plants. Line1 is T2-sensitive seedlings, line2 is positive control,
6 and line 3-5 is T2-resistant seedlings.

Figures

Figure 1

Determination of culture parameters in germination seeds and the optimal kanamycin screening concentration. a Seed germination rates in different light culture condition and imbibing time. b Seed germination rates in different kanamycin concentrations after 15-d cultivation. C Phenotypes in different kanamycin concentrations after 15-d cultivation. Each treatment was treated with 20 seeds. The error bars represent the standard error (n=3). Different letters indicate statistically significant differences at $P < 0.05$ as determined by Duncan's test.

Figure 2

Determination of factors influencing Agrobacterium-mediated transformation of germinating seeds. Infected seedlings after 3-d culture in MS-selection medium. b GFP expression in infected plants of Fig. 2a. c and e No GUS blue in the un-infected seedlings. d and f GUS blue in successfully-infected seedlings. c and d Infected seedlings after 3-d culture in MS-selection medium. e and f Infected seedlings after 2 weeks culture. g Effect of OD values on infection efficiency. h Effect of seed germination status on infection efficiency (1 represents the seed that has not yet exposed the radicle; 2 represents seed infected at the stage of radicle emergence; 3 represents that the seed has two cotyledons). i Effect of infection time on infection efficiency. Each treatment was treated with 20 seeds. The error bars represent the

standard error (n=3). Different letters indicate statistically significant differences at $P < 0.05$ as determined by Duncan's test. Bars = 0.2 cm.

Figure 3

Transformation flow chart of FveCHLH-RNAi T1 plants.

Figure 4

Expression of FveCHLH-RNAi T1 plants red fluorescent protein. a and b 3-day-old transgenic plants (left) and 3-day-old wild-type plants (right). c and d 7-day-old transgenic plants (left) and 7-day-old wild-type plants (right). e and f 3-day-old infected plants roots. g and h 3-day-old wild-type plants roots. i and j 3-day-old transgenic plants stolon. k and l 3-day-old wild-type plants stolon.

Figure 5

Phenotype of the *FveCHLH*-RNAi plants in strawberry. a Loss of green color in the *FveCHLH*-RNAi plants. b The 400-bp Kana products were detected in five *FveCHLH*-RNAi plants (lane2-6) but not detected in the wild-type plants (lane 1) . c The 964-bp DsRed products were detected in five *FveCHLH*-RNAi plants (lane2-6) but not detected in the wild-type plants (lane1). d *FveCHLH* gene expression of transgenic plants and wild type plants. e Proportion of infected seedlings showing phenotype 1 and 2 plants to total infected plants. These data are derived from 20 seedlings per replicate, three replicates. The error bars represent the standard error (n=3). Different letters indicate statistically significant differences at $P < 0.05$ as determined by Duncan's test. Bars = 0.5 cm.

Figure 6

Isolation and identification of T2-resistant seedlings. a The phenotype of T2-sensitive seedlings for 4 d and 10 d under the irrigation of 75 mg/L kanamycin nutrient solution (left); the phenotype of T2-resistant seedlings for 4 d and 10 d under the irrigation of 75 mg/L kanamycin nutrient solution (right). b DsRed gene (left) and Kanamycin resistance gene (right) of T2-resistant seedlings of FveCHLH-RNAi plants. Line 1 is T2-sensitive seedlings, line 2 is positive control, and line 3-5 is T2-resistant seedlings.