Plant Methods

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Overexpression of *Vitis GRF4-GIF1* improves regeneration efficiency in diploid *Fragaria vesca* Hawaii 4

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Abstract

Background Plant breeding played a very important role in transforming strawberries from being a niche crop with a small geographical footprint into an economically important crop grown across the planet. But even modern marker assisted breeding takes a considerable amount of time, over multiple plant generations, to produce a plant with desirable traits. As a quicker alternative, plants with desirable traits can be raised through tissue culture by doing precise genetic manipulations. Overexpression of morphogenic regulators previously known for meristem development, the transcription factors Growth-Regulating Factors (GRFs) and the GRF-Interacting Factors (GIFs), provided an efficient strategy for easier regeneration and transformation in multiple crops.

Results We present here a comprehensive protocol for the diploid strawberry *Fragaria vesca* Hawaii 4 (strawberry) regeneration and transformation under control condition as compared to ectopic expression of different *GRF4-GIF1* chimeras from different plant species. We report that ectopic expression of *Vitis vinifera VvGRF4-GIF1* provides significantly higher regeneration efficiency during re-transformation over wild-type plants. On the other hand, deregulated expression of *miRNA* resistant version of *VvGRF4-GIF1* or *Triticum aestivum* (wheat) *TaGRF4-GIF1* resulted in abnormalities. Transcriptomic analysis between the different chimeric *GRF4-GIF1* lines indicate that differential expression of *FvExpansin* might be responsible for the observed pleiotropic effects. Similarly, cytokinin dehydrogenase/oxygenase and cytokinin responsive response regulators also showed differential expression indicating GRF4-GIF1 pathway playing important role in controlling cytokinin homeostasis.

Conclusion Our data indicate that ectopic expression of *Vitis vinifera VvGRF4-GIF1* chimera can provide significant advantage over wild-type plants during strawberry regeneration without producing any pleiotropic effects seen for the *miRNA* resistant *VvGRF4-GIF1* or *TaGRF4-GIF1*.

Keywords Strawberry, Regeneration, Transformation, *GRF4-GIF1* chimera, Leaf development, Cytokinin

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Background

Somatic embryogenesis plays an essential role towards asexual propagation and regeneration of plants. Unlike organogenesis which requires a high cytokinin-to-auxin ratio [1, 2], somatic embryogenesis is mostly dependent on auxin [2–4]. In the last decade, concerted effort has been made to understand the molecular mechanisms that drive the transition of a vegetative cell into an embryogenic competent cell under the influence of auxin and cytokinin signalling. Understanding of the regeneration pathways leading to embryogenesis under the influence of phytohormones resulted in the development of in vitro techniques for tissue culture of several plant species [5] . The Rosaceae is one such family where sexual hybridization, asexual propagation, and genetic improvements have been pivotal for developing better varieties for quite some time [6]. It is a large and diverse family that includes several economically relevant food crops such as apple (*Malus*), plum, peach, almond, cherry (*Prunus*), pear (*Pyrus*), raspberry (*Rubus*), strawberry (*Fragaria*) and other species with economic value.

The cultivated strawberry, *Fragaria x ananassa*, is an octoploid species (2n=8x=56) derived from the hybridization between *F. chiloensis* and *F. virginiana* [7] . Although breeding and genetic engineering tools are available in *F. x ananassa* [8], the polyploid genome makes crop improvement in this species difficult. For that reason, the diploid woodland strawberry (*Fragaria vesca*) that holds close kinship to commercial strawberry is widely used as a genetic model [6]. *F. vesca* offers favourable attributes including a ~ 240 Mb reference genome (versus 157 Mb in *Arabidopsis thaliana*), short generation time, small plant size and a wide geographical distribution [7]. As a result, in the last few years, effective in vitro propagation, regeneration and transformation techniques have been developed for *F. vesca* to facilitate genetic engineering [9–11]. Despite the establishment of regeneration and transformation techniques in woodland strawberry, the potential of endogenous developmental regulators has only been recently investigated [12]. However, the potential of established heterologous developmental regulators in facilitating faster regeneration after re-transformation has not been explored.

Research in model organisms such as *A. thaliana* facilitated the identification of certain transcription factors that can integrate the signals leading to cellular reprogramming resulting in embryogenesis or meristematic fate [13]. These transcription factors are called developmental regulators as they coordinate spatial cellular distribution resulting in organ formation. For example, Somatic Embryogenesis Receptor Kinase (*SERK*), Leafy Cotyledon 1 (*LEC1*), Leafy Cotyledon 2 (*LEC2*), NiR, Baby Boom (*BBM*), Wound Induced Dedifferentiation 1 (*WIND1*), Wuschel (*WUS*) and *WOX5* have all been identified to be essential during somatic development [14–20]. Ectopic expression of these genes not only allowed regeneration of transformation-recalcitrant plant species but also increased regeneration efficiencies. However, overexpression of developmental genes like *WUS* and *BBM* induced pleiotropic effects, including callus necrosis, compromised differentiation of shoots and roots, reduced fertility of transgenic plants, and a variety of other aberrant phenotypes [21]. This necessitates the need for an alternative strategy to enhance regeneration without compromising the morphology of the plant. This search culminated with the finding that ectopic expression of a chimeric GRF-GIF protein complex could induce better regeneration of fertile cultivars [22].

The Growth-Regulating Factors (GRFs) are a small group of transcription factors that play an important role in plant development and are highly conserved in angiosperm, gymnosperm, and moss (bryophyte) lineages [23] . They encode proteins with conserved QLQ and WRC domains responsible for protein–protein and protein–DNA interactions, respectively. Many angiosperms and gymnosperm GRF genes carry the target site for microRNA 396 (*miR396*), which attenuates its activity [24]. The GRF proteins form complexes with their transcription cofactor GRF-Interacting Factors (GIFs) and forms a transcription activation complex [25]. In these GRF-GIF complexes, GIFs recruit chromatin remodelling complexes and GRFs remove the nucleosomes from chromatin by virtue of the QLQ motifs to activate expression of target genes [26]. In general, callus formation and subsequent plant regeneration are accompanied by epigenetic changes on the packaging of DNA involving formation of an open-chromatin state facilitating gene expression [27]. Hence, GRF-GIF complexes are thought to confer meristematic potential to proliferative and formative cells during organogenesis by inducing the open-chromatin state [28].

Here, we report that the ectopic expression of chimeric *GRF4-GIF1* from *Citrus*, *Triticum* and *Vitis* have differential effects in boosting regeneration and genetic transformation of diploid strawberry *F. vesca* Hawaii 4. We have also explored how the mutation of *miR396* site in the *VvGRF4* affects the activity of the *VvGRF4-GIF1* chimera during the regeneration of *F. vesca*. Henceforth, in the paper we will refer to the *GRF4-GIF1* chimeras as *CcGRF-GIF*, *VvGRF-GIF*, *TaGRF-GIF* and the *VvGRF-GIF* chimera mutated in the *miR396* site as *Vv miR GRF-GIF*. Transcriptomic analyses reveal several factors related to development and maturation differentially expressed by virtue of the transformation. We also report the increased potential of regeneration in *VvGRF-GIF* lines following re-transformation in comparison to wild-type plants.

Methods

Seed germination and meristem propagation

F. vesca Hawaii 4 seeds were harvested from the matured fruits and dried on filter paper at 37°C. Dried seeds were labelled and packed in envelops and stored at 4°C. Seeds were scarified with 70% ethanol followed by 1 M sulphuric acid (H_2SO_4) solution before they were thoroughly washed with water. To initiate germination, scarified seeds were plated on water agar and kept at 22°C. The germinated seedlings were propagated on nutrient rich soil in a glasshouse at 22–24°C under long day (16 h days – 8 h night) conditions to initiate runners. Fresh runners were harvested in water and transferred to the lab where, using a Leica stereomicroscope M165, the meristems were harvested using a scalpel. This tissue was immediately transferred to tubes containing strawberry propagation medium (SPM), taking special care to prevent desiccation. SPM is a MS-based medium (2.2 g/L) supplemented with 0.1 mg/L of BAP and 0.1 mg/L indole-3-butyric acid (IBA) and solidified with Daishin agar (Duchefa D1004, 9 g/L); the pH was adjusted to 5.8 before autoclaving. The tubes were maintained in a growth room at 20 °C under long day conditions until they regenerated into plantlets. Following shoot maturation, the plantlets were transferred to honey jars (HS French Flint Ltd, London, UK) containing SPM, where they were maintained for regular work.

