RESEARCH ARTICLE

Genomic structure and transcript analysis of the Rapid Alkalinization Factor (RALF) gene family during host-pathogen crosstalk in Fragaria vesca and Fragaria x ananassa strawberry

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Abstract

Rapid Alkalinization Factors (RALFs) are cysteine-rich peptides ubiquitous within plant kingdom. They play multiple roles as hormonal signals in diverse processes, including root elongation, cell growth, pollen tube development, and fertilization. Their involvement in host-pathogen crosstalk as negative regulators of immunity in Arabidopsis has also been recognized. In addition, peptides homologous to RALF are secreted by different fungal pathogens as effectors during early stages of infection. Previous studies have identified nine RALF genes in the diploid strawberry (Fragaria vesca) genome. This work describes the genomic organization of the RALF gene families in commercial octoploid strawberry (Fragaria x ananassa) and the re-annotated genome of F. vesca, and then compares findings with orthologs in Arabidopsis thaliana. We reveal the presence of 15 RALF genes in F. vesca genotype Hawaii 4 and 50 in Fragaria x ananassa cv. Camarosa, showing a non-homogenous localization of genes among the different Fragaria x ananassa subgenomes. Expression analysis of Fragaria x ananassa RALF genes upon infection with Colletotrichum acutatum or Botrytis cinerea showed that FanRALF3-1 was the only fruit RALF gene upregulated after fungal infection. In silico analysis was used to identify distinct pathogen inducible elements upstream of the FanRALF3-1 gene. Agroinfiltration of strawberry fruit with deletion constructs of the FanRALF3-1 promoter identified a 5’ region required for FanRALF3-1 expression in fruit, but failed to identify a region responsible for fungal induced expression.
Introduction

In plants, several small secreted peptides (SSPs) function as hormonal signalling molecules that respond to internal and external stimuli [1]. SSPs are known to be involved in different processes, ranging from organ growth to biotic and abiotic responses [2, 3].

Rapid alkalinization factors (RALFs) are cysteine-rich SSPs originally identified for their ability to rapidly alkalinize tobacco cell culture [4]. They are ubiquitous in the plant kingdom with 37 members identified in *Arabidopsis thaliana* genome alone [5, 6]. RALF genes are translated as pre-pro-proteins and are activated in the apoplast through proteolytic cleavage, generating ~50 amino acid long active peptides. Aside from the signal sequence necessary for extracellular extrusion, canonical RALF peptides contain distinctive amino acid motifs, such as the RRILA motif for S1P protease recognition [7] and the YISY motif, important for the activation of the signaling cascade [8, 9]. In addition, four conserved cysteines form two disulfide bonds that stabilise the mature RALF peptide. Based on these features, RALFs have been grouped into four major clades [6]; clades I, II and III contain typical RALF peptides, whereas clade IV includes the most divergent RALF proteins lacking both the RRILA and YISY conserved motifs and in some cases containing only three of the four cysteines.

RALF peptides bind to the *Catharanthus roseus* Receptor Like Kinases 1—like family proteins (CrRLK1L) known to be involved in cell expansion and reproduction throughout the plant kingdom [10]. The large CrRLK1L receptor family which includes FERONIA (FER) proteins, previously were reported to interact with *Arabidopsis RALF1* and *RALF23* [11, 12], BUDDHA'S PAPER SEAL 1 and 2 (BUPS1/2), ANXUR1, 2 (ANX1/2) proteins interact through their ectodomain and bind to RALF4 and 19 in the pollen tube [13] and THESEUS1 (THE1) the RALF34 receptor in the root [14]. Binding RALF peptides to CrRLK1L receptors also involves other interacting partners such as Lorelei-like-Glycosylphosphatidylinositol-Anchored proteins (LLG1,2,3) [9, 15] and Leucine-Rich Repeat Extensins (LRX). The latter has been reported to bind RALF4/19 in the pollen tube [16, 17] and to interact with FER, as part of cell wall sensing system responsible for vacuolar expansion and cellular elongation [18]. Binding of RALFs to their receptors leads to a number of different intracellular signaling events involving different molecular components, mostly still unidentified. However, it is known that in *A. thaliana* binding of RALF1 to FER receptor results in the phosphorylation of plasma membrane H(+) ATPase 2, inhibition of proton transport and subsequent apoplastic alkalinization [11].

RALF peptides regulate a variety of different functions such as cell expansion [11], root growth, root hair differentiation [19, 20, 21] stress response [22], pollen tube elongation and fertilization [13, 16, 22, 23]. In *A. thaliana*, RALF peptides can also act as negative regulators of the plant immune response following bacterial infection [12]. For example, RALF23 binding to the FER receptor inhibits physical interaction between the immune receptor kinases EF-TU RECEPTOR (EFR) and FLAGELLIN-SENSING 2 (FLS2) with their co-receptor BRASSINOSTEROID INSENSITIVE 1–ASSOCIATED KINASE 1 (BAK1), inhibiting immune signalling. Interestingly, biologically-active RALF homologs have also been identified in fungal plant pathogens, possibly following horizontal gene transfer, suggesting a role for fungal RALF genes as virulence factors [24]. In fact, a fungal RALF homolog is required for host alkalinisation and infection by *Fusarium oxysporum* [25]. Because alkalinisation is important for activation of virulence factors and successfully infection of plant tissues for many pathogenic fungi, secreted RALF peptides may promote host alkalinisation at early stage infection when hyphal biomass is not sufficient to secrete a large quantities of ammonia [24].

Strawberry is an important crop but is susceptible to fungal and bacterial pathogens that can have a significant effect on marketable yield [26]. Post-harvest molds are particularly
difficult to manage as infection initiates during flowering, and can become problematic after a long asymptomatic quiescent period on ripe fruits [27]. Severe strawberry fruit post-harvest molds are caused by *Colletotrichum acutatum*, causal agent of anthracnose disease, and *Botrytis cinerea*, causal agent of grey mold [28].

*F. × ananassa* fruits infected with *C. acutatum* show increase in the accumulation of a RALF transcript in the ripe susceptible fruit at an early stage of infection [29]. Overexpression of the *F. × ananassa* ortholog of *A. thaliana* RALF33, through transient agroinfiltration, increases strawberry fruit susceptibility to anthracnose, leading to increased fungal growth on fruits and the induction of the host immune response [30, 31]. Upregulation of RALF transcripts during plant infection has also been observed in mature red tomato fruits (*Solanum lycopersicum*) upon infection with *Colletotrichum gloeosporioides* [32] and in rice (*Oryza sativa*) upon *Magnaporthe oryzae* infection [31]. These findings suggest a role for RALF gene expression as a susceptibility factor in fungal infection. Furthermore Dobón et al. [33], studying the expression pattern of four *Arabidopsis* transcription factors mutants (at1g66810, pap2, bhlh99, zpf2) with increased susceptibility to *B. cinerea* and *Plectosphaerella cucumerina*, observed a coincidental increase of RALF23, RALF24, RALF32 and RALF33 transcripts.

The woodland strawberry RALF gene family members have been previously characterized based on the *F. vesca* genome annotation v1 [6], and nine *FveRALF* genes were identified and grouped in the four RALF clades. Analysis of an updated and annotated version of the *Fragaria vesca* genome (v4.0.a2) [34] and of the *Fragaria x ananassa* cv *Camarosa* genome sequence (v1.0.a1) [35, 36], allowed exploration of RALF gene family composition and genomic organization. Genome localization, phylogenetic and transcript analyses were conducted, based on available genomic and transcriptomic sources, with an aim to gain insights into the functional roles of specific RALF genes. Furthermore, the induction of RALF gene expression upon infection of *F. × ananassa* fruits with *C. acutatum* and *B. cinerea* was studied. *In silico* analysis of the FanRALF3-1 promoter was conducted in order to identify putative pathogen responsive motifs. We then tested *in vivo* if progressively truncated FanRALF3-1 promoter fragments could induce reporter genes expression in agroinfiltrated strawberry fruits infected with *C. acutatum*.

**Materials and methods**

**Identification of RALF family genes, phylogenetic analysis, and chromosome assignment**

*F. vesca* RALF genes identified in v1.1 genome and reported by Campbell and Turner [6] were detected in the Genome Database for Rosaceae (GDR; v4.0.a2) through keyword gene search 'RALF' and through a BLASTn search using the previously annotated RALF sequences against the *F. vesca* v4.0.a1 chromosome database. The v4.0.a2 [34] and the Blastn gene search outputs were then used to update the previous data on v1.1 genome. Nucleotide sequences of the 15 *F. vesca* RALF members (both previous and new found ones), were used as query for BLASTx on the *F. × ananassa* cv *Camarosa* Genome v1.0.a1 proteome [37] to find octoploid RALF orthologs. Sequences of *F. × ananassa* RALF peptides were aligned with MUSCLE [38] and the phylogeny was inferred using the Maximum Likelihood method and JTT matrix-based model [39]. The tree with the highest log-likelihood was chosen. The Initial tree was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with a superior log-likelihood value. Evolutionary analyses were conducted in MEGA X [40]. To define the classification of the new members in different clades, all the RALF protein sequences available from the Campbell and Turner annotation *F. vesca* genes were aligned with the newly identified RALFs and a
phylogenetic tree was constructed as mentioned above. *Fragaria x ananassa* genes annotation and position were retrieved from GDR (*F. × ananassa* cv. Camarosa genome v1.0.a1), and progenitor lineage were inferred according to [36]. Chromomap package in R [41] was used to create a *FanRALF* gene chromosome map.

**Expression profile of RALF family genes in *F. vesca***

Transcript accumulation of RALF family genes from different *F. vesca* tissues was depicted using heatmap3 package in R [42] from Transcripts Per Kilobase Million (TPM) values calculated by Li et al. [34, 43, 44, 45, 46].

