An effector-reporter system to study cellular cellular signal transduction in strawberry fruit (Fragaria ananassa)

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Abstract

Background:
An effector-reporter system is a powerful tool used to study cellular signal transduction; however, this technique has been traditionally adopted in protoplasts. A similar system to study cellular signal transduction in fruits has not yet been established. In this paper, we aim to establish an effector-reporter system for strawberry fruit, a model non-climacteric fruit.

Results: We compared four different reporters, including GUS, GFP, FLuc, and RLuc, as well as combination of two pairs of reporters, including GUS/GFP and RLuc/FLuc, by statistically analyzing the pattern of reporter activities exhibited among individual fruits. Among the reporters examined, GUS was the best choice. Further sampling indicated that a minimum of five mixed specimens with five fruits each is necessary for an assay. With the so-established system, FaCHS promoter demonstrated a sensitive response to FaMYB10 effector as well as to the external and internal cues implicated in the regulation of fruit ripening.

Conclusion: A step-by-step protocol was established to study cellular signal transduction in strawberry fruits. This finding will contribute to the molecular investigation of fruit ripening.

Keywords: Effector and Reporter, FaCHS, FaMYB10, Cellular signal transduction, Strawberry
Background

Fruit ripening is a complex process, which involves dramatic changes in various physiological and biochemical processes, such as the color, sugar, acid, aroma, and texture-associated metabolisms [1-5]. Based on the physiologically ripening characteristics, fleshy fruits can be categorized into two major groups, climacteric and non-climacteric [3,4]. Climacteric fruits exhibit a burst of respiration and ethylene production at the onset of ripening, whereas non-climacteric fruits do not. Tomato has been used as a model climacteric fruit while strawberry emerged as a model non-climacteric fruit [6-9]. The mechanisms underlying the regulation of fleshy fruit development and ripening have been a major focus of agricultural research. Both internal (e.g., phytohormones) and external (e.g., environmental factors) factors regulate fruit development and ripening. Studies have shown that the phytohormone abscisic acid (ABA) regulates fruit ripening in strawberry. Besides phytohormones, the role of various environmental stresses, such as light, heat, and osmotic stresses, in regulating strawberry fruit development and ripening has also been demonstrated [10-15].

Molecular studies have identified genes implicated in the regulation of fruit development and ripening; these mainly include structural genes, such as chalcone synthase (CHS), chalcone isomerase (CHI), dihydroflavonol 4-reductase (DFR), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), anthocyanidin synthase (ANS), UDP glucose-flavonoid 3-O-glucosyl transferase (UFGT), sucrose phosphate synthetase, and sucrose synthetase (SS), directly involved in physiological and biochemical metabolism. In recent years, researchers have focused on transcription factors that regulate fruit development and ripening. Many transcription factors (TFs), such as MYBs [16], NACs [17], HLH [18], and MADSs [19] family transcription factors, have been implicated in fruit development and ripening. Studies have demonstrated that MYB10 regulates anthocyanin biosynthesis in various plant species and organs via anthocyanin biosynthesis genes, such as CHS, F3H, and UFGT [20,21]. However, less is known about the specific targets of TFs and the response of
structural genes to the internal/external cues implicated in fruit development and ripening. This requires detailed study of the cellular signal transduction pathways.