Plant material and in vitro micropropagation

In vitro shoot cultures of *F. vesca* Hawaii 4 were subcultured at 4–6-week intervals, 5 per honey jar containing 50 ml medium. Strawberry multiplication medium (SMM) and SPM were alternated in each round of subculturing. Both basal culture media were composed of Murashige and Skoog (MS) macro and micro elements and vitamins, supplemented with sucrose (30 g/L) and 0.5 mg/L of 6-benzylaminopurine (BAP), solidified with Daishin agar (Duchefa D1004, 9 g/L) and the pH was adjusted to 5.8 before autoclaving.

Construct assembly and transformation into A *grobacterium tumefaciens*

The binary plasmid vector constructs *pL2B-pNOS-KantNOS-p35S-mCherry-t35S-54,122* and *pL2B-pNOS-Hyg-tmas-p35S-mCherry-t35S-5433* were assembled using Golden Gate cloning. The domesticated Level 0 constructs were synthesized by Thermo GeneArt and assembled into the Level 1 backbone using the *Bsa1* restriction enzyme. The different Level 1 constructs were assembled into the respective binary vector backbones using *Bbs1* restriction enzyme. The *GRF4-GIF1* chimera constructs were obtained from Addgene in *pDONR-zeo* backbone [22]. The individual *GRF4-GIF1* entry vectors: *TaGRF4-GIF1*, *VvGRF4-GIF1*, *VvmiRGRF4-GIF1* and *CcGRF4-GIF1* were recombined using LR clonase Gateway cloning kit (Invitrogen) into the *pK7WG2D* binary vector obtained from VIB Ghent [29].

Electrocompetent *Agrobacterium tumefaciens* strain EHA105 were mixed with 500 ng of binary vector constructs. The mixture was pipetted into an electroporation cuvette and loaded into the electroporator and pulsed for 2.5 s at resistance (200 ohm), capacitance (25 µFD) with pre-set voltage (Gene Pulser, Biorad). 500 µl of LB media (L1704, Duchefa) was added to the mixture of cells and plasmid after the shock and then transferred to a microfuge tube. Tubes were incubated in a shaker for 3 h at 200 rpm and 28°C. Cells were spread to LB + appropriate antibiotics and grown for 2 days at 28°C. Colonies were verified by PCR (Supplementary Table S1).

Transformation and regeneration of transgenic plants

Preparation of plant material for transformation

Petioles were harvested the day before the transformation from the youngest (most apical) leaves. The plant cultures used were four-six weeks old after the last subculture.

Transformation and regeneration

A. tumefaciens strain EHA105 with the binary vector were grown overnight (200 rpm, 28°C) in LB media with appropriate antibiotics. The culture was pelleted at 2,000 x g for 10 min and re-suspended in filter-sterilised liquid MS-based medium supplemented with glucose (30 g/L) and acetosyringone (100 µM), pH 5.2, to give OD 600 nm = 0.2–0.3. Petioles were cut into 4–5 mm pieces, submerged in the inoculum, and blotted on sterile filter paper to remove excess inoculum. The petiole pieces were then transferred to Strawberry Regeneration Medium (SRM) petri dishes (MS-based medium supplemented with 0.2 mg/L of α-naphthaleneacetic acid, 1 mg/L of thidiazuron (TDZ), 5 g/L of Agargel and 30 g/L of glucose and adjusted to pH 5.8). Petioles were co-cultivated in the dark for four days at 20 °C. After the incubation, explants were washed in a solution of filter-sterilised ticarcillin disodium/clavulanate potassium (TCA, Duchefa) (400 mg/L) in water for 4 h (60 rpm, 20°C), then blotted and transfered to T25 Cell Culture Flasks (Nunc) containing 15 ml of liquid SRM with antibiotic selection. Flasks were placed in a shaker at 60 rpm, 20°C, under low light intensity for 4 weeks, and then blotted and transferred to SRM selection petri dishes. Petioles were subcultured every 4 weeks until regeneration. Control (WT) shoots were regenerated using the same method, except that the explants were not co-cultivated with *A. tumefaciens*, and selection antibiotics were omitted from the culture media. The transformed shoots were transferred to 30 ml universal tubes (Fisher Scientific) containing 15 ml of rooting medium (Frag R) with selection. Frag R is MS-based medium (2.2 g/L) supplemented with 0.1 mg/L

of BAP and 0.1 mg/L IBA and 20 g/L of glucose, solidified with 9 g/L of Daishin agar (Duchefa) and adjusted to pH 5.8. After 4 weeks, shoots were moved to tubes containing SMT medium (MS-based medium supplemented with 0.225 mg/L of BAP, 0.2 mg/L IBA, 0.1 mg/L gibberellic acid (GA3) and 30 g/L of glucose, solidified with 7.5 g/L of Sigma-Aldrich agar and adjusted to pH 5.6 before autoclaving). In the next subculturing step, plants were changed to SMM tubes.

Mature transgenic plant propagation

After 4 weeks in SMM tubes, plants were mature enough to be moved to honey jars (5 plants per jar). Honey jars with SMM medium or Strawberry Medium for Rooting (SMR) were alternated at 4–6-week intervals. SMR medium is a MS-based medium supplemented with 0.4 mg/L of IBA, 0.1 mg/L gibberellic acid (GA3) and 30 g/L of glucose, solidified with 7.5 g/L of Sigma-Aldrich agar A1296 and adjusted to pH 5.6 before autoclaving.

Genotyping of transgenic lines

DNA were extracted from 50 to 100 mg of leaf tissue using an in-house protocol described subsequently. The frozen leaf tissue was ground with metal balls (IG100_5/32_PK1000; Simply Bearings Ltd., Leigh, UK) using a mechanical pulveriser (MiniG from Spex) at 1200 rpm for 30 s. 500 µl of the extraction buffer (1.25% sodium dodecyl sulphate (SDS); 100 mM Tris HCl pH 8.0; 50 mM EDTA pH 8.0 and 25 mg PVP) were added to the disrupted tissue. The samples were mixed and incubated at 65 °C for 30 min, inverting the tubes each 5 min. Samples were cooled placing them in ice for around 5 min and then 250 µl of chilled 5 M NaCl, mix and incubate in ice for 15 more min. The samples were centrifuged for 10 min at 20,000 g. Supernatant was transferred into a new tube containing 360 µl of isopropanol. Samples were vortexed and incubated for 30 min or overnight at -20 °C to allow DNA to precipitate. The samples were centrifuged for 20 min at 15,700 g. Supernatant was discarded and pellet was washed in 500 µl of 70% ethanol. The samples were centrifuged for 20 min at 15,700 g and supernatant was discarded. Washing step was repeated once more and supernatant was discarded. Each pellet was resuspended in 50 µl TE buffer (10 mM Tris HCl pH 8.0; 1 mM EDTA pH 8.0). PCR amplification was performed using gene specific primers and PCR-BIO Taq Mix Red (PCR Biosystems) following the manufacturer's guidelines.

RNA extraction

RNA was extracted from 100 to 200 mg of leave tissue using an in-house protocol [30]. RNA integrity was assessed using the Agilent TapeStation system using RNA screen tape. Library preparation and paired-end RNA sequencing was performed by Novogene (Cambridge, UK) on an Illumina NovaSeq 6000 platform. Sequencing data were deposited at the NCBI under the Bioproject ID PRJNA986313.