**Infection of *Fragaria x ananassa* fruits**

*F. × ananassa* cv. Alba plants were grown in the greenhouse at 25˚C and 16 h of light. White (21 d after anthesis) and red fruits (28 d after anthesis) were harvested and infected. Each treatment contained at least three biological replicates. *Colletotrichum acutatum* (Isolate Maya-3, from CRIOF-UniBo fungi collection) and *Botrytis cinerea* strain B05.10 were grown on PDA plates for 15 d. Detached fruits were dipped for 30 s in a 10^6 conidia per mL suspension or in water as negative control and incubated in plastic bags for 24 h at approximately 21˚C (room temperature) as reported in Guidarelli et al. (2011) [29].

**RNA extraction and qRT-PCR analysis**

The surface of experimental fruits was excised with a scalpel and immediately frozen in liquid nitrogen. RNA was extracted according to Gambino et al. [47], run on an 2% agarose gel and quantified with NanoDrop™ 3300 for integrity and quality control, respectively. The cDNA was prepared from 1 μg of RNA using Promega ImProm-II™ Reverse Transcription system. Quantitative RT-PCR analysis was performed using ThermoFisher MAXIMA SYBR GREEN/ROX QPCR 2x supermix. The relative accumulation of RALF transcripts was calculated using standard curve method and *Elongation Factor 1* gene as reference (*XM_004307362* [48]). Primers for RALF gene expression analysis were designed on *F. × ananassa*, *F. vesca* subgenome sequences and specificity was verified by observing a single peak in the dissociation curve for each primer pair. All primers used for gene expression analyses are listed in S2 Table.

**Statistical analyses**

RALF and eGFP reporter transcripts were quantified as the average of three independent biological replicates, each formed by a group of at least three treated fruits. For eGFP quantification, all mean normalized expression values were expressed relatively to the negative empty pKGWFS7 vector infiltrated fruits. Student t-test was used to assess statistical significance between samples and controls.

**In silico prediction of regulatory elements in *FanRALF3-1* and *FanRALF6-1* promoters**

Analysis of regulatory elements required for pathogen-induced transcripts was performed by examining pathogen-upregulated and downregulated transcripts found in datasets of red strawberry (*F. × ananassa*) fruits 24 h post infection with *C. acutatum* [29] and *B. cinerea* [49]. Promoters of 87 upregulated and 36 downregulated genes were analysed in response to *C. acutatum* infection, whereas 97 upregulated and 16 predicted promoters were analysed *B. cinerea* infected fruits. For each gene, 1500 bp upstream the ATG start codon were analyzed from the *F. vesca* genome v4.0.a1 assembly. MotifLab software v1.08 [50] was used for *in silico* analysis.
using PLACE database for Motif Scanning [51], and AlignACE algorithm for Motif Discovery [52] and motif similarity analysis algorithm for motifs comparison. Briefly, Motif Scanning was performed using the Simple Scanner program with default parameters. A regulatory element was considered statistically significant to expression by performing the same Motif scanning program on randomly generated DNA sequences starting from input predicted promoter sequences using a third order background model. The frequency measured for each cis-acting element on random DNA was used as background occurrence for statistical significant evaluation using a binomial test with p-value threshold of 0.05. Motif Discovery was performed using AlignACE method with default parameters and motif significance were calculated as mentioned above for Motif scanning method.

*Fragaria × ananassa* RALF3 promoter characterization

Preliminary assessment of putative promoter allelic variants were conducted on *F. × ananassa* cv. Alba and cv. Florida Elyana. Genomic DNA was extracted from leaves using Wizard® Genomic Purification kit (Promega). The Plant Tissue Protocol (Manufacturer protocol 3.E.) was modified adding two consecutive chloroform:isoamyl alcohol (24:1) purification steps after Protein Precipitation solution was added and before 2-propanol precipitation. The *FanRALF3-1* putative promoter was amplified using primers For 5’-TGCATCTGGTACATCATCCCTTG-3’ and Rev 5’-GTAGTCGACTCTCCCATCCTTG-3’, cloned into pGEM®-T easy vector (Promega). Five clones for each variety were sequenced and aligned with the *F. × ananassa* cv. Camarosa genomic sequence available from GDR, using Clustal Omega.

Cloning of progressively truncated promoters and *agrobacterium*-mediated transient transformation

The upstream sequence of *FanRALF3-1* was PCR-amplified starting from *F. × ananassa* cv. Elyana genomic DNA, using primer 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTNNCTGAAGGACAAACATTTTCTC-3’ as the reverse primer for all promoter fragments, 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTNNCTGATTACATCATCCCTTG-3’ as the forward primer for the complete promoter fragment (T6), 5’-GGGGACAACTTTGTACAAGAAAGCTGGGTNCTGAAGGACAAACATTTTCTC-3’ as the forward primer for the 400 bp fragment (T4) and 5’-GGGGACAACTTTGTACACAAAAAGGCTNNCTGATTACATCATCCCTTG-3’ as the forward primer for the 200 bp fragment (T2). Truncated *FveRALF3-1* promoter constructs and a double p35S promoter used as a positive control were cloned into pDONR222 using the Gateway BP reaction and consequently cloned into pKGWFS7 (S1 Fig) vector by LR Reaction. The resulting plasmids were then introduced into chemically competent *Agrobacterium tumefaciens* strain EHA105 by heat shock transformation. Briefly, liquid nitrogen-frozen cells were thawed, and then incubated for 5 min at 37˚C with 1 μg of plasmid DNA. The cells were then incubated at 30˚C for 2 h in LB medium with shaking and plated. Positive colonies were then grown in selective media (Rifampicin 100 μg/mL and spectinomycin 50 μg/mL) until the culture reached an OD$_{600}$ of 0.8. Cells were collected by centrifugation and resuspended in fresh MS medium (Murashige and Skoog Basal Medium 4.4 g/L plus 20 g/L sucrose) and grown to OD$_{600} = 2.4$. At the end, acetosyringone (4’-Hydroxy-3’,5’-dimethoxy-yacetophenone) was added to the culture to final concentration of 200 μM. At least three white attached fruits for each condition were injected with about 3–5 mL of the *Agrobacterium* solution using a needle and syringe until culture filled strawberry fruit tissues. Five days after agroinfiltration, fruit were harvested and infected with *C. acutatum* conidial suspension or mock-inoculated with water, as was described above. After 24 h the fruits were dissected and
one half was used for RNA extraction and eGFP transcript analysis, and the other half was used for histochemical assay of GUS activity.

**Histochemical GUS assay**

Surface tissue and longitudinal sections of infected and mock-infected fruits were cut with a razor blade and dipped in GUS staining solution (50 mM Na-phosphate (pH 7.5), 10 mM EDTA, 1 mM 5-bromo-4-chloro-3-indolyl-glucuronide (X-gluc), 0.1% Triton X-100, 0.5 mM potassium ferricyanide and 5% (w/v) polyvinylpyrrolidone-40 (PVP)). Strawberry tissues were incubated overnight at 37 °C, and then maintained at 4 °C in absolute ethanol until being photographed.

**3D modelling of FanRALFs interaction with MRLK and LLG2 proteins**

Homology models of FanRALF3, in complex with FERONIA and LLG2 were generated using Modeller [53](v9.19) package using the complex of *A. thaliana* RALF23, LLG2 and FERONIA (PDB: 6a5e) as the template. ClustalX was used to create alignments of different components of the complex: FanRALF3 (GDR: snap_masked-Fvb2-2-processed-gene-47.50-mRNA-1), *F. × ananassa* FERONIA MRLK47 (GDR: Uniprot: A0A1J0F5V4) and *F. × ananassa* LLG2 (GDR: maker-Fvb3-4-snap-gene-34.65-mRNA-1) with the *A. thaliana* proteins in the *A. thaliana* complex. PyMOL Molecular Graphic Systems (Schrödinger, LLC) was used for the analysis of the homology models and generation of the figures.

**Results and discussion**

**Identification of RALF gene family members in *fragaria vesca***

RALF proteins belonging to 51 plant species have been previously classified in four clades based on sequence conservation [6]. Typical distinctive amino acid sequence motifs, such as the RRILA proteolytic cleavage site [7] and the YISY receptor binding site, are present in RALF peptides of clade I to III, and missing in clade IV, which contains more divergent members of the family. Nine RALF genes have been previously identified in the *Fragaria vesca* v1 genome.

In order to classify the members of RALF gene family in *Fragaria vesca*, the recent genome annotation (v4.0.a2) [34] was searched using 'RALF' as keyword gene name, revealing the presence of 13 RALF genes. These *F. vesca* RALF (*FveRALF*) [54] genes are named with progressive numbers according to their chromosome position, from Chr1 to 6 (Table 1), with one RALF gene in Chr1, two in Chr2, -3, and -5, and three in Chr4, and -6. No RALF genes are found in Chr7. Out of the nine RALF genes previously reported by Campbell and Turner [6], eight genes are confirmed both for identity and chromosome position. These are the gene08146 (corresponding to *FveRALF2*), gene10567 (*FveRALF3*), gene02376 (*FveRALF4*), gene02377 (*FveRALF5*), gene06579 (*FveRALF6*), gene06890 (*FveRALF8*), gene10483 (*FveRALF9*), gene22211 (*FveRALF13*) (Table 1). The gene00145, previously annotated as gene encoding for a protein with the typical RALF motifs RRILA and YISY [6], was discarded since in the new v4.0.a2 annotation its sequence corresponds to gene FvH4_6g07633 encodes for a shorter protein lacking most of the conserved motifs.