Researchers have extensively studied the cellular signal transduction pathways in vegetative organs; however, study in fleshy fruits faces many challenges. A major limiting factor in the study of cellular signaling in fruits is the shortage of related techniques and systems. Effector-reporter analysis technique has been used as a powerful tool to study the cellular signal transduction pathways underlying fruit development and ripening [22,23]. This technique is based on transient gene expression in plant cells [24-27]. Since the development of protoplast transient expression [28], cellular signal transduction has been extensively studied with the aid of effector/reporter analysis in protoplasts. Earlier studies focused on responsive promoters, such as ABA-responsive Em promoter in rice protoplasts [29], GA-responsive amylase gene promoter in oat and barley aleurone protoplasts [30-32], and abiotic and biotic stress-responsive chalcone synthase (CHS) promoter [33-35]. In recent years, protoplast-based effector/reporter analysis has been increasingly employed to unravel different signaling cascades. Two-component signaling circuit [36] and oxidative stress-activated MAPK cascade in Arabidopsis protoplast; auxin-triggered MAPK signaling cascade [37] and stress-induced CDPKs cascade in Maize protoplast [38], and more recently, [27] in vitro reconstitution of abscisic acid signaling in Arabidopsis. Protoplast-based effector/reporter systems have facilitated rapid discovery of cellular signal transduction pathways in various biological processes; however, limitations exist [22]. First of all, it is not possible to isolate active protoplasts from each plant cell type or under all growth conditions. In addition, protoplasts cannot exhibit the biological processes of different cell types [22,23]. So far, the protoplast-based effector/reporter system has been mainly used in maize and Arabidopsis.

Besides protoplast-based transient gene expression, transient expression has been increasingly reported in the fruits of many plant species, among which strawberry may be the one that was most extensively studied [6,21,39-41]. We previously
conducted a comprehensive study on transient gene expression to optimize and standardize this technique in strawberry fruits [39]. Based on the optimized conditions, the present study aims to develop an easy and reliable effector-reporter system to study cellular signal transduction in strawberry fruits. However, unlike that in protoplasts, the level of transient expression varies among fruits (Referred as sampling variability; SV). To overcome this problem, the present study investigates the characteristics of different reporters as regard to their variabilities among individual fruits. Further, to reduce the workload and cost involved, we also evaluated the minimum sample size with an acceptable margin of error. A step-by-step protocol using CHS promoter-driven GUS as the reporter and MYB10 transcription factor as the effector is provided.

**Results**

**Pattern of sample variability**

The transient gene expression, expressed as relative fluorescent unit (RLU), varied largely among the individual fruits (referred as sample variability; SV). To characterize this variability in relation to the employment of different reporters, we examined four commonly used reporters, including GUS, GFP, FLuc, and RLuc. Fig. 1a shows a general pattern of variability as indicated by GFP fluorescence image in a sample size 24. While strong GFP fluorescence could be clearly observed in some fruits, such as fruit No. 3, 14 and No.19, the GFP fluorescence might be much weak in others, such as fruit No.4, 5,7 and No. 21, and even hardly observed in some fruits, such as No. 13. The specific pattern of the SV of different reporters is shown in Fig.s b to e (vertical coordinate denotes the number of specimens and horizontal coordinate denotes their values located in the corresponding range). All the four reporters exhibited high variability (0–1500 for GUS, Fig. 1b; 0–1500 for GFP, Fig. 1c; 0–150 for FLuc, Fig. 1d; and 0–800 for RLuc, Fig. 1e). This large SV implies that a relatively large number of fruits would be required to satisfy the need for
the significance test of difference among different treatments in a study. Coefficient of
to assess the pattern of SV [42,43]. In the present study, we
determined the percentage of the two parameters (percentage of CV, PCV and
percentage of SEM, PSEM) to assess the variability. Specifically, according to the
formulas: 
\[
SD = \sqrt{\frac{1}{N-1} \sum_{i=1}^{N} (x_i - \bar{x})^2},
\]
where SD is the standard deviation, \(x_i\) is one sample
value; \(\bar{x}\) is the sample mean, and \(N\) is the sample size; and
\[
SE = \frac{SD}{\bar{x}},
\]
where SE is the standard error, SD is the standard deviation, and \(n\) is the number of observations of
the sample, PCV and PSEM were respectively determined as follows: PCV=SD/ \(\bar{x}\)×
100%, and PSEM=SE/ \(\bar{x}\)×100%. As PSEM might be caused by biological or technical
replicates, to understand the precise contribution of biological and technical replicates
to the PSEM value, we further examined the SV due to technical replicates.
Variability due to the technical replicates was smaller than that due to the biological
replicates. Meanwhile, the PSEM for GFP was much larger than that for other
reporters (Table 1). As shown in Table 1, the PSEM value was 13.22%, 56.45%,
14.17%, and 7.44% for GUS, GFP, FLuc, and RLuc, respectively, indicating that
GUS and RLUC could be priority selections for the technical development of cellular
signal transduction.