RNA sequencing analysis

Raw reads were quality controlled using FastQC v0.11.9 [31], and adapters and low-quality regions were trimmed using Trimmomatic v0.39 using a sliding window of 4 and minimum PHRED score of 20 [32]. The first 10 nucleotides were trimmed and reads less than 100 nucleotides and unpaired reads were discarded. *GRF-GIF* transgene sequences (Supplementary File S1) were concatenated with the *F. vesca* v4.0.a1 genome. Assemblies were indexed and reads were aligned using HISAT2 v2.2.1 using the default settings for paired end reads [33]. Annotations for the GRF-GIF transgenes were generated using StringTie 2.1.7 and these were merged with the *F. vesca* v4.0.a2 gene annotations [34]. Quantification was performed using featureCounts v2.0.1 [35] and differential expression analysis was performed with the R package DESeq2 v3.17 [36]. Comparisons were made between the empty vector control (*pK7WG2D*) and each GRF-GIF construct. The Benjamin and Hochberg approach for control of the false discovery rate was used and an adjusted p-value below 0.05 was used to identify differentially expressed genes (DEGs). For visual inspection of samples distances, variance stabilizing transformation (VST) was used to normalise the raw read counts and a principal component analysis (PCA) was performed using R. KEGG and InterProScan functional annotations from the Genome Database for Rosaceae (GDR) [37] were used to annotate DEGs. A Venn diagram of shared DEGs was plotted using the R package ggvenn v0.1.10 (https://cran.r-project.org/web/packages/ggvenn) and heatmaps of DEG log2 (fold change) (log2FC) were produced using the python library seaborn v0.12.0 [38]. Heatmap clustering was performed using hierarchical clustering based on Euclidean distance. To visualise the expression of individual DEGs, raw read counts were TPM normalised using bioinfokit v2.1.0 and plotted using seaborn v0.12.0 [38].

Imaging

Pictures of the plates were taken with a Canon DSLR camera EOS4000D. Visualization of mCherry fluorescence and eGFP-ER in plant tissue and pictures of the calli, shoots and plants were performed using a Leica Stereomicroscope M165. The leaves were scanned using an EPSON flatbed scanner. The images were assembled using Inkscape and Adobe Photoshop.

Statistical analysis

Histograms and statistical analyses were performed with R (2023.03.0 Build 386 © 2009–2023 Posit Software,

PBC). Statistical differences were tested by performing (i) non-parametric Kruskal-Wallis test and the differences among samples were determined using pairwise comparisons with Wilcoxon rank sum test with continuity correction and (ii) ANOVA followed by Tukey's HSD (honestly significant differences) test.

Sequence alignments and phylogenetic tree

Gene and protein sequences were obtained using NCBI (https://www.ncbi.nlm.nih.gov/) and OrthoDB (https://www.orthodb.org/?ncbi=18049678). Accession numbers and protein names used for the phylogenetic tree are available in the phylogenetic tree in Supplementary Figure S1 and Supplementary Table S2. Protein FASTA sequences were aligned using MUSCLE method with MEGA11 (11.03.13 Built 11220624 © 2013–2023). The phylogenetic tree was built in MEGA11 using the Maximum Likelihood method and JTT matrix-based model. The bootstrap consensus tree inferred from 1000 replicates. All positions with less than 95% site coverage were eliminated.

Results

Establishing *F. vesca* stock plants and a regeneration protocol

To establish a uniform population of *F. vesca* Hawaii 4 plants, surface sterilized and scarified seeds were germinated on water agar plates. Following their germination, the plantlets were propagated on soil mix in glasshouses. After 4–5 weeks growth in the glasshouse, the plants started to produce runners (Fig. 1a). The runners allow vegetative propagation of strawberry by producing a 'clone-plant' with each runner tip containing an apical meristem that can develop into a new plant (Fig. 1b). Apical meristem tissue was collected from the growing runner and propagated in Shoot Propagation Media (SPM) in tubes (Fig. 1c). Meristem culture is the most prevalent mode of vegetative propagation for strawberry as it allows selection of disease-free plants [39]. Subsequently, the plants growing from the meristem were moved into jars for shoot multiplication (Fig. 1d). 4–6 weeks post propagation into SPM, the plants produced enough petioles for the establishment of the regeneration experiment. Young petioles were harvested from the jars and sliced into 4–5 mm pieces under sterile condition to initiate regeneration (Fig. 1e). The petiole pieces were transferred to liquid shoot regeneration media (SRM) in flasks

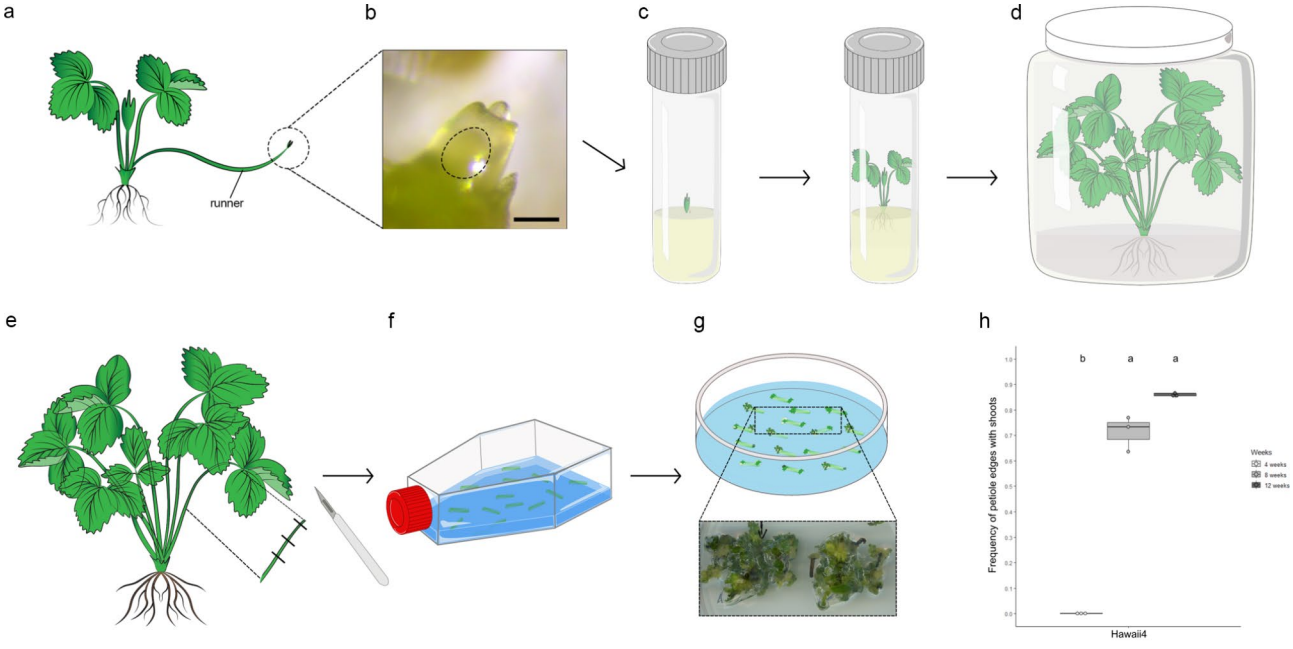

Fig. 1 Establishment of a tissue culture stock of *F. vesca* Hawaii 4 to use as a starting material in transformations with *A. tumefaciens*. (**a**) Schematic representation of a F. *vesca* Hawaii 4 runner and (**b**) stereo-micrograph of a meristem, (c-d) Whole plant regeneration going through shoot (**c**) and root (**d**) development. (**e-g**) Schematic representation of *F. vesca* Hawaii 4 regeneration process using petioles as an explant going from petiole harvest (**e**), callusing (**f**) and shoot induction (**g**). (**h**) Boxplot represents the regeneration efficiency of *F. vesca* petioles at 4-, 8- and 12- weeks post transfer to the regeneration media (SRM) where the box extends from the 25th to 75th percentiles; whiskers, 1.5 interquartile range points out of the whiskers. Statistical analysis is performed using Kruskal-Wallis test where $n = 50$ and $p < 0.05$. Scale bar = 500 µm

and maintained at 22 °C with regular shaking under long day conditions (Fig. 1f). 2 weeks into the SRM, the petioles started to form callus at both the cut ends when they were transferred to SRM plates (Fig. 1g). Regeneration efficiency of plants from the callus were assessed at 4-, 8- and 12-weeks following incubation in SRM for 50 petiole edges (Fig. 1h). After 4 weeks, all petioles had calli on both edges, that started to produce shoots by 8 and 12 weeks with an efficiency of 71% and 86%, respectively. Thus, we could produce a running stock of Hawaii 4 plants and establish an efficient platform for regeneration that could be further exploited to produce transgenic plants.