In order to find new, previously unannotated RALF genes, a BLASTn analysis was performed on the genome of the *F. vesca* (v4.0.a1 assembly) [55], using the 13 known *FveRALF* genes as query sequences. The BLAST search identified a putative new *FveRALF* gene located on chromosome 2 (Fvb2:15886205..15886408) and homolog to *FveRALF10* and coding for a protein lacking the RRILA cleavage site and the YISY active site, and having only two out of four cysteines at conserved positions, but presenting the RALF conserved domain in the C-terminal part of the protein sequence (S2 Fig). This hypothetical new RALF gene was named...
FveRALF14 (Table 1). Furthermore the BLASTn analysis revealed the presence of another predicted new FveRALF gene, with two RALF conserved domains both homolog to Arabidopsis AtRALF19 and alternatively translated in frame 1 or frame 2. This gene was annotated also in the previous V4.0.a1 version of FvH4_3g15010 and here named FveRALF15 (Table 1).

The FveRALF protein members were aligned using Clustal Omega (S3 Fig) and classified in clades according to Campbell and Turner [6] (Table 1). Overall, FveRALF clustered in three major clades (Table 1) instead of four, since FveRALF proteins of clade I and II were not distinguishable: namely clade I-II (FveRALF3, FveRALF7, FveRALF8, FveRALF13), clade III (FveRALF1, FveRALF2, FveRALF4, FveRALF5, FveRALF9 and FveRALF15), clade IV (FveRALF6, FveRALF9, FveRALF10, FveRALF11, FveRALF12, FveRALF14). The FveRALF include two members that aligned well with Arabidopsis AtRALF32 (FveRALF11, FveRALF6), two with similarity to AtRALF25 (FveRALF10, FveRALF14), three closely matching AtRALF19 (FveRALF1, FveRALF2, FveRALF4), four similar to AtRALF33 (FveRALF3, FveRALF8, FveRALF13, FveRALF9), and one respectively to AtRALF4 (FveRALF2), AtRALF5 (FveRALF12), AtRALF24 (FveRALF7) and AtRALF34 (FveRALF1). All FveRALF genes are predicted to contain a single exon and no introns, except for predicted FveRALF15 which possesses two exons and one large predicted intron. Interestingly, the transcript FveRALF3 originates from a putative Natural Antisense Transcript (NAT) generating region and its complementary sequence encodes for the 3’ untranslated region of a heat shock factor binding protein gene (FvH4_2g25350).

Identification, evolution and chromosome organization of RALF genes in F. x ananassa

F. vesca RALF gene sequences were used as query against a database of predicted proteins (v1.0.a1 Proteins source [35]) in F. x ananassa cv. Camarosa (Fxa). Fifty RALF members were identified in the Fxa octoploid strawberry (S1 Table). Paralogous genes (orthologs to particular
Five RALF genes were identified in the Fxa subgenomes from the alignment and phylogenetic analysis (S4 Fig and Fig 1A). Progenitors lineage was inferred from chromosome localization (Fig 1B) according to the prediction by Edger et al. [35]. Fourteen genes out of these 50 are localized in the F. vesca subgenome, 15 members in the F. nipponica subgenome, 13 in the F. iinumae subgenome and only eight in the F. viridis subgenome (Fig 1C). It is important to note that these assignments were based on the best subgenome match in the ‘Camarosa’ genotype. This is one accession and octoploid strawberry subgenomes structure may not be the same among all genotypes at this ploidy.

Fxa RALF genes (FanRALF) were named based on corresponding FveRALF orthologs and the subgenome localization, with progressive numbers from 1 to 4 to indicate F. vesca, F. iinumae, F. nipponica and F. viridis progenitors, respectively (accordingly to Edger et al.[35]), and progressive letters to nominate genes orthologous to the same FveRALF genes and localized on the same chromosome (e.g. FanRALF8-2a is one of the two Fxa genes orthologous to FveRALF8 mapping on F. iinumae subgenome).

Gene homology and chromosome localization analysis showed that only four out of 13 FveRALF genes, namely FveRALF1, FveRALF4, FveRALF7, FveRALF9, have orthologs in all the four subgenomes. For all the other cases the FveRALF orthologs are not represented in all the

Fig 1. Fragaria x ananassa (Fxa) RALF genes phylogenetic analysis, evolution and chromosome organization. (a) Phylogenetic tree was built aligning 50 FanRALF protein sequences using MUSCLE. The tree is drawn to scale, with branch lengths measured in the number of substitutions per amino acidic site in the protein sequences. Gene annotations refer to F. × ananassa cv. Camarosa v1.0.a1 and are listed in S1 Table. Progenitor lineage were inferred from gene chromosome location (b), and pink was used for F. vesca subgenome, green for F. nipponica, light blue for F. viridis and orange for F. iinumae. The color legend also reports the code number used to name different FanRALF genes according to subgenome lineage, as is reported in S1 Table. (b) FanRALF genes chromosome spatial organization in the octoploid genome. (c) Pie chart showing total FanRALF gene members present in the four subgenomes.

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different *Fxa* subgenomes, probably due to gene loss events occurred during evolution or polyplody adjustment (Fig 1A). In particular, the *F. viridis* derived subgenome has the lowest number of RALF gene members and is lacking genes orthologous to *FveRALF2*, *FveRALF5*, *FveRALF6*, *FveRALF8*, *FveRALF10*, *FveRALF11* and *FveRALF14*. Similarly to *F. vesca*, no RALF genes are localized in Chr7 of the different progenitors. It is likely that in the *Fxa* genome some of the RALF genes are the result of duplication events. For example *FanRALF11-1a* and *FanRALF11-1b* are both orthologous to *FveRALF11* and are positioned close together on Chr6 (*F. vesca* subgenomes Fig 1B). Another atypical gene organization is found for *FanRALF15* and *FanRALF4-1* genes, located on Chr3 (*F. vesca* subgenome), which are annotated as single genes but contain two tandem RALF conserved domains, suggesting that a duplication event occurred during genome evolution. In addition, the *FanRALF7-4* gene is predicted to encode a 325 aa protein containing a conserved RALF domain within the first 104 amino acids and a domain homologous to chloroplastic NADPH-dependent aldehyde-reductase like protein, from aa 124 to 325. There are many potential explanations for how this novel domain architecture could occur, and it will be interesting to test for functional relevance of this particular variant.

Genes *FanRALF9-3a* and *FanRALF9-3b*, occur as NAT element on Chr 5 (*F. nipponica* subgenome), as was observed in *F. vesca* for *FveRALF3*. The *F. viridis* derived subgenome contains the fewest detected putative RALF genes. This was also the case for R gene family in *Fxa* [35]. However, in contrast to the R gene family, there is not a clear dominance of *F. vesca* progenitor in the *FanRALF* gene family composition, since genes are similarly distributed in the *F. iinumae*, *F. nipponica* and *F. vesca* subgenomes (Fig 1C). As was speculated by Edger et al. [35] the lack of RALF genes in *F. viridis* subgenome could be related to the higher TE content of this subgenome which can cause both higher mutation rates and gene loss. *Fxa* RALF gene classification in specific clades agrees with the structure in diploid woodland strawberry, with 21 genes in clade IV, 17 genes in clade III, 12 in clade I-II (S1 Table).

**Transcriptome dataset analysis of RALF genes in *F. vesca***

To provide insights into the RALF gene members functions in strawberry, RNA-seq datasets that have been mapped onto the new genome annotation v4.0.a2 by Li et al. [34] were analyzed in different tissues and developmental stages. RALF members were grouped based on similar expression profile and hierarchical clustering resulted in four major RALF expression groups (Fig 2): i) RALF genes specifically expressed in mature male gamete (*FveRALF4*, *FveRALF5*, *FveRALF10*, *FveRALF11*); ii) a gene expressed only in two anther developmental stages (*FveRALF2*); iii) *FveRALF3* and *FveRALF12* genes mainly expressed in roots, and in roots after two days after infection with *Phytophthora cactorum*; iv) and genes mainly expressed in different fruit developmental stages (*FveRALF1*, *FveRALF6*, *FveRALF7*, *FveRALF8*, *FveRALF9* and *FveRALF13*).

Contrary to what has been observed in *Arabidopsis*, where clade IV RALF genes were highly expressed in flower tissues [6], the woodland strawberry *FveRALF* transcripts included in each of the four expression groups belong to different clades, suggesting that members of the same clade are have different roles in different contexts. The highly specific expression of four *FveRALF* genes in male gamete and late stage of anther development (*FveRALF4*, *FveRALF5*, *FveRALF10*, *FveRALF11*) shown by the heatmap (Fig 2), suggests a role for these RALF genes in the ovule-pollen, cell-cell communication during the sequence of events precisely regulated during fertilization. A recent study reports that in *Arabidopsis* *AtRALF34* gene, expressed in the ovule, competes with *RALF4* and *RALF19*, expressed in the pollen tube, for binding to BUPs.
and ANXs receptors [13]. The interaction between AtRALF34, expressed by the female gamete, and receptor complex formed by BUPS1/2 and ANX1/2, present on pollen tube membrane leads to pollen tube rupture and sperm release [13]. The competitive binding of AtRALF4 and AtRALF19 to this receptor complex suggest that they have a redundant function in regulating pollen tube growth and integrity [16]. It is possible that these RALF genes functional redundancy is conserved in woodland strawberry.