Sampling analysis in relation to the sample size
Because PSEM is determined by both sample size ‘N’ and SE as described above, to
determine the sample size for a given PSEM, we evaluated the relationship between
sample size and PSEM with the GUS reporter (better choice).

We first conducted GUS analysis using a large sample size (80 individual fruits), and
by random sampling, we then examined the change in PSEM with increase in sample
size. As shown in Fig. 2, compared to the sample size 30 (Fig. 1a), the data
distribution of the sample size 80 looks more like the normal distribution. The
calculated PSEM was 7.41%, 19.42%, and 11.42% for samples size 5, 10, and 30, respectively (Table 2). Supposing that a PSEM below 15% can be acceptable [42-47], the sample size should be more than 30 to satisfy the need of the technical development. We further used mixed specimens with either three or six individual fruits to reduce the PSEM. For the mixed specimen with three fruits, we evaluated PSEM for sample size 5 and 10. The obtained PSEM value was 14.60% for sample size 5 and 10.84% for sample size 10. For the mixed specimen with six fruits, we evaluated PSEM for sample size 5, and the obtained PSEM value was 10.33% (Table 2), which indicates that by using the mixed specimen, a sample size as low as 5 was enough to satisfy the test of the significance of difference. Based on these findings, we used mixed specimens with a sample size 5 for further experiments to study cellular signal transduction in strawberry fruits.

Comparison between single and double reporters

To characterize the effect of a ratio approach on sampling SV, we first determined the ratios of different reporter combination for about 30 individual fruits (GUS to GFP and RLuc to FLuc), and by random sampling, we evaluated the change in PSEM with a change in the sample size. Fig. 3 shows the pattern of variability for the different combinations. Compared with the single reporters (Fig. 1), data with both reporters at different ratios was closer to the normal distribution for RLuc to FLuc, but not for GUS to GFP. As shown in Table 3, the ratio PSEM for RLuc to FLuc was 7.71%, which was smaller than that of the single reporters with the same sample size (10.6573% for RLuc and 14.17% for FLuc, sample size 30; Table 1). A PSEM value of 0.7393% (Table 3) was obtained with sample size as low as 10 for RLuc and FLuc combination. This was much smaller than the PSEM value for the sample size 30 with single reporters (Table 1). These results suggested that use of ten individual fruits could satisfy the need for the study of cellular signal transduction in strawberry fruits.
Application of the so-established technique to study cellular signal transduction
The ratio method with low SV proved relatively better; however, this approach involves relatively high technical requirements and cost. Therefore, to test the application of the so-established technique, we evaluated GUS reporter in sample size 5, meanwhile, with a mixed 5 individual fruits as an independent sample (i.e. specimen), as described above. With GUS driven by FaCHS promoter as the reporter and FaMYB10 as the effector, we investigated the response of the GUS reporter to FaMYB10 and to ABA and mannitol treatment. The reporter activity significantly increased with either ABA or mannitol treatment (Fig. 4). Co-transformation of FaMYB10 resulted in an increase in reporter activity for 7.33 folds in comparison with control without FaMYB10. The related PSEM value varied from 7.79% to 12.86% among the different treatments. The detailed statistical parameters of this experiment are shown in Table 4. Collectively, these results suggest that the so-established technical system can be easily applied to the study on cellular cellular signal transduction in strawberry fruits. A step-by-step protocol of this technical system is provided in the ‘material and method’.