Selection of stable transgenics using antibiotic and fluorescent cassettes

To successfully raise transgenic plants, it is important to select the positive lines from the wild type revertant. Antibiotics such as kanamycin and hygromycin previously allowed efficient selection of transgenic strawberry plants [40]. To compare the transformation efficiency of different antibiotic selection cassettes, 50 petiole pieces from 4-week-old Hawaii 4 crowns grown on SPM medium were infected with *A. tumefaciens* strain EHA105 containing plasmids carrying hygromycin or kanamycin selection cassettes (Fig. 2a). Negative control and non-transformed petioles did not survive the treatment with either antibiotic, turning brown by 4 weeks indicating senescence (Fig. 2b). Transformation efficiency was assessed at 4-, 8- and 12-weeks post transformation (WPT) and was estimated as frequency of petiole edges with regenerating shoots (Fig. 2b-c). At all 3 time points, transformation efficiency of the hygromycin and kanamycin selection cassettes were found to be comparable (Fig. 2c). As a transformation marker, the plasmids were carrying mCherry fluorescent protein driven

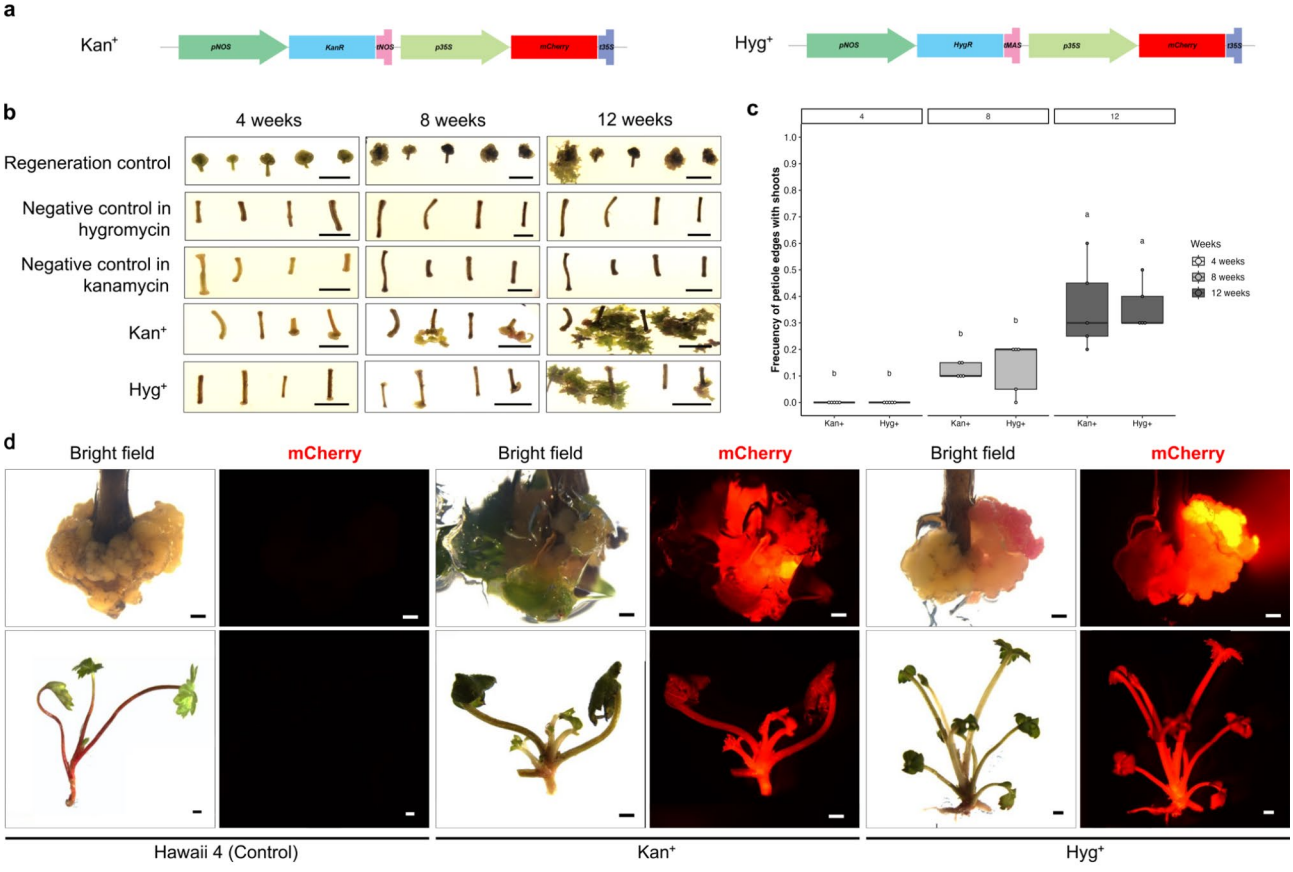

Fig. 2 Effect of kanamycin and hygromycin selection cassette on *F. vesca* Hawaii 4 regeneration at 4-, 8- and 12- weeks after transformation with *A. tumefaciens* EHA105. (**a**) Schematic representation of Kan$^+$ (kanamycin selection) and Hyg$^+$ (Hygromycin selection) constructs. Regeneration of petiole with shoots studied at 4-, 8- and 12-weeks post transfer to the regeneration media where (**b**) micrograph represents regenerating petiole with shoots. Scale bar = 1 cm and (**c**) Boxplot representing the regeneration efficiency where the box extends from the 25th to 75th percentiles; whiskers, 1.5 interquartile range; points out of the whiskers. Statistical analysis is performed using ANOVA followed by Tukey's HSD test where $n = 100$ and $p < 0.05$. (**d**) Representative images of Kan$^+$ and Hyg$^+$ calli at 12- weeks and regenerating plantlets under bright field and corresponding mCherry filter (red). Scale bar = 1 mm

downstream to promoter 35 S. Regenerating fluorescent shoots on the selection media were subsequently transferred to shooting and rooting media over the course of 12 weeks to facilitate development of stable plantlets with root systems (Fig. 2d). The uniform mCherry expression in all plant tissues throughout development indicated the lack of chimeric transformants. Moreover, the healthy physiology of the plants suggested the absence of any pleiotropic effects from the antibiotic selection cassettes (Fig. 2d). Thus, the antibiotic and visual fluorescent markers cassette provides a dual selection for positive transformants all through the regeneration process.

GRF4-GIF1 chimeras from different species increase regeneration efficiency

Introduction of developmental genes has resulted in faster regeneration of callus for several plant species including certain recalcitrant plants [14–20] [14–20]. To improve strawberry transformation efficiency, *GRF4-GIF1* chimeras from different species were tested for their effect on strawberry transformation. Petiole pieces from 4-week-old Hawaii 4 crowns were infected with *A. tumefaciens* strain EHA105 carrying *GRF4-GIF1* chimeras from *Vitis vinifera* (constitutive, *VvGRF-GIF* and *miR396*-resistant version, *Vv miR GRF-GIF*), *Citrus clementina* (*CcGRF-GIF*) and *Triticum aestivum* (*TaGRF-GIF*) (Supp Fig. S1-S2). Transformation efficiencies were assessed at 4-, 8- and 12- WPT and were estimated as frequency of petiole edges with shoots (Fig. 3b-c). At 4 WPT, regeneration efficiency of petioles transformed with *VvGRF-GIF*, *CcGRF-GIF* and *TaGRF-GIF* chimeras were comparable to the empty vector transformed petioles (Fig. 3c). At the same time point, *Vv miR GRF-GIF* showed regeneration efficiency comparable to 8 WPT for the empty vector control indicating a 4-week faster regeneration of shoot from callus which is evident in having much mature plantlets by 12 weeks (Fig. 3c). This is concomitant to the previous report where the *miRNA* resistant variety of GRF resulted in an increase in cell number and leaf size in *Arabidopsis* [41]. The *Vv miR GRF-GIF* construct contained four synonymous mutations to prevent the binding of *miRNA396*, which regulates *GRF4* expression (Supp Fig. S3). *miRNA396* is known to target *GRF1-4* family transcripts, controlling the activity of *AtGRF3* during leaf development [41, 42]. At week 8- and 12- WPT, all *GRF4-GIF1* chimeras showed significantly higher shoot regeneration compared to the empty vector controls (Fig. 3b-c).