Among the FveRALF transcripts expressed in flower and fruit organs, FveRALF1 and FveRALF7 are the most abundant at the early stage of development in shoot apical meristem (SAM), floral meristem (FM) and receptacle meristem (RM), with FveRALF1 also being the

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**Fig 2. Detection of RALF transcripts in F. vesca.** The heatmap reflects the expression profile (as Transcription per Kilobase Million (TPM)) of FveRALF members (rows) in different tissues and developmental stages (column) of F. vesca. Labels at the bottom specify the tissues and the stages, whereas labels at the top group tissues and stages for organs. Dendrogram on the left shows rows relationship according to similar expression profile. RALF members classification in clades are shown: in green clade I, in blue clade II, in light blue clade III and magenta clade IV. SAM (shoot apical meristem), FM (floral meristem), REM (receptacle meristem), Anther7-8 (identified by stomium development and appearance of a preliminary lobed structure), Anther9 (microspore mother cells start meiosis), Anther10 (microspores are loose in the locale after calluse wall holding tetrads disaggregeates), Anther11 (pollen mitotic division occurs), anther12 (no visible change in anther development), Carpel7/8 (round carpel primordial reach the receptacle apex), Carp19 (bowling pin shaped carpel primordial), Carp110 (carpel is dived in almost equal apical and basal part by a central constriction), Carp111 (style is elongated and became twice in length than the ovary base), Carp112 (carpels have music note shape and styles are separated from each other), Cortex1 and Pith1 (flower just opened), Cortex2 and Pith2 (at about 3 DPA, when pollination occurs), Cortex3 and Pith3 (at about 6 DPA), Cortex4 and Pith4 (at about 9 DPA), Cortex5 and Pith5 (at about 12 DPA), Rg15D and Rg22D (Red Ruegen receptacle tissue at 15 DPA and at 22 DPA), Yw15D and Yw22D (Yellow Wonder receptacle tissue at 15 DPA and at 22 DPA), Embryo4 and Ghost4 (at about 9DPA, with immature cotyledons), Embryo5 and Ghost5 (ad about 12 DPA, mature embryo which fill up entire ovules), Leaf (young trifoliate leaves), Ovule1 and Pollen (collected from just open flower), Seed2 (complete achene from mature fruit), Seedling (complete seedling at 10 days post germination), Style1 (style and stigma from just open flowers), Style2 (style from flower at about 3 DPA), Wall1 (carpel wall from just open flower), Wall2 (carpel wall at about 3 DPA), Wall3 (carpel wall at about 6 DPA), Wall4 (carpel wall at about 9 DPA), Wall5 (carpel wall at about 12 DPA), Root (collected from 7 week old plants grown in aerated hydroponic culture) and Root_P (after 2 days of inoculation with Phytophthora cactorum).

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family member most highly expressed in the leaves. As for fruit, FveRALF8 is the gene most highly expressed in mature fruits, whereas FveRALF6, FveRALF7, FveRALF9 are detected during fruit growth and in the mature organ at 15 d post anthesis (15 DPA) both in Yellow Yonder and Red Rugen genotypes (the two F. vesca genotypes used for RNA seq) and FveRALF3 and FveRALF13 transcripts are most abundant in the mature fruits at 20 DPA. FveRALF1, FveRALF6, FveRALF7 and FveRALF13 expression decreases during fruit development both in the inner and the outer tissues of fruit while FveRALF9 expression gradually increases with fruit development.

In a recent work, Jia et al. [56] analyzed the expression of the woodland strawberry (F. vesca) Malectin Receptor Like Kinases (MRLK) also known as the Catharanthus roseus RLK-like proteins (RLK1Ls). F. vesca MRLKs are encoded by more than 60 genes, and more than 50% of these are expressed during fruit development. The majority of fruit FvMRLK genes are expressed at high level only at the early stage of fruit ripening, and decrease at ripe stages. Transiently silencing and overexpression of MRLK47 in strawberry fruit, severely affects ripening regulation [50]. Moreover, a recent report describes how MRKL47 changes the sensitivity of ripening-related genes to ABA, a key hormone for strawberry fruit ripening [57]. Consistently, both RALF and ABA were found to be FERONIA-mediated cross-talk signals in stress-response and cell growth in Arabidopsis [58]. FERONIA receptor is known to be important for cell-wall integrity and in Ca^{2+} signaling [59], both known to be important during fruit growth and ripening [60]. The similar expression profile of FveMRLKs to the one we observe here for FveRALF1, FveRALF6, FveRALF7 and FveRALF13 supports the idea that the RALF-MRLK signalling plays an important role for also for strawberry fruit development. Future studies will test the interaction between RALF peptides and FveMRLK in vivo.

Expression profile of RALF genes in Fragaria x ananassa fruit and induction upon pathogen infection

RALF peptides are known to play a role in plant-pathogen interaction [24] since they were found to negatively regulate plant immunity response in Arabidopsis [12]. They were also found to be secreted by fungal pathogen as crucial virulence factors [25, 61]. Furthermore, it was reported that genes homologous to AtRALF33 were upregulated both in tomato (Solanum lycopersicon) and commercial strawberry (F. x ananassa) susceptible ripe fruits interacting with C. gloeosporioides and C. acutatum, respectively [29, 31, 32].

For this reason, the transcript levels of FanRALF genes were assessed in F. x ananassa fruit at two different ripening stages and upon infection with two different fungal pathogens, C. acutatum or B. cinerea. Fruit FanRALF gene targets were chosen based on the FveRALF gene homologs expressed in fruit (Fig 2) since the F. vesca progenitor is reported to have the most dominant transcripts detected among the different subgenomes [35]. Therefore, primers were designed to amplify genes that are expressed in fruit (FanRALF1-1, FanRALF3-1, FanRALF6-1, FanRALF7-1, FanRALF8-1, FanRALF9-1 and FanRALF13-1), which included orthologs to AtRALF33 (FanRALF3-1, FanRALF8-1, FanRALF9-1 and FanRALF13-1).

FanRALF3 transcripts show a significant increase in abundance upon infection with either pathogen in the susceptible ripe stage, whereas FanRALF9 expression decreases in white fruits upon C. acutatum but not upon B. cinerea infections (Fig 3). The expression of FanRALF8 and FanRALF13 were not affected by infection, neither in white nor in red fruits (Fig 3). Out of the other FanRALF genes analyzed, only FanRALF6 shows a clear downregulation in infected fruits at both ripening stage and in response to both pathogens, while FanRALF1 and FanRALF7 gene expression is significantly decreased only in red ripe stage with B. cinerea and in white stage with C. acutatum, respectively.
The expression profiles of fruit RALF genes on Fxa strawberry fruits infected with two post-harvest pathogens are consistent with our previous results [30]. This report showed that transient overexpression of a FanRALF33-like gene (here now named FanRALF3) affected disease susceptibility. Notably, the FanRALF3 and FanRALF13 genes encode mature peptides differing only by two amino acids (S2 Fig) but accumulate differently to pathogen infection, suggesting that the genes possess subfunctionalized promoters that allow responses to different stimuli, or that the two amino acid difference leads to contrasting roles for the two different peptides.

Furthermore, N-terminal sequence alignment of FanRALF1-1, FanRALF3-1, FanRALF7-1, FanRALF8-1a and FanRALF13 peptides with AtRALF23 (Fig 4A) shows that residues directly involved in AtRALF23 binding with AtLLG2 are conserved, suggesting that FanRALF peptides in strawberry fruits may be the strawberry orthologs in the LLG-MRLK heterotypic complex. Consistently, the homology models of FanRALF3-1 peptide interaction with FanMRLK47—
the MRLK mostly expressed in Fxa fruits [56] and with the Fxa LLG2 homolog (maker-Fvb3-4-snap-gene-34.65), show that the structural components necessary to bind MRLK47 and LLG2 are conserved in FanRALF3 (Fig 4B and 4C), suggesting a similar binding mechanism and complex formation. In Arabidopsis, AtRALF23 binding to FERONIA and LLG proteins leads to negative immunity response regulation, and this hypothesis can be tested by analysis of FanRALF3-1 binding to MRLK47 in fruits.

Finally, the expression of FanRALF1, FanRALF6 and FanRALF9 decreases during infection, thus it is possible that these FanRALF gene members may play different roles than RALF3 homologs during plant-pathogen interaction and immune response.

**FanRALF3 promoter analysis in Fragaria x ananassa subgenomes and varieties**

As shown above and reported in previous studies, RALF gene expression is triggered by different biotic and abiotic stimuli, however the signaling events regulating its expression are not yet known. Identification of the promoter elements necessary for gene induction by fungal pathogens could provide a basis to identify the transcription factors and other mechanisms involved in immunity signaling and ultimately provide the necessary knowledge to develop synthetic pathogen-responsive promoters to fight infections. Among FanRALF family genes, FanRALF3 shows increases in transcripts in response to pathogen, and its overexpression in strawberry fruits is related to susceptibility [30]. For this reason FanRALF3 was chosen for promoter truncation analysis.

To study FanRALF3 putative promoter function in F. × ananassa, the upstream sequence conservation among the Fxa subgenomes was assessed. FanRALF3-1 sequence from the 3'UTR of upstream flanking gene (annotated as 'maker-Fvb2-2-augustus-gene-47.69-mRNA-1') and its ATG (590 bp) was used as input for BLASTn analysis against Fragaria x ananassa cv. Camarosa v1.0.a1. Five sequences were retrieved on F. iinumae Chr2-4, F. nipponica Chr2-1 and F. viridis Chr 2–3 (S4 Fig), indicating that the putative FanRALF3 homoeologous promoter sequences in the the Fxa subgenomes are highly similar, except for F. viridis, already reported to be the most divergent and silent in octoploid genome [35].

To study allelic variability, the FanRALF3-1 putative promoter sequence similarity was assessed also in genomes of F. × ananassa varieties with different susceptibility to fungal pathogens, the cv. Florida Elyana from Florida (U.S.A.) which is resistant to anthracnose disease [62], and cv. Alba, an italian variety which is highly susceptible to C. acutatum infection (https://plantgest.imagelinenetwork.com/it/varieta/frutticole/fragola/alba/59). The promoters were amplified with specific primers and five clones for each variety were sequenced and aligned with that of the v1.0.a1 genome sequence cv. Camarosa (S5 Fig). Only a single nucleotide polymorphism was detected between cvs. Alba and Florida Elyana. This suggests that the function associated with the FanRALF3-1 5’ upstream sequence in octoploid strawberry may be based on small differences in sequence, or that factors upstream of the promoter are the basis of the mechanism of resistance.