Discussion
Transient gene expression is a powerful tool used to study cellular signal transduction in plant cells [22]; however, there are limitations for its use in fleshy fruits. In this study, we aim to establish transient gene expression in strawberry fruits that will provide the foundation to develop such a technical system. Transient gene expression in strawberry fruits is influenced by various factors, such as gene delivery method, fruit developmental stage, and environmental factors [39]. The influence of these factors leads to high variability in the level of gene expression (i.e. SV). In the present study, we examined the pattern of SV of different reporters. In the case of GUS reporter, the difference between the minimum and maximum values of gene expression was more than 50 folds (Fig. 1), which made it difficult to study cellular
signal transduction in strawberry fruits.

Study of cellular signal transduction essentially relies on a significance test of difference among samples (between samples with and without stimulation). In a protoplast-based system, a sample size of 3–5 (biological replicates) is usually used. This provides an acceptable PSEM for the significance test of difference (<15%) [24-26]. In the present study, we set an acceptable PSEM about 10% and assessed the number of fruits required for the significance test of difference. We propose that at least 30 individual fruits are required to attain a significant difference (Table 1). This implies the need of several hundreds of fruits in a study with various treatments. Such large number of fruits makes a study quite difficult to be performed. Therefore, we evaluated the minimum sample size with which a system can be established to study cellular signal transduction in strawberry fruits.

The number of fruits required to develop a system is essentially determined by the statistical parameters, SD and SE, which are associated with SV. Due to the difference in the absolute value of SE among samples, we adopted a relative SM (Percentage of SM to the Mean; designated as PSEM) in the present study. To investigate the relationship between PSEM and sample size, we adopted random sampling strategy based on a relatively large sample group that was practically used to measure the reporter activity in each individual fruit. In random sampling, a small sample (5–30 specimens) was randomly chosen from a large sample group (50–80 specimens). Theoretically, result of random sampling is identical with that of the practically measured sample. Transient gene expression in strawberry fruits was manipulated via three major steps, including gene delivery, material grinding, and reporter activity analysis. To reduce the workload and cost involved in material grinding, we used mixed specimens. Non-mixed specimens with 30 individual fruits demonstrated a PSEM of 11.423% (Table 2), whereas mixed specimens (each specimen consisted of six fruits and a sample size 5 was adopted) with only 5 specimens produced a PSEM value of 10.333% (Table 2). Although in both cases the total number of fruits was similar, the use of mixed specimens reduced workload and cost by five-sixth.
The ratio method has been traditionally thought to be better than the single reporter method. In the present study, we evaluated the ratio of two pairs of reporters, GUS to GFP and RLuc to FLuc. Use of RLuc and FLuc resulted in a lower PSEM (7.71%) compared with the four signal reporters (13.22% for GUS, 56.45% for GFP, 14.17% for FLuc, 10.65% for RLuc; sample size about 30, Table 1). Surprisingly, combination of GUS and GFP did not result in lower PSEM (11.97%). Green fluorescence (GFP), examined with TBS-380 Fluorometer (Turner Biosystems), appeared to be unstable, which was reflected by high PSEM (56.45%; Table 1). This finding implies that the use of two reporters in combination must be based on an accurate determination of both reporters; an error derived from any single reporter will result in a larger error in their ratio.

Use of double reporter method is better due to low PSEM; however, there are disadvantages in its practical use. In addition to the cost and equipments involved, one major disadvantage is the difficulty involved in vector construction. In protoplasts, transient gene expression can be performed by co-transformation of different vectors, with the effector gene and the reporter gene cloned separately into two different vectors. By contrast, co-transformation of different vectors is impossible due to the large SV in strawberry fruits. To study cellular signal transduction, several genes need be cloned into a single vector. In case of a single reporter, it is easy to clone an effector gene into the reporter vector. In the ratio approach, at least three genes (two reporters plus at least one effector) need to be cloned into the same vector, and this makes vector construction difficult (due to limited cleavage sites). These disadvantages of double reporters can be avoided by the use of a single reporter, which can produce a significance test of difference with an acceptable sample size. Use of mixed specimens with six individual fruits and a sample size as low as 5 could result in a satisfied PSEM (e.g.10%). We therefore propose the use of GUS reporter alone.