While the introduction of the *GRF4-GIF1* chimeras resulted in efficient regeneration, their constitutive expression also induced several pleiotropic effects on the plants [43]. To study the effect of *GRF4-GIF1* chimeras on regenerating plant physiology, shoots were taken at 12 weeks and grown until rooted plantlets were established (Fig. 3d). As all the constructs were carrying eGFP transformation reporter, its expression was monitored throughout the experiment, from callus to regenerating plantlets, to ensure no chimeric plants were selected (Fig. 3d). Severe pleiotropic effects were observed for *Vv miR GRF-GIF* plants where, despite being more vigorous in regeneration, the plants failed to show the canonical leaf expansion and elongation that are hallmarks for proper development in strawberry (Fig. 3d-e). Similar observations were made for rice where the *miRNA396* resistant variety of *Vv miR GRF-GIF* resulted in formation of large calli without proper regeneration [22] . As compared to the *miRNA* resistant version, *VvGRF-GIF* and *CcGRF-GIF* showed proper regeneration with healthy adult plants established under lab condition (Fig. 3d). One of the reasons behind the better health of the *VvGRF-GIF* and *CcGRF-GIF* could be the presence of the *miRNA396* target site where *F. vesca miRNA396* could bind to regulate the expression of the *GRF4* gene expression (Supp Fig. S3). *TaGRF-GIF* plants also exhibited aberrant leaf development in the regenerated plants. 3 out of 5 lines showed leaves with more lobes that were more serrated with sharp edges compared to empty vector transformed plants (Fig. 3e). Multiple protein alignment showed that *TaGRF4* shares much less homology compared to the dicot GRFs (40% vs.~85%), but like its dicot counterparts still retains the *miRNA396* binding site (Supp Fig. S4-S5). The presence of the *miRNA* target site suggests canonical transcriptional regulation by *miRNA396*, but divergent protein structure of *TaGRF4* might be activating phytohormone responses resulting in the aberrant leaf morphology. In a previous observation, *OsbZIP48* from rice could complement an *Arabidopsis Athy5* mutant but caused pleiotropic effect like semi-dwarfism [44]. Thus, our observation indicates that cross species activation of *GRF4-GIF1* chimera can induce pleiotropic effects due to possibly mis-regulation at the transcriptional or translational level.

Transcriptomic analysis of the *GRF4-GIF1* chimeras shows differential gene activation

All the chimeric *GRF4-GIF1* produced a positive effect on regeneration efficiency irrespective of their source. But the pleiotropic effects in *Vv miR GRF-GIF* and *TaGRF-GIF* in strawberry indicates the presence of complex transcriptional landscape under different chimeric conditions. To investigate the issue, transcriptomic analysis was performed for each condition with leaf extracted RNA. Significantly high expression of the *GRF4-GIF1* chimeras were observed for each of the transgenic lines assayed (Fig. 4a). All the differentially expressed genes (DEGs) as compared to empty vector control represented in any one of the GRF-GIF chimera datasets are projected in the form of a heatmap (Fig. 4b).

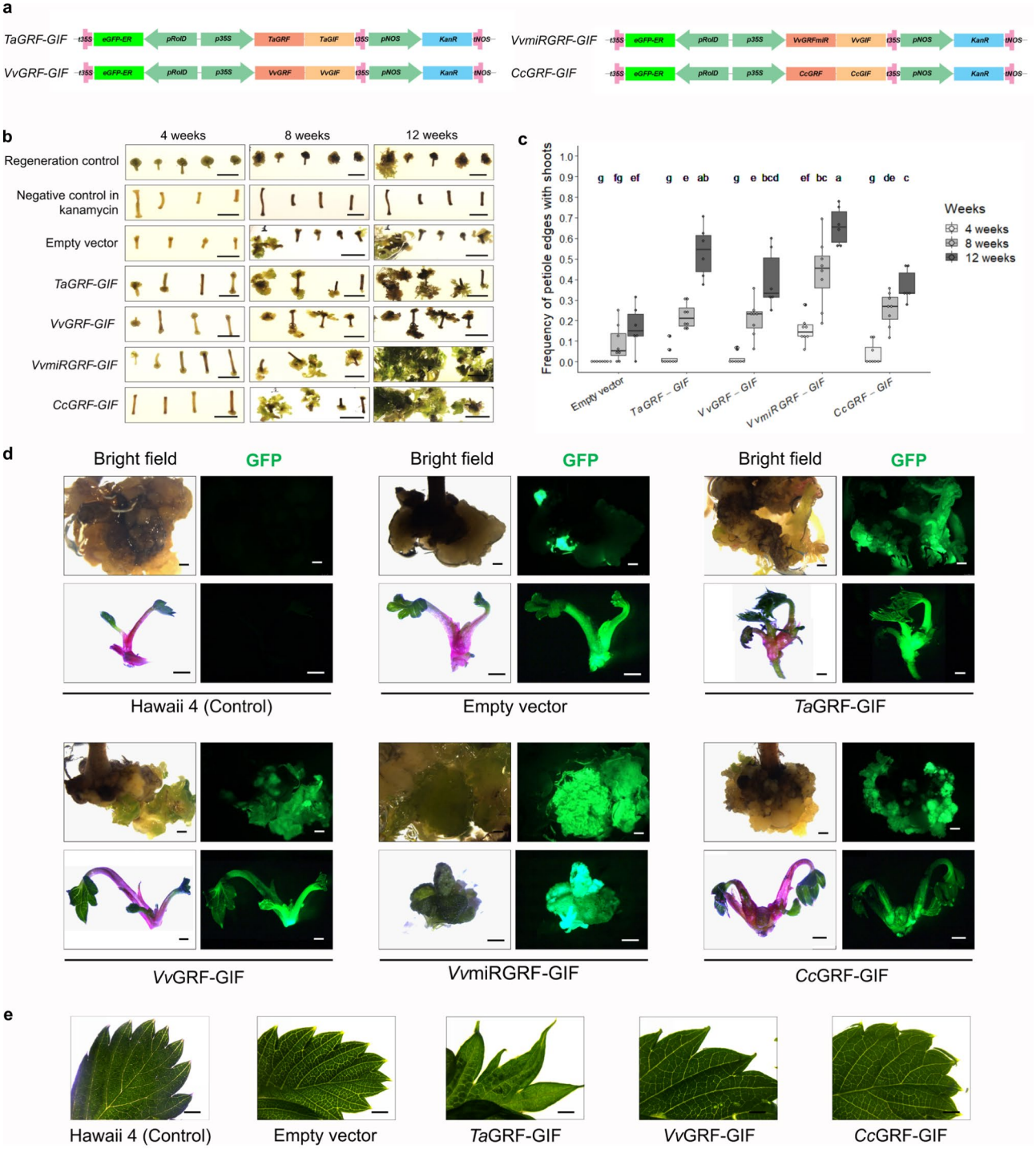

Fig. 3 Effect of *GRF-GIF* chimeras in *F. vesca* Hawaii 4 regeneration at 4-, 8- and 12- weeks after transformation with *A. tumefaciens* EHA105. (**a**) Schematic representation of *GRF-GIF* chimeras constructs. Regeneration of petiole with shoots studied at 4-, 8- and 12- weeks post transfer to the regeneration media where (**b**) micrograph represents regenerating petiole with shoots. Scale bar = 1 cm and (**c**) Boxplot representing the regeneration efficiency where the box extends from the 25th to 75th percentiles; whiskers, 1.5 interquartile range; points out of the whiskers. Statistical analysis is performed using Kruskal-Wallis test where $n = 100$ and $p < 0.05$. Representative images of control (Hawaii4) and *GRF-GIF* chimeras (**d**) calli at 12- weeks and regenerating plantlets under bright field and corresponding eGFP filter (green). Scale bar = 1 mm. and (**e**) leaf margins under stereomicroscope. Scale bar = 1 mm