**Prediction of FveRALF3 promoter pathogen-responsive regulatory elements**

The FanRALF3-1 (from F.vesca subgenome) putative promoter sequence was chosen for pathogen-responsive regulatory element analysis because of the reported F. vesca subgenome dominance in octoploid genome [35]. Since Fa- and FveRALF3 upstream putative regulatory sequences share 99% level of identity and the available Fxa pathogen-responsive transcriptome data have all been mapped onto F. vesca genome, the analysis of the FanRALF3-1 promoter
regulatory elements responsive to pathogen infection were carried out on F. vesca genome FveRALF3 promoter.

The 656 bp genomic sequence upstream of FveRALF3 ATG start codon was analysed using truncation analysis. FveRALF3 putative promoter sequence was compared with known transcription factor binding sites of genes known to be regulated in Fxa strawberry fruit upon C. acutatum and B. cinerea infections [29, 49] in the PLACE database [51] (Motif Scanning analysis). These latter sequences and FveRALF3 putative promoter were then scored for motif frequency (Motif Discovery analysis) (Fig 5).

Motif scanning analysis of cis-acting elements enriched for fungal-induced transcripts revealed the presence of an element initially identified as initiator of PsaDb gene promoter (INRNTPSADB, PLACE ID: S000395). The putative promoter lacks a TATA-box, which is common to FveRALF3 and the majority of the 5′ upstream sequence (78 out of 87 (90%)) of the C. acutatum responsive genes promoter sequences and 92 out of 97 (94%) of B. cinerea genes (Fig 5). Other TATA-like elements such as TATABOX2 (PLACE ID: S000109) and TATAPVTRNALEU (S000340), which have the role of recognition and initiator of transcription complex [63, 64] were also found in several putative promoter sequences (59 (67%) from C. acutatum upregulated genes and in 41 (42%) of B. cinerea induced genes). Among the group of genes upregulated by C. acutatum, 33 (37%) and 47 (57%) predicted promoters

Fig 5. In silico analysis of predicted regulatory elements in pathogen-induced fragaria genes. (A) From the top: FveRALF3 gene putative promoter, Motif Scanning (MS) and Motif Discovery (MD) outputs resulting from the analyses of C. acutatum-induced genes, and MS and MD outputs from the analysis of B. cinerea-induced genes 24 h post-infection. Small colored boxes in MS indicate known regulatory elements from PLACE database found to be significantly abundant in the group of sequences analyzed and in FveRALF3 putative promoter, while large coloured boxes in MD represent sequences found significantly enriched in the upstream sequences of genes analyzed. (B) and (C) are the color key respectively for C. acutatum and B. cinerea reported in (A). Codes and names of regulatory elements are reported, together with elements percentage abundance among sequences analyzed (Sequences), total count of elements found (total), element background frequency calculated as number of elements found in a third order random generated sequences (Exp), ranking score values calculated by MotifLab software using a binominal test with p-value threshold of 0.05 (Score). Consensus sequence of each element is shown along the position of the element in the putative FveRALF3 promoter (Position bp) and the strand in which it is found.

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proteins are a large family of transcription factors involved in regulation of many processes in plants, such as phenylpropanoid metabolism [67], ABA and JA signaling [68], responses to abiotic and biotic stress [69], cell death and output from the circadian clock. MYB proteins generally interact with basic helix-loop-helix (bHLH) family member and are regulated by cytosolic WD40 repeat proteins through formation of MYB/bHLH/WD40 dynamic complexes, which regulate various gene expression pathways [70]. Interestingly, MYB46 is involved in enhancing B. cinerea resistance through down-regulation of cell wall associated genes (CESA) during early stage infection [71]. Arabidopsis T-DNA insertion mutants of genes regulated by MYB46, such as the a zinc-finger containing protein gene zfp2, the Basic Helix-Loop-Helix TF bhlh99, the AUX/IAA-type transcriptional repressor pap2 and the At1g66810 gene coding for a Zinc Finger Transcription Factor, showed enhanced susceptibility to the necrotrophic fungal pathogens B. cinerea and P. cucumerina [33]. The transcript analysis of these four mutants revealed a coincident upregulation of RALF23, RALF24, RALF32 and RALF33 [33] showing that MYB46 and RALF genes are part of a co-expression network, and possibly functionally related.

A Motif Discovery analysis was performed using AlignACE algorithm [52] on the putative promoter sequences of both FveRALF3 and the identified C. acutatum and B. cinerea strawberry upregulated genes, and significantly overrepresented motifs were assessed. The motifs identified using MotifLab software are named ‘AlignACE’ followed by progressive numbers. For C. acutatum genes group the AlignACE000085 element (consensus GxTxxXTGTGxxTTG) was found in 20 (23%) predicted promoters, and is partially overlapping with MYBZM elements at the position between bases 57 and 62 of FveRALF3 putative promoter. The AlignACE00098 (xxTGxxCTTGG) element was found in the 14 (16%) C. acutatum upregulated genes and align to the elements AlignACE00021 (TTGxxTTGG) found in B. cinerea upregulated group (19% of sequences analyzed). It is tempting to speculate that the position of this element might be a regulatory component important for FveRALF3 fungal-induced expression. Furthermore in B. cinerea gene group the AlignACE00040 (AAxxTGTGxxGxxAA), AlignACE00048 (TxGAAxxGGTC) and AlignACE00034 (AxTGAAxxTG) motifs, located between bases 262 and 317 in FveRALF3 putative promoter, were found significantly overrepresented.

Besides upregulation of FanRALF3, both pathogen infections led to the downregulation of FanRALF6 transcript accumulation (Fig 3), suggesting a role also for this gene during fruit defence response. In order to explore why these two genes showed opposite behaviour and to highlight the presence of similar cis-regulatory elements in FanRALF3 and FanRALF6 promoters, the predicted promoter sequence of FanRALF6 was also scored for the presence of annotated cis-acting elements and motif discovery by analysing sets of downregulated genes in datasets from C. acutatum and B. cinerea infected fruit tissues (Fig 6A).

Similar to the FanRALF3-1 predicted promoter, the FanRALF6-1 upstream sequence (F. vesca progenitor) has very high identity (99%) with F. vesca (data not shown), thus the regulatory element analysis was performed considering 1500 bp from ATG of FveRALF6 gene. Contrary to the high numbers of genes found upregulated upon C. acutatum and B. cinerea infections, examined in the FveRALF3 promoter analysis, the number of genes downregulated similarly to FveRALF6 is limited to 36 for C. acutatum and 16 for B. cinerea. The motif scanning analysis revealed that 100% of downregulated genes during C. acutatum early infection (Fig 6B) contain the INRNTPSADB element (S000395, [63]), found also in upregulated genes analysis (Fig 5), and known to particiapte in transcription initiation. Analysis also uncovered
the "REalpha" element (REALPHALGLHCB, S000362), a feature initially identified in Lhcb21 gene promoter of duckweed (Lemna gibba) [72, 73], that is involved in phytochrome regulation. This element is present in 30 out of 36 (83%) sequences analyzed with the consensus sequence AACCAA, and shares high similarity with sequences recognized by MYB family TFs. The motif has been identified in promoters of stress responsive genes, such as MYB1AT (S000408, aAACAA) and MYBATRD22 (S000175, CTAACCA) (S3 Table). MYB binding motifs (MYBPLANT and MYBZM) were also found in FanRALF3 and other genes upregulated upon fungal pathogen infection (Fig 5B). These preliminary analyses suggest a role of these conserved sequences in pathogen responsive gene regulation. On the other hand, the motif scanning analysis of B. cinerea downregulated genes did not show any significant result (Fig 6C).

In addition, the motif discovery analysis revealed the occurrence of conserved motifs among C. acutatum downregulated genes (Fig 6B) and in particular AlignACE00001 (AAAAAXAAAX) motif was found in 28 (77%), AlignACE00020 (TTTggATGAA) in 12 (33%), AlignACE00045 (TGxagAxtGAAa) in 16 (44%), AlignACE0004 (GAGAGAaAxxa) in 18 (50%) and AlignACE00026 (ctCGCGGxga) in 9 (25%) out of 36 sequences analyzed. The AlignACE00001 consensus motif (AAAAAXAAAX) is a A-reach sequence that has been reported to correlate with scaffold attachment regions [73] and in chromatin loop domain organization and transcription [74]. On the other hand, the AlignACE00004 motif (GAGAGAaAxxa) shows the typical sequences recognized by GAGA-binding proteins involved in chromatin silencing modification [75]. Motif discovery analysis performed on B. cinerea induced-downregulated genes revealed the enrichment of AlignACE00015 motif (gxTTTtxATGaxg) which was found in 9 out of 16 (56%) sequences analyzed.

Fig 6. FanRALF6 In silico analysis of predicted regulatory elements in pathogen-induced fragaria genes. (A) From the top: the FanRALF6 gene putative promoter, Motif Scanning (MS) and Motif Discovery (MD) outputs resulting from the analyses of C. acutatum-induced genes, and MS and MD outputs from the analysis of B. cinerea-induced genes 24 h post-infection. Small colored boxes in MS indicate known regulatory elements from PLACE database found to be significantly abundant in the group of sequences analyzed and in FanRALF6 putative promoter, while large colored boxes in MD represent sequences found significantly enriched in the upstream sequences of genes analyzed. (B) and (C) are the color key respectively for C. acutatum and B. cinerea reported in (A). Codes and names of regulatory elements are reported, together with elements percentage abundance among sequences analyzed (Sequences), total count of elements found (total), element background frequency calculated as number of elements found in a third order random generated sequences (Exp), ranking score values calculated by MotifLab software using a binomial test with p-value threshold of 0.05 (Score). Consensus sequence of each element is shown along the position of the element in the putative FanRALF6 promoter (Position bp) and the strand in which it is found.