FaCHS, gene encoding chalcone synthase, controls color development [48] and has been a gene of interest. Transcriptional regulation of CHS was achieved by a complex
of R2R3-MYB and basic helix–loop–helix (bHLH) transcription factors (TFs) [49]. In strawberry, FaMYB10 regulates FaCHS [40, 50]. Considering this regulatory role, we evaluated an effector-reporter system with FaMYB10 as the effector and GUS driven by FaCHS promoter as the reporter to study cellular signal transduction in strawberry fruits. The highly sensitive response of GUS reporter to FaMYB10 effector as well as ABA and mannitol stimuli indicates the reliability and practicability of the so-established system. The findings of this study will help explore fruit development and ripening in strawberry.

**Conclusion**

With GUS gene as reporter in combination with mixed specimen, an effector/reporter analytical technique was established. This technique can be applied to and easily performed for the study on cellular signal transduction in strawberry fruits.

**Materials and methods**

**Plant materials**

Octoploid strawberry plants (Fragaria × ananassa Duch., Benihoppe) were grown in a greenhouse at 18–28 °C and 75%–90% humidity under 8 h/16 h dark/light cycle. Strawberry fruits were classified into six developmental stages as follows: small green fruit (SG), mid-sized green fruit (MG), large green fruit (LG), white fruit (W), turning fruit (T), and fully reddened fruit (FR). Fruits at LG to W stages were used for the study.

**Reagent and buffers**

Agar, casein tryptone, and yeast extract were derived from OXOID (UK). NaCl, MgCl₂, EDTA, glycerin, Triton X-100, DTT, Tris-HCl (pH 8.0), 4-MUG, ethanol, NaH₂PO₄·2H₂O, and Na₂HPO₄·2H₂O were purchased from SIGMA (USA). Antibiotics including Amp, Kan, Sp, and Rifampicin were purchased from SIGMA (USA). TransStart® FastPfu PCR SuperMix (high-fidelity enzyme) was purchased from TransGen Biotech (China). Restriction endonuclease and T4 DNA ligase were
purchased from NEB (USA). DH5a Large intestine competence and EHA105
Agricultural rod competence stains were purchased from TransGen Biotech Company. 
GV3101 (pSoup) chemically competent cells were purchased from ZOMANBIO (China). TransDetect Double-Luciferase Reporter Assay Kit was purchased from TransGen Biotech (China).

The main buffer was prepared from four reagents (Luciferase Reaction Buffer; Luciferase Reaction Substrate (Lyophilized); Luciferase Reaction Buffer II; and Luciferase Reaction Substrate II (50X)) in the LUC test kit; Buffer I: Luciferase Reaction Reagent Luciferase Reaction Substrate was completely dissolved in Luciferase Reaction Buffer (5 mL Buffer + 1 vial Substrate) and was stored in the dark; Buffer II: Luciferase Reaction Reagent II Luciferase Reaction Substrate II and Luciferase Reaction Buffer II were mixed at a ratio of 1:50, and the reagent was stored in the dark after packaging.

**Vector construction**

**Construction of single reporters**

Vector pCAMBIA1301, which carries GUS reporter gene driven by cauliflower mosaic virus 35s promoter, was adopted to construct GUS reporter. This vector was purchased from YouBio (China).

Vector pH7WG2D.1, which carries eGFP reporter gene driven by Agrobacterium rhizogenes plasmid proID promoter, was adopted to construct GFP reporter. This vector was purchased from BioVector NTCC Inc. Vector pGreenII 0800-LUC, which carries both FLuc and RLuc driven by mosaic virus 35s promoter, was adopted to construct the single reporter (FLuc or RLuc). This vector was purchased from YouBio (China).