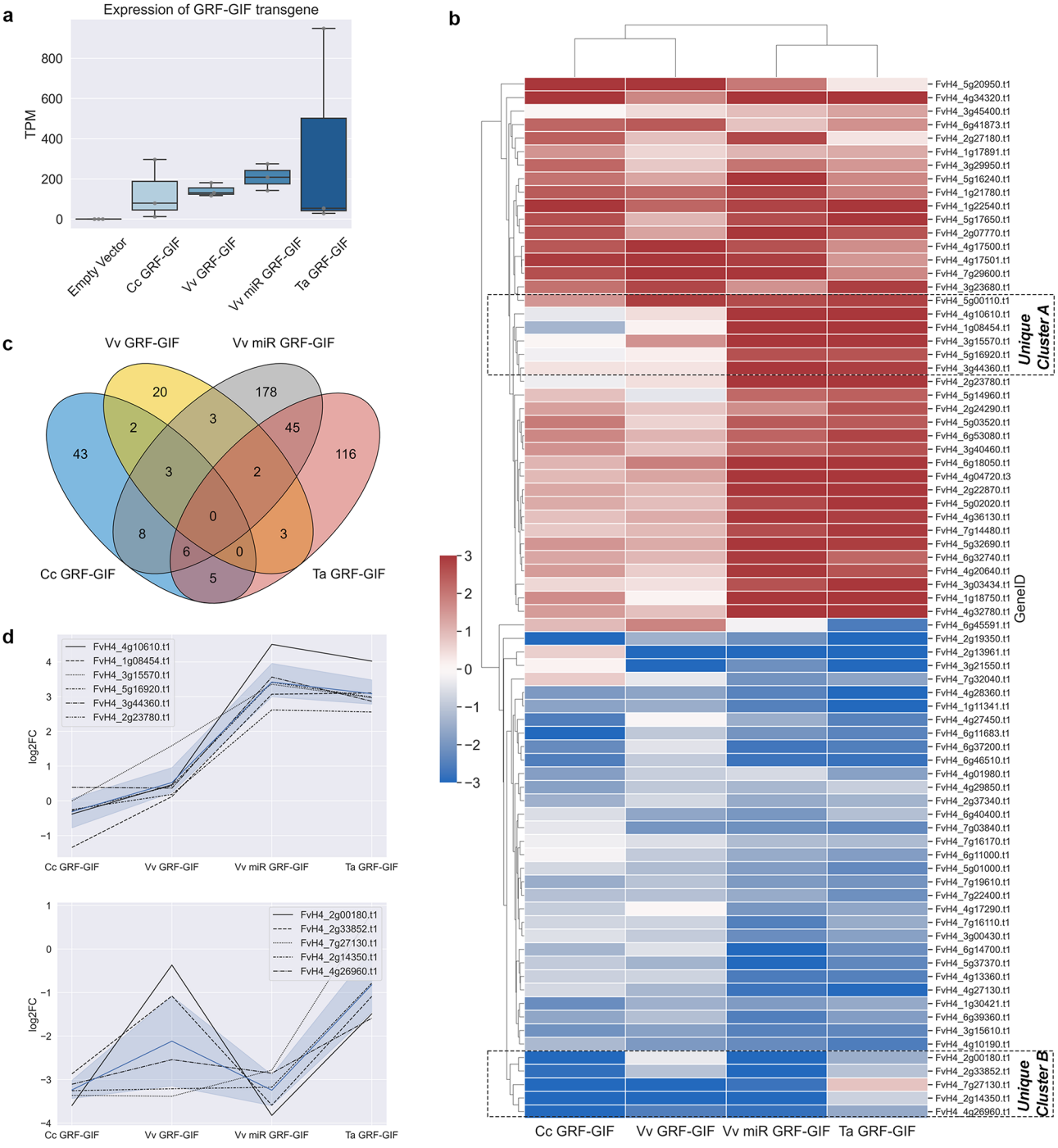

Fig. 4 Transcriptome analysis of the *GRF-GIF* chimeras in *F. vesca* Hawaii 4. (**a**) Relative expression of the *GRF-GIF* transgenes in the leaves of three independent lines for each construct. (**b**) Heatmap showing the log2 transformed fold change (log2FC) of all differentially expressed genes (DEGs) shared across two or more *GRF-GIF* chimeras. Two unique clusters of expression patterns are highlighted by hatched boxes. (**c**) Venn diagram representation of the total number of DEGs shared between the different *GRF-GIF* chimeras. (**d**) Plots showing the log2FC of the unique cluster DEGs identified in b

Concomitant to the aberrant phenotypes, both *Vv miR GRF-GIF* and *TaGRF-GIF* lines shows 178 and 116 DEGs that were not represented in the other data sets (Fig. 4c). A particular group of DEGs showed very high expression in both *Vv miR GRF-GIF* and *TaGRF-GIF* as compared to the *CcGRF-GIF* and *VvGRF-GIF* identified as Unique cluster A. *FvH4_3g44360*, encoding a peroxidase from this cluster showed ~ 3.5-fold higher expression in *Vv miR GRF-GIF1* compared to control plants (Fig. 4b-d; Supp Table S3). A previous report in *Nicotiana benthamiana* showed that overexpression of peroxidase leads to developmental abnormalities with retarded root

development [45]. Another gene that is significantly upregulated in *VvGRFmiR-GIF* is *FvH4_4g10610* encoding a EP3-like endochitinase (Fig. 4b-d; Supp Table S3). Endochitinase is an extracellular protein secreted by the non-embryogenic cells in the medium inducing somatic embryogenesis [46, 47]. It could be possible that the higher expression of EP3-like endochitinase induced more somatic embryos in *Vv miR GRF-GIF1* lines, but the sustained expression of the gene resulted in lack of regeneration. Interestingly, this gene is also upregulated in *TaGRF-GIF* lines indicating that the possible phenotypic effect of EP3-like endochitinase in plant development ranges from somatic embryogenesis to proper plant development (Supp Table S3). *TaGRF-GIF* lines showed an exclusive upregulation of *FvH4_7g27130* encoding an expansin gene from the Unique cluster B where a cluster of DEGs show significantly higher expression in *TaGRF-GIF* as compared to the others (Fig. 4b-d). A recent report in Poplar showed that overexpression of *GRF5* resulted in increased leaf size, and transcriptomic analysis assisted with DAP-seq showed significant representation of cell cycle and expansin gene families [48]. This paper also reported that poplar *PpnGRF5* binds to promoter of Cytokinin oxidase/dehydrogenase (*pPpnCKX*) and negatively regulate its expression resulting in elevated cytokinin levels in the cells. Conversely, our transcriptomic data indicated ~ 3-fold increase of *FvH4_2g39230* encoding Cytokinin oxidase/dehydrogenase in the *TaGRF-GIF* lines (Supp Table S3). While an increase in the expression of CKX should ideally result in decrease of the cytokinin level, the transcriptome of *TaGRF-GIF* shows ~ 2-fold increase in *FvH4_5g16240* expression encoding a type-A two-component response regulator (RRs) (Supp Table S3). Type-A response regulators act downstream to cytokinin signalling where upon activation, negatively regulate the pathway [49]. It could be possible that the deformed leaflet formation observed in *TaGRF-GIF* lines is due to abnormal cell division caused by deregulated levels of cytokinin. Differential activation of the cytokinin pathway is further evident by the fact that different sets of two-component RRs are activated in *CcGRF-GIF* and *Vv miR GRF-GIF* lines (Supp Table S3). Thus, our data indicates that *TaGRF-GIF* chimera could differentially activate the cytokinin oxidase and response regulator genes along with expansin genes to affect the developmental processes in strawberry as compared to the *VvGRF-GIF* chimera which did not show any abnormalities.