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**FveRALF3 promoter-reporter assay**

In order to explore the functional relevance of the FanRALF3 putative promoter elements identified, three progressive truncated fragments of the FanRALF3-1 upstream sequence were cloned into pKGWFS7 vector and fused to two tandem reporter genes eGFP and β-glucuronidase (GUS) (S1 Fig). Deletions were 200 bp (T4) and 400 bp deletion (T2) (Fig 7A). *Agrobacterium*-mediated transient transformation of white *Fxa* fruits was performed through injection of bacteria transformed with each of the three constructs. Fruits were infected with *C. acutatum* and analyzed for both reporter genes activity at 24 hpi, through quantification of eGFP transcript in qRT-PCR and histochemical GUS assay for β-glucuronidase activity. GUS reporter activity, visualized as blue color of fruits, showed great variability among infected and mock-infected fruits (Fig 7B). Consistent with this, no significant difference was shown in the eGFP transcript level quantified in *C. acutatum* infected versus control fruit (Fig 7C). This is possibly due to the fruit response to *Agrobacterium* itself, independently from the fungal pathogen. Indeed *Agrobacterium* can be perceived as a pathogen by the fruit and stimulate similar responses as *C. acutatum*, including those leading to FanRALF3 expression. The variability affecting strawberry *Agrobacterium*-mediated transformation depending on technical and environmental conditions has recently been described [76]. It was shown that the expression level of a reporter gene is normally distributed in a population of 30 treated fruits, with huge variation among different fruits. Other important factors affecting agroinfiltration methodology are the quantity of bacteria injected for each fruits, the stage of fruit ripening and the temperature and incubation time after transient transformation. Perhaps for all of these reasons it was not possible to infer the identity of FanRALF3-1 promoter elements inducible by fungal pathogens such as *C. acutatum* in these experiments. However, the truncated promoter tests were informative. The GUS activity and eGFP expression were measurable and comparable.

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**Fig 7. Dissection of FanRALF3-1 promoter and agrobacterium-mediated reporter assay.** (A) Schematic representation of the promoter fragments used in this study. Pink squares indicate putative MYB-related regulatory elements, yellow squares indicates putative TATA-box related transcriptional activation elements. Histochemical GUS staining to detect β-glucuronidase activity in longitudinal fruit sections of Agroinfiltrated fruits. The table shows fruits transformed with negative (empty vector) and positive (double tandem p35S, 2x35S) controls. Fruits transformed with T6, T4 and T2 truncated promoter fragments were treated with *C. acutatum* or mock-treated and collected 24 h post-infection. (B) Histochemical GUS staining of fruits to detect β-glucuronidase activity in longitudinal fruit sections of Agro-infiltrated fruits. The table shows fruits transformed with negative (empty vector) and positive (double tandem p35S, 2x35S) controls. Fruits transformed with T6, T4 and T2 truncated promoter fragments were treated with *C. acutatum* or mock-treated and collected 24h post-infection. Three replicates for each conditions are shown. (C) Histogram showing qRT-PCR quantitative analysis of eGFP reporter expression. Each bar represents the average of three biological replicates. Expression values were normalized to pKGWFS7 empty vector infiltrated fruits. (C) Mock- treated samples, (I) *C. acutatum* infected.

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from the T4 and T6 sequences, while reporter activity from the T2 element was almost undetectable. These findings suggest that T4, comprising 400 bp sequence upstream FanRALF3-1 ATG, contains the necessary promoter sequence elements necessary to drive reporter gene expression in strawberry fruits and that the 200 bp region is not sufficient. The full-length upstream region which includes the 3’-UTR of the adjacent gene, shows no more activity that the 400 bp sequence, suggesting this region lacks additional elements to affect transcript accumulation over the T4 construct. According to Motif Scanning analysis, the T4 promoter fragment includes at least two regulatory elements known to be recognized by transcriptional activation complex, (TATA-boxes and Initiator of activation in TATA-less promoter) (Figs 5 and 7A).

Conclusions

Rapid Alkalinization Factors are small signal peptides with multiple roles in plant growth, fertilization and disease response. RALF genes are upregulated in different plant hosts upon pathogens attack and sequences similar to RALF genes are also expressed by many fungal pathogens as virulence factors, suggesting a role as susceptibility factors during plant pathogen interaction. The present work aimed to characterize the RALF gene family in F. vesca woodland and F. x ananassa octoploid strawberries according to tissue specific expression and similarity to Arabidopsis RALFs. The RALF gene family members distribution among F × ananassa subgenome is consistent with octoploid genome evolution, which is characterized by different transposable element activity in the different subgenomes and consequently differential gene distribution. A putative involvement of a MYB transcription factor as regulator of Fan-RALF3-1 infection-inducibility is speculated based on in silico promoter prediction and MYB motif recognition. This element is present in the 400 bp upstream the start codon of Fan-RALF3-1 gene sequence. Because FanRALF3-1 contains the same conserved N-terminal sequence as AtRALF23 it is speculated that FanRALF3-1 interaction with receptor FERONIA (MRLK47) and the coreceptor FanLLG2 may follow the Arabidopsis complex interaction structure. Future efforts will seek to identify specific pathogen-responsive promoter elements and their role in strawberry disease resistance.

Supporting information

S1 Fig. Map of pKGWFS7 plasmid used for promoter reporter assay. Sm/Spr, spectinomycin resistance. Kan, kanamycin resistance. FveRALF3 promoter, T6,T4,T2, empty or 2x35S promoter according to experimental procedure. eFGPand GUS, chimera reporter gene formed by eGFP and β-glucoronidase ORFs in frame. T35S, terminator. (PNG)

S2 Fig. F.vesca RALF peptide sequences aligned in clustal omega. Black boxes highlight conserved domains RRILA cleavage site, YISY activation site, and conserved cysteines (*). The two proteins coded by FveRALF15 in different frames were annotated as FveRALF15-F1 (5’-3’ Frame1) and FveRALF15-F2 (5’-3’ Frame2). (PNG)

S3 Fig. FveRALF phylogenetic analysis and clades determination. Phylogenetic tree shows the classification of FveRALF genes in three clades, which were named according to sequence feature similarity with Campbell and Turner clades classification. clade IV (magenta), clade III (light blue) and clade I-II (blue). Neighbour-joining tree values are listed near genes name. (PNG)

S4 Fig. F.vesca and Fragaria x ananassa RALF peptide sequences aligned in clustal omega. Black boxes highlight conserved domains RRILA cleavage site, YISY activation site, and
conserved cysteines (\(\cdot\)).

S5 Fig. Blastn output of \textit{FanRALF3-1} upstream sequence against \textit{Fragaria x ananassa cv. Camarosa} v1.0.a1 pseudomolecule using GDR.

S6 Fig. \textit{FanRALF3-1} putative promoter sequence alignment in different \textit{Fragaria x ananassa} varieties. It was considered \textit{Fragaria x ananassa cv. Florida Elyana} from Florida (U.S.A), the Italian variety \textit{cv. Alba} and the sequenced \textit{cv. Camarosa (v1.0.a1)}.

S1 Table. List of \textit{Fragaria x ananassa} RALF genes identified through \textit{FveRALFs} BLASTx (GDR). In the table are listed for each gene, Chromosome localization ‘Chr’, subgenome localization ‘Subg.’, gene identification number used to name FanRALF in this work ‘geneID’, ‘\textit{Fragaria x ananassa cv. Camarosa} transcript v1.0.a1 annotation’, ‘Genome location’ coordinates, classification in clades according to Campbell and Turner (2017) ‘clade’, \textit{F. vesca} orthologous gene ‘Fv.Ort.’, ‘E-value’ and identity rate ‘identity’. ‘Gene ID’ column presents ‘\textit{v1short name}’ reporting gene v1 annotation abbreviation used to identify genes in Fig 1A and ‘orthology based’ nomenclature, assigned according to \textit{FveRALF} orthology and chromosome lineage: -1, -2,-3,-4 after \textit{FveRALF} orthologous name were used respectively to indicate \textit{F. vesca}, \textit{F. iinnu-mae}, \textit{F. nipponica} and \textit{F. viridis} progenitors, progressive letters was used to name genes orthologous to the same \textit{FveRALF} gene.

S2 Table. List of primers used for RALFs qRT-PCR expression analysis In \textit{Fragaria x ananassa} infected fruits, and for reporter gene expression in transient transformed fruits.

S3 Table. Motif similarity analysis comparing target motif "S000362" against motifs from "PLACE". The table shows MotifLab ‘motif similarity analysis’ output listing: PLACE motif identification code (Motif ID), Motif abbreviated name (Name), Average Log-Likelihood Ratio values (ALLR), Chi-squared values (Chi2), Kullback-Leibler Divergence values (KLD), Pearson’s correlation values (Corr), Pearson’s correlation weighted values (Corrw), sum of squared distances values (SSD) and consensus sequence (Logo). Motifs are sorted according to ALLR values.

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References

1. Olsson V, Joos L, Zhu S, Gevaert K, Butenko MA, De Smet I. Look Closely, the Beautiful May Be Small: Precursor-Derived Peptides in Plants. Annu Rev Plant Biol. 2019; 70(1):153–86. https://doi.org/10.1105/tpc.112.099010 PMID: 22932676

2. Murphy E, Smith S, De Smet I. Small Signaling Peptides in Arabidopsis Development: How Cells Communicate Over a Short Distance. Plant Cell. 2012 Aug; 24(8):3198–217. https://doi.org/10.1105/tpc.112.101161 PMID: 22932676

3. Tavormina P, De Coninck B, Nikonorova N, De Smet I, Cammue BPA. The Plant Peptidome: An Expanding Repertoire of Structural Features and Biological Functions. Plant Cell. 2015; https://doi.org/10.1105/tpc.15.00440 PMID: 2715375

4. Pearce G, Moura DS, Stratmann J, Ryan CA. RALF, a 5-kDa ubiquitous polypeptide in plants, arrests root growth and development. Proc Natl Acad Sci. 2001; 98(22):12843–7. https://doi.org/10.1073/pnas.201416998 PMID: 11675511

5. Sharma A, Hussain A, Mun BG, Imran QM, Falak N, Lee SU, et al. Comprehensive analysis of plant rapid alkalization factor (RALF) genes. Plant Physiol Biochem. 2016; 106:82–90. https://doi.org/10.1016/j.plaphy.2016.03.037 PMID: 2715375

6. Campbell L, Turner SR. A Comprehensive Analysis of RALF Proteins in Green Plants Suggests There Are Two Distinct Functional Groups. Front Plant Sci. 2017; 8(January):1–14.