**Construction of double reporters**

Vector pH7WG2D.1 that carries eGFP driven by proID promoter was used as a backbone to construct the GUS/GFP double reporter. GUS gene was first amplified from pCAMBIA1301 using sense (5’-AAAAAGCAGGCTATGGTAGATCTGAGGTT-3’) and antisense
(5′-AGAAAGCTGGGTTTCACACGTGGTGGT-3′) primers. The PCR product was
introduced into the lethal region of \textit{ccdB} gene of pH7WG2D.1 vector by Gateway
technology to obtain GUS/GFP double reporter designated as p35s::GUS-proID::GFP
vector. pGreenII 0800-LUC vector (purchased from YouBio) that carries \textit{RLUC} gene
driven by the mosaic virus 35s promoter as well as \textit{FLUC} gene with multiple cloning
sites was used as a backbone to construct the RLuc/FLuc double reporter. Cauliflower
mosaic virus 35s promoter was digested from pCAMBIA1301 and cloned between
\textit{HindIII} and \textit{NcoI} sites of pGreenII 0800-LUC vector to obtain RLuc/FLuc double
reporter designated as p35s::RLUC-p35s::FLUC vector.

\textbf{Construction of effector and reporter}

Vector pCAMBIA1301 that carries \textit{GUS} gene and a multiple cloning site in front of
the \textit{GUS} gene was used as a backbone to construct \textit{CHS} reporter. \textit{CHS} promoter was
amplified from the genome of \textit{Fragaria × ananassa} Duch. (Benihoppe) using sense
(5′-AAGCTTTTATGCTGATTTGATTATGTGT-3′) and antisense (5′-
CCATGGTTTGATTTCTCAGAGAAGTGTC-3′) primers. The PCR products were
cloned into \textit{HindIII} and \textit{NcoI} sites of pCAMBIA1301 vector to obtain \textit{CHS} reporter
designated as pCHS::GUS vector.

pCHS::GUS vector was used as a backbone to construct \textit{FaMYB10} effector. The
full-length coding sequence of \textit{FaMYB10} was amplified from the cDNA of
\textit{Fragaria × ananassa} Duch. (Benihoppe) using sense
(5′-GGATCCATGGAGGGTTATTTCGGTGT-3′) and antisense
(5′-GAGCTCAATTTTCTAATTGTAGAGTCTGTGG-3′) primers. The PCR products
were cloned into pBI121 with \textit{BamHI} and \textit{SacI} to fuse with 35S promoter. The
expression cassette was amplified using sense
(5′-GTCGACTGAGACTTTTCAACAAAGG-3′) and antisense (5′-
CCCCGGGGATCTAGTAAACATAGATGA-3′) primers, and the PCR product was
cloned into \textit{SalI} and \textit{SmaI} sites of pCHS::GUS vector to obtain the effector/reporter
construct designated as p35s::MYB10-pCHS::GUS.
Sampling strategy

The relationship between PSEM and sample size was established by both practical measurement and random sampling. We first measured the reporter activity of each fruit using a relatively large sample size (e.g., sample 81 for non-mixed specimen, sample size 50 for mixed specimen of three fruits and sample size 49 for mixed specimen of six fruits for the GUS reporter). We evaluated PSEM for sample size 5, 10, and 30 in non-mixed specimens, for sample size 5 and 10 in mixed specimens of three fruits, and for sample size 5 in mixed specimens of six fruits. Random sampling was performed 27–31 times to ensure a sampling PSEM below 15% using Microsoft Excel ‘RAND function’.

Statistical analysis

Biological or technical replicates were set from 5 to 80 according to different aims of the experiment as described above. Statistical parameters as well as the analysis of significance of difference were conducted using SigmaPlot (Systat Software, Inc.) for Windows.

GFP fluorescence image analysis

Analysis of GFP fluorescence image was conducted by an independently developed apparatus with an argon laser, a 488-nm excitation filter, and a 507-nm emission filter for analyzing and imaging objects up to 100 cm² in surface area.