VvGRF-GIF plants show better regeneration efficiency following re-transformation

Morphogenic regulators not only facilitate recalcitrant plants to undergo somatic embryogenesis but also allow better regeneration efficiency for plants that are already known to undergo somatic embryogenesis [21, 22, 43]. By virtue of their faster regeneration efficiency, we investigated whether *GRF4-GIF1* stable lines in strawberry perform better during re-transformation when compared to the empty-vector transformed lines and the wild-type plants. Due to the pleiotropic effects in *Vv miR GRF-GIF* and *TaGRF-GIF* lines, only *VvGRF-GIF* and *CcGRF-GIF* were considered for re-transformation with a vector carrying hygromycin resistance gene and mCherry fluorescent marker (Fig. 5a). As the transformed plants already had the *GRF4-GIF1* chimeric cassette with kanamycin selection and eGFP marker, the vector with hygromycin selection cassette was chosen that was previously used in Fig. 2. 50 pieces of petioles from each line were infected and transformation efficiency were assessed at 4-, 8- and 12- WPT as before (Fig. 5b-c). At week 4, transformation efficiency was 0% in all the samples. By week 8- and 12-, the average transformation efficiency of two of the *VvGRF-GIF* lines were ~ 40% higher compared to the empty vector transformed petioles and wild-type plants (Fig. 5c). It is important to note that petiole regeneration following re-transformation is usually slower than single transformation possibly due to presence of multiple antibiotic selection. The shoots of these two *VvGRF-GIF* lines looked bigger and healthier by 12 weeks compared to the empty vector transformed lines (Fig. 5b). At week 8- and 12-, no significant difference in regeneration efficiency was noted for *CcGRF-GIF* lines (Fig. 5b-c). The calli and the regenerating plants were checked for fluorescence where the eGFP fluorescence indicated the consistent expression of the *GRF4-GIF1* cassette and the mCherry indicated double transformation events (Fig. 5d). The double fluorescent calli were transferred to selection media for the propagation of transformed plants. Although, both *VvGRF-GIF* and *CcGRF-GIF* lines looked healthy at their rooted plantlet stages (Fig. 3), the differences in their regeneration efficiency after re-transformation is difficult to explain. One interesting difference is the upregulation of 3 cytokinin responsive RR genes *FvH4_2g27180*, *FvH4_5g16240* and *FvH4_6g25290* in *CcGRF-GIF* as compared to *VvGRF-GIF* lines (Supp Table S3). Along with this difference, there are several other genes that were differentially regulated in the *CcGRF-GIF* lines which could contribute to their lack of regeneration phenotype (Fig. 4c). Thus, ectopic expression of *VvGRF-GIF* chimera could be a useful tool for expediting strawberry transformation without incurring unwanted pleiotropic effect.

Discussion

Strawberry is a commercially important crop where several desirable agronomic traits define its value in the market. But fundamentally, the presence of physiological or genetically linked trade-offs limits the possibility for certain combinations of phenotypes to occur [50]

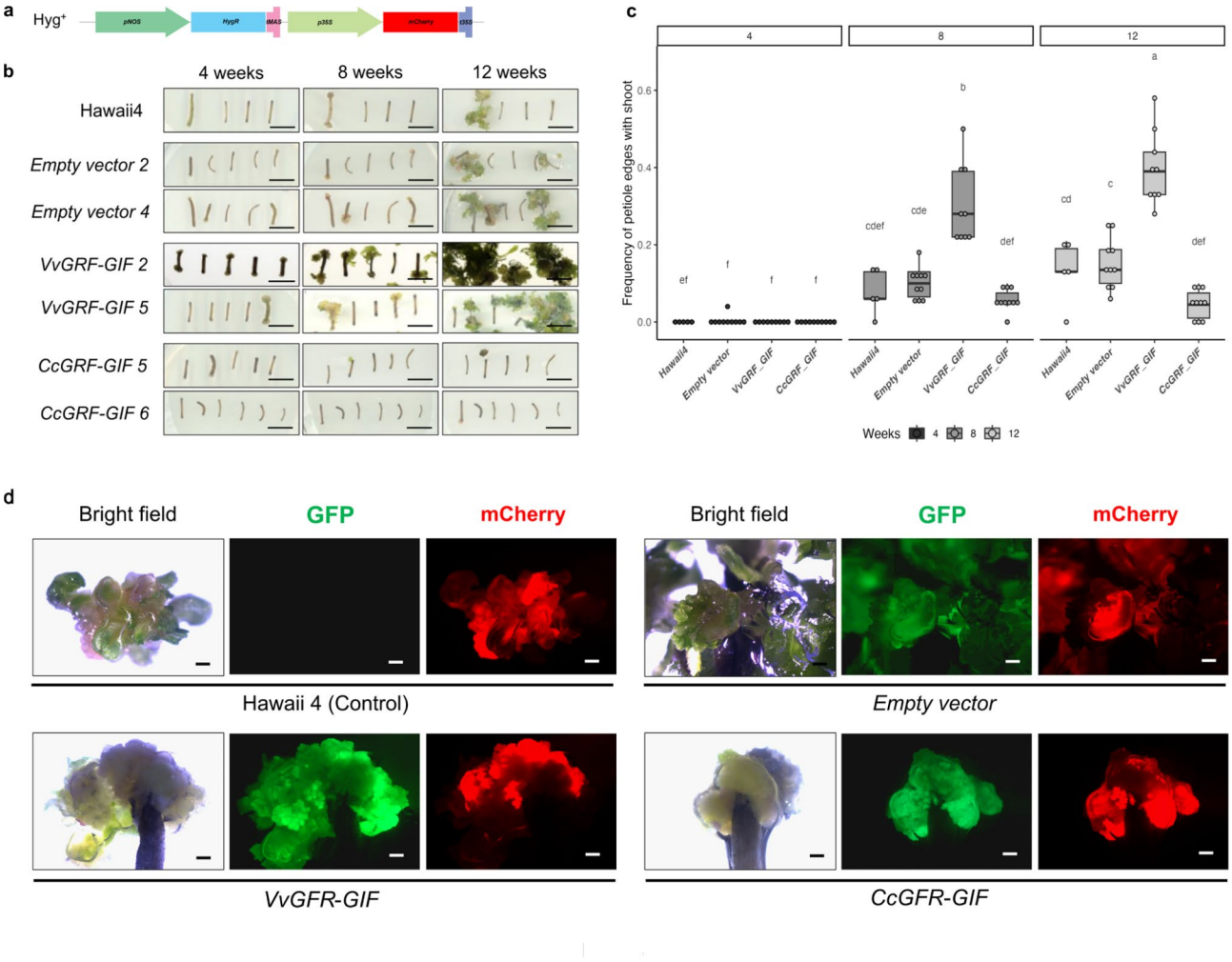

Fig. 5 Effect of re-transformating on *F. vesca GRF-GIF* chimeras at 4-, 8- and 12- weeks after transformation with *A. tumefaciens* EHA105. (a) Schematic representation of Hyg + constructs transformed. (b) Regeneration of petiole with shoots studied at 4-, 8- and 12- weeks post transfer to the regeneration media where (b) micrograph represents regenerating petiole with shoots. Scale bar = 1 cm and (c) Boxplot representing the regeneration efficiency where the box extends from the 25th to 75th percentiles; whiskers, 1.5 interquartile range; points out of the whiskers. Statistical analysis is performed using Kruskal-Wallis test where $n = 100$ and $p < 0.05$. (d) Representative images of control (Hawaii4), empty vector, *VvGRF-GIF* and *CcGRF-GIF* chimera callus at 12- weeks and regenerating plantlets under bright field mCherry (red) and GFP (green) filter. Scale bar = 1 mm

. As often these viable traits are diametrically opposed, genetic engineering over normal breeding provides an opportunity to overcome the genetically linked traits [51]. In this study, we present an efficient strategy to expedite transformation in the diploid strawberry model with the introduction of *GRF4-GIF1* chimeras (Figs. 1, 2 and 3). Woodland strawberry has 10 GRF and 2 GIF genes, which show complex regulation and functional redundancy [52]. To avoid investigating multiple combinations to identify the most efficient pairings, we instead explored the regeneration potential of the available GRF-GIF chimeric toolkit, which has previously proven to be effective in heterologous system [22]. Using different types of *GRF4-GIF1* chimeras coming from multiple plant species allowed us to explore the best possible chimera as complicated regulation of GRFs resulted in pleiotropic effects for *Vv miR GRF-GIF1* and *TaGRF-GIF* (Fig. 3). Transcriptomic analysis of the different lines provided necessary insight into the possible causes of the pleiotropic effects and the complex regulation for *GRF4-GIF1s* (Fig. 4). We also found *VvGRF-GIF* lines has much better efficiency after re-transformation as compared to the empty vector or wild-type plants (Fig. 5).