7. Srivastava R, Liu JX, Guo H, Yin Y, Howell SH. Regulation and processing of a plant peptide hormone, ATRALF23, in Arabidopsis. Plant J. 2009; 59(6):930–9. https://doi.org/10.1111/j.1365-313X.2009.03296.x PMID: 19473327

8. Pearce G, Yamaguchi Y, Munske G, Ryan CA. Structure-activity studies of RALF, Rapid Alkalization Factor, reveal an essential—YSY—motif. Peptides. 2010; 31(11):1973–7. https://doi.org/10.1016/j.peptides.2010.08.012 PMID: 20806938

9. Xiao Y, Stegmann M, Han Z, DeFalco TA, Parys K, Xu L, et al. Mechanisms of RALF peptide perception by a heterotopic receptor complex. Nature. 2019; https://doi.org/10.1038/s41586-019-1409-7 PMID: 31291624

10. Galindo-Trigo S, Gray JE, Smith LM. Conserved Roles of CrRLK1L Receptor-Like Kinases in Cell Expansion and Reproduction from Algae to Angiosperms. Front Plant Sci. 2016; https://doi.org/10.3389/fpls.2016.01269 PMID: 27621737

11. Haruta M, Sabat G, Stecker K, Minkoff BB, Sussman MR. A peptide hormone and its receptor protein kinase regulate plant cell expansion. Science (80-). 2014; 343(6169):408–11.

12. Stegmann M, Monaghan J, Smakowska-Luzan E, Rozenich H, Lehner A, Holton N, et al. The receptor kinase FER is a RALF-regulated scaffold controlling plant immune signaling. Science (80-). 2017; 355(6322):287–9.

13. Ge Z, Bergonci T, Zhao Y, Zou Y, Du S, Liu MC, et al. Arabidopsis pollen tube integrity and sperm release are regulated by RALF-mediated signaling. Science (80-). 2017;

14. Gonneau M, Desprez T, Martin M, Doblas VG, Bacete L, Miart F, et al. Receptor Kinase THESEUS1 Is a Rapid Alkalization Factor 34 Receptor in Arabidopsis. Curr Biol. 2018; 28(15):2452–2458.e4. https://doi.org/10.1016/j.cub.2018.05.075 PMID: 30057301

15. Li C, Yeh F-L, Cheung AY, Duan Q, Kita D, Liu M-C, et al. Glycosylphosphatidylinositol-anchored proteins as chaperones and co-receptors for FERONIA receptor kinase signaling in Arabidopsis. Elife. 2015 Jun 8; 4. https://doi.org/10.7554/eLife.06587 PMID: 26052747

16. Mecchia MA, Ringi C, Boisson-Dernier A, Santos-Fernandez G, Martinez-Bernardini A, Grossniklaus U, et al. RALF4/19 peptides interact with LRX proteins to control pollen tube growth in Arabidopsis. Science (80-). 2017; 355(6322):287–9.

17. Moussu S, Broyart C, Santos-Fernandez G, Augustin S, Wehrle S, Grossniklaus U, et al. bioRxiv [preprint]. Structural basis for recognition of RALF peptides by LRX proteins during pollen tube growth. bioRxiv.2019; 695874. [posted 2019 Aug 5] available from https://www.biorxiv.org/content/10.1101/695874v1
18. Dünser K, Gupta S, Herger A, Feraru MI, Ringli C, Kleine-Vehn J. Extracellular matrix sensing by FERONIA and Leucine-Rich Repeat Extensins controls vacuolar expansion during cellular elongation in Arabidopsis thaliana. EMBO J. 2019; 38(7):1–12.

19. Wu J, Kurten EL, Monshausen G, Hummel GM, Gilroy S, Baldwin IT. NaRALF, a peptide signal essential for the regulation of root hair tip apoplastic pH in Nicotiana attenuata, is required for root hair development and plant growth in native soils. Plant J. 2007; https://doi.org/10.1111/j.1365-313X.2007.03289.x PMID: 17916115

20. Du C, Li X, Chen J, Chen W, Li B, Li C, et al. Receptor kinase complex transmits RALF peptide signal to inhibit root growth in Arabidopsis. Proc Natl Acad Sci. 2016; https://doi.org/10.1073/pnas.1609626113 PMID: 27930296

21. Atkinson NJ, Lilley CJ, Urwin PE. Identification of Genes Involved in the Response of Arabidopsis to Simultaneous Biotic and Abiotic Stresses. Plant Physiol. 2013; 162(4):2028–41. https://doi.org/10.1104/pp.113.222372 PMID: 23800991

22. Covey PA, Subbaiah CC, Parsons RL, Pearce G, Lay FT, Anderson MA, et al. A Pollen-Specific RALF from Tomato That Regulates Pollen Tube Elongation. Plant Physiol. 2010; 153(2):703–15. https://doi.org/10.1104/pp.110.155457 PMID: 20388667

23. Ge Z, Cheung AY, Qu LJ. Pollen tube integrity regulation in flowering plants: insights from molecular assemblies on the pollen tube surface. New Phytologist. 2019. https://doi.org/10.1111/nph.15645 PMID: 30556141

24. Fernandes TR, Segorbe D, Prusky D, Di Pietro A. How alkalization drives fungal pathogenicity. PLOS Pathog. 2017; 13(11):e1006621. https://doi.org/10.1371/journal.ppat.1006621 PMID: 29121119

25. Masachis S, Segorbe D, Turrà D, Leon-Ruiz M, Fürst U, El Ghalid M, et al. A fungal pathogen secretes plant alkalinizing peptides to increase infection. Nat Microbiol. 2016; 1(6). https://doi.org/10.1038/nmicrobiol.2016.43 PMID: 27572834

26. Boa E. Compendium of Strawberry Disease. American Phytopathological Society Press. Plant Pathol. 2001 Feb; 50(1):138–138.

27. Prusky D. PATHOGEN QUIESCENCE IN POSTHARVEST DISEASES. Annu Rev Phytopathol. 1996; 28.

28. Feliziani E, Romanazzi G. Postharvest decay of strawberry fruit: Etiology, epidemiology, and disease management. J Berry Res. 2016; 6(1):47–63.

29. Guidarelli M, Carbone F, Bourjailles JF, Bertolini P, et al. Colletotrichum acutatum interactions with unripe and ripe strawberry fruits and differential responses at histological and transcriptional levels. Plant Pathol. 2011; 60(4):685–97.

30. Merino MC, Guidarelli M, Neuman F, De Biase D, Pession A, Baraldi E. Induced expression of the Fragaria × ananassa Rapid alkalinization factor-33-like gene decreases anthracnose ontogenetic resistance of unripe strawberry fruit stages. 2019;1–12.

31. Wang P, Yao S, Kosami K, Guo T, Li J, Zhang Y, et al. Identification of endogenous small peptides involved in rice immunity through transcriptomics- and proteomics-based screening. Plant Biotechnol J. 2019;1–14. https://doi.org/10.1111/pbi.13208 PMID: 31301098

32. Alkan N, Prusky D, Fluhr R, Friedlander G, Ment D. Simultaneous transcriptome analysis of Colletotrichum gloeosporioides and tomato fruit pathosystem reveals novel fungal pathogenicity and fruit defense strategies. New Phytol. 2014; 205(2):801–15. https://doi.org/10.1111/nph.13087 PMID: 25377514

33. Dobón A, Canet JV, García-Andrade J, Angulo C, Neumetzler L, Persson S, et al. Novel Disease Susceptibility Factors for Fungal Necrotrophic Pathogens in Arabidopsis. PLoS Pathog. 2015; 11(4):1–30.