GUS and GFP analyses

Approximately 10 µL of the protein extract was mixed with 100 µL of reaction buffer (10mM Tris-HCl (pH 8.0), 2mM MgCl₂, 1mM 4-MUG) in a 2 mL vial and was incubated at 37 °C for 60 min. To this, 900 µL of 0.2 M Na₂CO₃ was added to stop the reaction. This reaction mixture was transferred to a colorimetric cup adapted to a TBS-380 mini fluorometer with two excitation modes (UV; 365–395 nm and blue; 465–485 nm), which produce two emission spectra (440–470 nm for UV and 515–575
nm for blue). GUS activity was measured using excitation set to UV mode and emission at 365 nm. This activity was expressed as relative fluorescence unit (RLU). For GFP measurement, the protein extract was transferred into the colorimetric cup and the excitation was set to blue and emission to 465 nm.

**RLuc (renilla luciferase) and FLuc (firefly luciferase) analyses**

RLuc and FLuc were analyzed using the Double-Luciferase Reporter Assay Kit following the manufacturer’s protocol. Luciferase Reaction Reagent I (100 μL) was equilibrated to room temperature and was thoroughly mixed with 20 μL of the protein extract. The reaction mixture was transferred into a 1.5 mL vial, and was measured immediately with TD20-20 Luminometer. The FLuc activity was expressed as relative fluorescence unit (RLU). Further, 100 μL of Luciferase Reaction Reagent II was added to the reaction mixture, and was measured immediately with TD20-20 Luminometer. The RLuc activity was expressed as relative fluorescence unit (RLU).

**Step-by-step protocol to study cellular signal transduction**

**Step 1. Preparation of fruits**

We used octoploid strawberry, *Fragaria × ananassa* Duch. (Benihoppe) in this study. Plants were grown in a greenhouse and fruits were divided into six developmental stages as described above. Fruits at large green to white stages were chosen for the study, and fruits at a uniform stage were used for each experiment. The selection of fruits was based on achene color (breaking stage). Number of fruits per experiment was decided as described below. Fruits were harvested by cutting from the petiole and were immediately brought to the laboratory for infection.

**Step 2. Sampling design**

Each fruit was individually infected, and five infected fruits were pooled for grinding to obtain the mixed specimens. At least five biological replicates were maintained, and so, for each treatment 25 fruits were maintained. For control, fruits were infected with an empty vector (no effector/reporter).
**Step 3. Preparation of the bacterium carrying the target vector**

(1) Bacterial culture

Liquid culture medium and antibiotic was used based on the target vector. Kanamycin was used for pCAMBIA1301, pCHS::GUS, and p35s::RLUC-p35s::FLUC and streptomycin for pH7WG2D.1. The agrobacterium strain EHA105 carrying the target vector was first cultured in 20 mL of the medium at 200 rpm and 28 °C until OD600 reached 0.6. This culture was further grown under the same conditions until OD600 reached 0.6 in a large volume, which was determined by the number of fruits required for the infection. Normally, each individual fruit needs 1–3 mL.

(2) Strain activation

The bacterium was collected by centrifugation at 4000 r for 10 min and resuspended in activation medium (10 mM MgCl₂, 200 μM acetosyringone, and 10 mM MES, pH 5.6) to obtain OD600 of 0.5–0.8. The culture after 3–4 h incubation at 28 °C was used for infection. The activation medium cannot be stored, fruits should be prepared in advance.

(3) Fruit infection

Agrobacterium suspension was injected into the fruits using 1 mL syringe. The needle tip was inserted into the fruit center from either the top or the bottom, and the suspension was slowly and evenly injected into the fruits until the whole fruit got fully infiltrated. If 1 mL was not enough to fully infiltrate, fruits were injected more than once. The infiltrated fruits were incubated in dark at 20–25 °C temperature and 90% humidity for 4 days. These infected fruits were either used immediately for the different experiments or frozen in liquid nitrogen and kept at -80 °C for further use.