Diploid strawberry is an attractive system to study functional genomics in Rosaceae due to its small genome size, short life cycle and facile vegetative and seed propagation [10]. But the biggest bottle neck for doing any forward or reverse genetics is an efficient protocol to raise stable transgenics. In the last couple of decades there has been a considerable effort to establish various protocols for raising successful strawberry transgenics with various level of efficiencies ranging from 63 to 68% [10, 53]. Here,

we have presented a comprehensive protocol for preparing a uniform line of clean stock plants using meristem culture which can be used for raising stable transgenics (Fig. 1). Strawberry is generally propagated using stolon which runs the risk of getting infected material into tissue culture spreading through vascular tissues [54]. Meristem culture on the other hand allows an alternative strategy to obtain large quantities of virus free material due to active cell division and lack of differentiation [55] . Moreover, tissue culture of strawberry leads to somaclonal variation which can be avoided by meristeming and thus allowing true-to-type plants [56]. The stability of the background is revealed by ~90% regeneration efficiency with very little variability (Fig. 1d).

Growth regulating factors (GRFs) are a small family of transcription factors that play important role in plant development by controlling various aspects of leaf developmental [41, 48, 57], stem development, apical meristem development [58, 59] and root development [60]. GRFs form complexes with GRF interacting proteins (GIFs) which act as transcriptional coactivators [61]. From an evolutionary perspective, all land plants encode for GRF proteins except green algae, whereas GIFs are universally present [23]. Several studies have shown that GRFs are active at the sites of active growth and differentiation, with expression gradually decreasing in the matured tissues [24]. In *A. thaliana*, this expression regulation is primarily carried out by the *miR396a* and *miR396b* which shows near perfect sequence alignment with the transcripts of several *GRFs* [24, 62]. *F. vesca* encodes for *miR396* gene which also shows sequence conservation with *AtmiR396* indicating that *miRNA* mediated control of GRFs is highly conserved across the plant kingdom (Supp Fig. S3). This is in consonance to a previous report where ectopic expression of *AtmiR396* resulted in reduction in gene expression of *GRFs* in *N. benthamiana* [63]. *Vitis* belonging to the order Vitales is phylogenetically closest to the *Fragaria* sharing maximum homology to *FvGRF4* followed by *Citrus* from Sapindales and *Triticum* from Poales (Supp Fig. S4). Provided that phylogenetic proximity often results in similar regulation, *VvGRF-GIF* transformation resulted in ~50% callus regeneration in 'Hawaii4' (Fig. 3c), which is close to what has recently been reported for *FvGRF3-GIF1* chimera in a thoroughly inbred diploid cultivar of strawberry 'YW5AF7' [64]. Using the *GRF4-GIF1* chimeras from both eudicot and monocot species allowed us to dissect the functional diversity within the GRF4 family in a heterologous system. *miR396* expression and *GRF4* expression work reciprocally, whereby *GRF4* expression decreases in mature leaves with an increase in *miR396* expression [41]. The importance of the transcriptional control of *GRF4* by *miR396* is revealed in the pleiotropic phenotype of the *Vv miR GRF4-GIF1* lines (Fig. 3). While facilitating more regeneration events, the sustained expression of the *GRF4-GIF1* chimera in the *Vv miR GRF4-GIF1* lines prevented most of these plants to reach proper tissue differentiation (Fig. 3b). Transcriptome analysis showed that there were many genes that were differentially upregulated in the *Vv miR GRF4-GIF1* lines as compared to the *VvGRF4-GIF1* and *CcGRF4-GIF1* (Fig. 4; Supp Table S3). Upregulation of certain vital genes required for the transition from cell division to differentiation like endochitinase and peroxidases might have played a significant role (Fig. 4; Supp Table S3). This agrees with a previous observation in *Citrus* where inactivation of peroxidase activity was shown to be important for in vitro plant differentiation [65].

The expansion of the GRF family transcription factors happened due to large scale genome duplication [23]. In case of the eudicots, a whole genome triplication event in the ancestor led to the formation of several *GRF* genes. Like eudicots, a similar duplication event led to formation of the monocot *GRFs*. The *TaGRF4-GIF1* showed only 40% protein sequence homology to the eudicot GRF4-GIF1s compared to ~85% within the eudicots (Supp Fig. S4-5). As genome duplication events are directly related to neofunctionalization [66], it could be possible that during evolution, GRFs gained functions in monocots that are different from eudicots. This is evident from our observation of the leaf phenotype in strawberry lines where ectopic expression of *TaGRF4-GIF1* caused leaf deformations (Fig. 3e). Transcriptome analysis showed that in these leaf tissue there was a significant increase in the expression of an expansin gene *FvH4_7g27130* (Fig. 4b). Previously in *N. benthamiana*, local expression of expansin recapitulated leaf formation from a meristem and could also alter the shape of the leaf lamina [67]. GRF transcriptional activity tightly controls the cytokinin concentration in plants, which in turn is responsible for cell division and expansion [48]. *TaGRF-GIF* lines showed higher expression of cytokinin responsive RRs in the abnormal leaves (Fig. 4; Supp Table S3). During the leaf expansion phase, an increase in cytokinin concentration can lead to abnormal leaf development [68]. Thus, the abnormality in the strawberry leaves in *TaGRF-GIF* lines could be due to misexpression of cytokinin responsive and expansin genes.

The benefits of the developmental genes during transformation first came into prominence for their ability to jump start somatic embryogenesis [43]. Re-transformation of multiple genes in a desirable background, or 'stacking' of genes, has always been challenging for multiple reasons [69]. Although a recent report in *Fragaria vesca* cultivar YW5AF7 showed better regeneration after β-estradiol induction of *Fragaria vesca GRF3-GIF1*, its efficacy during re-transformation is yet to be explored [12]. Here, we show that the introduction of

the *VvGRF-GIF* in strawberry gives the plants certain advantages during re-transformation where the transformation efficiency increases by ~40% as compared to the empty vector transformed plants (Fig. 5). Moreover, the expression of both visual fluorescent transformation markers ensured that both the cassettes were properly transformed. But surprisingly, the *CcGRF-GIF* lines did not show any significant improvement during the re-transformation experiment (Fig. 5). Transcriptome analysis indicated significant differences between *VvGRF-GIF* and *CcGRF-GIF* lines despite the lack of any phenotypic discrepancies (Fig. 4c, Supp Table S3). Cytokinin is intrinsically linked to regeneration of plants and GRF lines were shown to behave very differently during regeneration experiments depending upon its availability [22] . As several cytokinin responsive RRs genes are activated in the *CcGRF-GIF* lines, it could be possible that disproportionate cytokinin levels affected its regeneration.

Conclusions

A comprehensive protocol for strawberry transformation will turn out to be extremely beneficial for understanding genetics within the Rosaceae family, which includes several economically important horticultural crops. The re-transformation protocol that we present here can be utilized in the future to raise stable transgenics of mutant backgrounds where faster screening strategies, such as virus-induced gene silencing (VIGS) can be introduced to study viable traits. Overall, not only have we presented *VvGRF-GIF* to be an effective *GRF4-GIF1* chimera for enhancing regeneration in a strawberry transformation system, but also highlighted the pitfalls of using the wrong chimeras. At the same time, the global perturbation of the hormonal pathway in the transgenic lines poses a challenge to study the role of any developmental or hormonal pathway genes. But despite such challenges, the ability of GRF-GIF chimeras in enhancing regeneration could be beneficial for economically important octoploid strawberry varieties where transformation efficiencies are low [70]. Thus, our study provides an overarching scope for bringing more such important horticultural Rosaceae crops under tissue culture following the strawberry footsteps.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13007-024-01270-8.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

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Author contributions

A.K. and R.H. conceived the idea. E.R.S and A.K. designed the experiments. K.M. provided her expertise to micro-propagate the strawberry cultivar in the tissue culture. E.R.S. and F.M carried out the experiments. E.R.S analysed the regeneration efficiency data and J.P. carried out the transcriptome analysis. A.K. supervised the project. A.K. wrote the manuscript with support from E.R.S and R.J.P.

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Data availability

Sequencing data were deposited at the NCBI under the Bioproject ID PRJNA986313.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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