34. Li Y, Pi M, Gao Q, Liu Z, Kang C. Updated annotation of the wild strawberry Fragaria vesca V4 genome. Hortic Res. 2019; 6(1). https://doi.org/10.1038/s41438-019-0142-6 PMID: 31069085

35. Edger PP, Poorten TJ, VanBuren R, Hardigan MA, Colle M, McKain MR, et al. Origin and evolution of the octoploid strawberry genome. Nat Genet. 2019; 51(3):237–45. https://doi.org/10.1038/s41588-019-0356-4 PMID: 30804557

36. Folta KM, Barbey CR. The strawberry genome: a complicated past and promising future. Hortic Res. 2019; 6(1):97. https://doi.org/10.1038/s41438-019-0181-z PMID: 31645955

37. Yuan H, Yu H, Huang T, Shen X, Xia J, Pang F, et al. The complexity of the Fragaria × ananassa (octoploid) transcriptome by single-molecule long-read sequencing. Hortic Res. 2019; 6(1). https://doi.org/10.1038/s41438-019-0126-6 PMID: 30962939

38. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004; https://doi.org/10.1093/nar/gkh340 PMID: 15034147

39. Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. Bioinformatics. 1992; https://doi.org/10.1093/bioinformatics/8.3.275 PMID: 1633570
40. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018; 35(6):1547–9. https://doi.org/10.1093/molbev/msy096 PMID: 29722887

41. Anand L. chromoMap: An R package for Interactive Visualization and Annotation of Chromosomes. bioRxiv 2019 [preprint]. [posted 2019 Apr 11]. https://www.biorxiv.org/content/10.1101/605600v1;

42. Zhao S, Guo Y, Sheng Q, Shyr Y. Heatmap3: an improved heatmap package with more powerful and convenient features. BMC Bioinformatics. 2014; https://doi.org/10.1186/1471-2105-15-s10-p16

43. Kang C, Darwish O, Geretz A, Shaham R, Alkharouf N, Liu Z. Genome-Scale Transcriptomic Insights into Early-Stage Fruit Development in Woodyland Strawberry Fragaria vesca. Plant Cell. 2013 Jun 1; 25 (6):1960–78. https://doi.org/10.1105/tpc.113.111732 PMID: 23898027

44. Hollender CA, Kang C, Darwish O, Geretz A, Matthews BF, Slovin J, et al. Floral transcriptomes in woodland strawberry uncover developing receptacle and anther gene networks. Plant Physiol. 2014; 165(3):1062–75. https://doi.org/10.1104/pp.114.237529 PMID: 24283807

45. Hawkins C, Caruana J, Li J, Zawora C, Darwish O, Wu J, et al. An eFP browser for visualizing strawberry fruit and flower transcriptomes. Hortic Res. 2017; https://doi.org/10.1038/hortres.2017.29 PMID: 28674614

46. Toljamo A, Blande D, Kärenlampi S, Kokko H. Reprogramming of Strawberry (Fragaria vesca) Root Transcriptome in Response to Phytophthora cactorum. Gijzen M, editor. One PLoS. 2016 Aug 12; 11 (8):e0161078. https://doi.org/10.1371/journal.pone.0161078 PMID: 27518577

47. Gambino G, Perrone I, Gribaudo I. A rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. Phytochem Anal. 2008; https://doi.org/10.1002/pca.1078 PMID: 18618437

48. Amil-Ruiz F, Garrido-Gala J, Blanco-Por tales R, Folta KM, Muñoz-Blanco J, Caballero JL. Identification and Validation of Reference Genes for Transcript Normalization in Strawberry (Fragaria × ananassa) Defense Responses. PLoS One. 2013; https://doi.org/10.1371/journal.pone.0070603 PMID: 23940602

49. Xiong JS, Zhu HY, Bai YB, Liu H, Cheng ZM. RNA sequencing-based transcriptome analysis of mature strawberry fruit infected by necrotrophic fungal pathogen Botrytis cinerea. Physiol Mol Plant Pathol. 2018; 104(August):77–85.

50. Klepper K, Drablès F. MotifLab: a tools and data integration workbench for motif discovery and regulatory sequence analysis. 2013; https://doi.org/10.1186/1471-2105-14-9

51. Higo K, Ugawa Y, Iwamoto M, Higo H. PLACE: a database of plant cis-acting regulatory DNA elements. 1998; 26(1):358–9.

52. Hughes JD, Estep PW, Tavazoie S, Church GM. Computational identification of Cis-regulatory elements associated with groups of functionally related genes in Saccharomyces cerevisiae. J Mol Biol. 2000; 296(5):1205–14. https://doi.org/10.1006/jmbi.2000.3519 PMID: 10698627

53. Sali A, Blundell T. Sali A. & Blundell T. L. Comparativ e modelling by satisfaction of spatial restraints. J. Mol. Biol. 234, 779–815. Journal of molecular biology. 1994. https://doi.org/10.1006/jmbi.1993.1626 PMID: 8254673

54. Jung S, Bassett C, Bielenberg DG, Cheng CH, Dardick C, Main D, et al. A standard nomenclature for gene designation in the Rosaceae. Tree Genet Genomes. 2015; https://doi.org/10.1007/s11295-015-0931-5

55. Edger P, VanBuren R, Collé M, Poorten TJ, Wai CM, Niederhut h CE, et al. Single-molecule sequencing and optical mapping yields an improved genome of woodland strawberry (Fragaria vesca) with chromosome-scale contiguity. GigaScienc., Volume 7, Issue 2, February 2018, gix124, https://doi.org/10.1038/giga/2019/gix124

56. Jia M, Ding N, Zhang Q, Xing S, Wei L, Zhao Y, et al. A FERONIA-Like Receptor Kinase Regulates Strawberry (Fragaria × ananassa) Fruit Ripening and Quality Formation. Front Plant Sci. 2017; 8 (June):1–14.

57. Moya-león MA, Mattus-araya E, Herrera R. Molecular Events Occurring During Softening of Strawberry Fruit. 2019; 10(May). https://doi.org/10.3389/fpls.2019.00615 PMID: 31156678

58. Chen J, Yu F, Liu Y, Du C, Li X, Zhu S, et al. FERONIA interacts with ABI2-type phosphatases to facilitate signaling cross-talk between abscisic acid and RALF peptide in Arabidopsis. 2016;55:19–27.

59. Feng W, Kita D, Peaucelle A, Cartwright HN, Doan V, Duan Q, et al. The FERONIA Receptor Kinase Maintains Cell-Wall Integrity during Salt Stress through Ca2+ Signaling.Curr Biol. 2018; https://doi.org/10.1016/j.cub.2018.01.023 PMID: 29456142

60. Forlani S, Masiero S, Mizzotti C. Fruit ripening: the role of hormones, cell wall modifications, and their relationship with pathogens. J Exp Bot. 2019; https://doi.org/10.1093/jxb/erz112 PMID: 30854549

61. Thynne E, Saur IML, Simbaqueba J, Ogivie HA, Gonzalez-Cendales Y, Mead O, et al. Fungal phytopathogens encode functional homologues of plant rapid alkalinization factor (RALF) peptides. Mol Plant Pathol. 2017; 18(6):811–24. https://doi.org/10.1111/mpp.12444 PMID: 27281634
62. Seijo TE, Chandler CK, Mertely JC, Moyer C, Peres NA. Resistance of strawberry cultivars and advanced selections to Anthracnose and Botrytis fruit rots. Proc Florida State Hortic Soc. 2008; 121:246–8.

63. Nakamura M, Tsunoda T, Obokata J. Photosynthesis nuclear genes generally lack TATA-boxes: A tobacco photosystem I gene responds to light through an initiator. Plant J. 2002; https://doi.org/10.1046/j.0960-7412.2001.01188.x PMID: 12602222

64. Grace ML, Chandrasekharan MB, Hall TC, Crowe AJ. Sequence and Spacing of TATA Box Elements Are Critical for Accurate Initiation from the β-Phaseolin Promoter. J Biol Chem. 2004;

65. Merida A, Mackay S, Culianez-Macia FA, Parr A, Roberts K, Martin C, et al. The AmMYB308 and AmMYB330 Transcription Factors from Antirrhinum Regulate Phenylpropanoid and Lignin Biosynthesis in Transgenic Tobacco. Plant Cell. 2007; https://doi.org/10.1038/s3870694

66. Grotewold E, Drummond BJ, Bowen D, Peterson B. The myb-homologous P gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. Cell. 1994; https://doi.org/10.1016/0092-8674(94)90117-1

67. Liu J, Osbourn A, Ma P. MYB transcription factors as regulators of phenylpropanoid metabolism in plants. Mol Plant. 2015; 8(5):689–708. https://doi.org/10.1016/j.molp.2015.03.012 PMID: 25840349

68. Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell. 2003; https://doi.org/10.1105/tpc.006130 PMID: 12509522

69. Ramirez V, Agorio A, Coego A, García-Andrade J, Hernández MJ, Balaguer B, et al. MYB46 Modulates Disease Susceptibility to Botrytis cinerea in Arabidopsis. Plant Physiol. 2011; https://doi.org/10.1104/pp.110.171843 PMID: 21282403

70. Pireyre M, Burrow M. Regulation of MYB and bHLH transcription factors: A glance at the protein level. Mol Plant. 2015; 8(3):378–88. https://doi.org/10.1016/j.molp.2014.11.022 PMID: 25667003

71. Ramirez V, García-Andrade J, Vera P. Enhanced disease resistance to botrytis cinerea in myb46 arabidopsis plants is associated to an early downregulation of CesA genes. Plant Signal Behav. 2011; https://doi.org/10.4161/psb.6.6.15354 PMID: 21617373

72. Degenhardt J, Tobin EM. A DNA binding activity for one of two closely defined phytochrome regulatory elements in an Lhcb promoter is more abundant in etiolated than in green plants. Plant Cell. 1996; https://doi.org/10.2307/3870066

73. Breney P, van Montagu M, Gheysen G. The role of scaffold attachment regions in the structural and functional organization of plant chromatin. Transgenic Res. 1994; 3(3):195–202. https://doi.org/10.1007/bf01973987 PMID: 8025597

74. Pascuzzi PE, Flores-Vergara MA, Lee TJ, Sosinski B, Vaughan MW, Hanley-Bowdoin L, et al. In vivo mapping of Arabidopsis scaffold/matrix attachment regions reveals link to nucleosome-disfavoring poly (dA:dT) tracts. Plant Cell. 2014; 26(1):102–20. https://doi.org/10.1105/tpc.113.121194 PMID: 24488963

75. Hecker A, Brand LH, Peter S, Simoncino N, Killian J, Harter K, et al. The Arabidopsis GAGA-binding factor BASIC PENTACYSTEINE6 recruits the POLYCOMB-REPRESSIVE COMPLEX1 component LIKE HETEROCHROMATIN PROTEIN1 to GAGA DNA motifs. Plant Physiol. 2015; 168(3):130–41.

76. Zhao Y, Mao W, Chen Y, Wang W, Dai Z, Dou Z, et al. Optimization and standardization of transient expression assays for gene functional analyses in strawberry fruits. Hortic Res. 2019; 6(1). https://doi.org/10.1038/s41438-019-0135-5 PMID: 31069083