**Step 4. Fruit treatment to study cellular signal transduction**

To study the response of the reporter to different stimuli, fruits were treated with the corresponding stimuli. In the present study, each fruit was cut into four equal parts and were incubated with 100 or 700 mM mannitol for 5 h at room temperature. If the treatment is necessary in some researches, the infected fruits can be treated as described in step 3.
Step 5. Protein extraction for reporter analysis

Fruits were frozen in liquid nitrogen. Five fruits were mixed and ground into a fine powder with a mortar and pestle. For each assay, 100 mg of the fine powder was weighed and added into a 2.0 mL vial containing 500 µL of extraction buffer (1mM EDTA, 10% glycerol, 0.5% Triton X-100, 1mM DTT, 100mM Na2HPO4-NaH2PO4; pH 6.8). This mixture was incubated in a shaking incubator at 4 °C for 1 h and centrifuged for 10 min at 13000 rpm and 4 °C. The supernatant was used to analyze the reporter activity.

Authors’ contributions

JY and WJ designed the research. BZ, TL, WW and ZD performed the experiment. JL, ZX and KJ participated in part experiments. XY and BL provided key technique mentoring. WJ prepared the manuscript.

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Competing interests

The authors declare that they have no competing interests

Availability of data and materials

All data generated or analysed during this study are included in this published article. Materials are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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**Figure legends**
Fig. 1  Data distribution of different reporter, showing the variability of each reporter.

a GFP fluorescence images, showing the variability in the level of GFP protein among individual fruits in sample size 24. The number above each image denotes a random order in the sample. b to e Quantitative determination of the activity of different reporters. For each reporter, the data distribution was shown based on a specific sample group consisted of 30 individual fruits. Fruits at the LG stage were fully injected with the agrobacterium carrying the different single reporter construct, and on the fourth after the injection, the reporter activity was measured. The vertical coordinate denotes the number of fruits with their reporter activities located within each corresponding region shown in horizontal coordinate. b, GUS; c, GFP; d, FLuc; d, RLuc; e, GFP fluorescence image. More detailed statistic parameters are shown in table 1.
Fig. 2 Data distribution of the GUS reporter, showing the sampling effect on its data variability.

Fruits at the LG stage were fully injected with the agrobacterium carrying the *GUS* reporter construct, and on the fourth after the injection, the reporter activity was measured. The vertical coordinate denotes the number of fruits with their reporter activities located within each corresponding region shown in horizontal coordinate. a, sample group consisted of 80 specimens and each specimen represents an individual fruit; b, sample group consisted of 50 specimens and each specimen represents a mixture of 3 individual fruits; c, sample group consisted of 50 specimens and each specimen represents a mixture of 6 individual fruits. More detailed statistic parameters are shown in table 2.
Fig. 3  Data distribution, showing the variability in ratio of double reporters.

Fruits at the LG stage were fully injected with the agrobacterium a carrying double reporter construct, and on the fourth after the injection, the reporter activity was measured and the ratio of each individual fruit was calculated. The vertical coordinate denotes the number of fruits with their ratio values located within each corresponding region shown in horizontal coordinate. a, ratio of GUS to GFP with the sample group consisted of 30 individual fruits; b, ratio of RLUC to FLUC with the sample group consisted of 33 individual fruits. More detailed statistic parameters are shown in table 3.
Fig. 4. Analysis of signal transduction by using the effector/reporter technical system in strawberry fruits.

*FaMYB10* driven by 35S promoter (i.e. effector) and *GUS* driven by the *FaCHS* promoter (i.e. reporter) were cloned into a same vector. Fruits at the LG stage were fully injected with the agrobacterium a carrying ‘effector/reporter’ construct, or single reporter of *GUS* driven by the *FaCHS* promoter. On the fourth day after the injection, fruits were treated with 100 ABA or 700 mM mannitol for 5 hours and then measured for the GUS activity. Each treatment included five specimens and each specimen was a mixture of 5 individual fruits. Different letters denote statistical significances with $P < 0.05$ and the same letter shows no significant differences among groups ($P \geq 0.05$).
