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Induced expression of the *Fragaria* × *ananassa Rapid alkalinization factor-33-like* gene decreases anthracnose ontogenic resistance of unripe strawberry fruit stages

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SUMMARY

Rapid alkalinization factor (RALF) genes encode for ubiquitous small peptides that stimulate apoplastic alkalinization through interaction with malectin-like receptor kinase. RALF peptides may act as negative regulators of plant immune response, inhibiting the formation of the signal receptor complex for immune activation. Recently RALF homologues were identified in different fungal pathogen genomes contributing to host infection ability. Here, *FaRALF-33-like* gene expression was evaluated in strawberry fruits inoculated with *Colletotrichum acutatum, Botrytis cinerea*, or *Penicillium expansum* after 24 and 48 h post-infection. To investigate the role of *FaRALF-33-like* in strawberry susceptibility, transient transformation was used to overexpress it in white unripe fruits and silence it in red ripe fruits. Agroinfiltrated fruits were inoculated with *C. acutatum* and expression, and histological analysis of infection were performed. Silencing of *FaRALF-33-like* expression in *C. acutatum*-inoculated red fruits led to a delay in fruit colonization by the fungal pathogen, and infected tissues showed less penetrated infective hyphae than in wild-type fruits. In contrast, *C. acutatum*-inoculated white unripe fruits overexpressing the *FaRALF-33-like* gene decreased the ontogenic resistance of these fruits, leading to the appearance of disease symptoms and penetrated subcuticular hyphae, normally absent in white unripe fruits. The different response of transfected strawberry fruits to *C. acutatum* supports the hypothesis that the *FaRALF-33-like* gene plays an important role in the susceptibility of fruits to the fungal pathogen *C. acutatum*.

Keywords: *Colletotrichum acutatum*, *Fragaria* × *ananassa*, fungi, RALF, ripening.

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INTRODUCTION

Plants are naturally provided with very sophisticated and strong immune mechanisms and the development of diseases is generally dependent on the 'switch-off' state of these mechanisms or the 'switch-on' state of susceptibility mechanisms. Besides suppression or plant immunity evasion, pathogens, in particular biotrophs and hemibiotrophs, require cooperation of the host for the establishment of a compatible interaction. Accommodating the pathogen involves enabling it to establish feeding structures inside the host cell to obtain nutrients. All plant genes that facilitate infection and support compatibility can be considered susceptibility genes. Mutation or silencing of a susceptibility gene can therefore limit the ability of the pathogen to cause disease (van Schie and Takken, 2014). Lately, the possibility of controlling susceptibility genes has emerged as a more promising approach to develop resistant crops than the control of resistance genes. However, knowledge of the susceptibility factors present in plants favouring pathogen invasion and/or growth remains limited.

Small secreted peptides have achieved significant relevance as signalling molecules in plants since they play a key role in the regulation of plant defence and development. Among them, the members of the rapid alkalinization factor (RALF) family were first identified through their ability to trigger a rapid increase in extracellular pH when added to plant cell suspensions (Pearce *et al.*, 2001). RALF peptides have been linked to several physiological and developmental processes, including cell expansion (Haruta *et al.*, 2014), lateral root development (Murphy *et al.*, 2016), root hair growth (Wu *et al.*, 2007), pollen tube elongation (Covey *et al.*, 2010), nodulation in legumes (Djordjevic *et al.*, 2015) and stress (Atkinson *et al.*, 2013). *In silico* analysis of the *Arabidopsis thaliana* genome identified 37 genes belonging to the RALF family, named RALF1 to RALF37 (Olsen *et al.*, 2002), and the homologues identified in other species have been named according to their similarities to the members of the

A. thaliana family (Campbell and Turner, 2017). The RALF pleiotropic role indicates that these peptides are key players in plant development. Dobón *et al.* (2015) showed that *zfp2, bhlh99, pap2* and *At1g66810 Arabidopsis* mutants, which are susceptible to *Botrytis cinerea and Plectosphaerella cucumerina*, share a common transcriptional signature of 77 up-regulated genes, including RALF. These genes function as positive regulators of disease susceptibility, and their expression is induced during the course of a pathogen infection.

Colletotrichum acutatum, the hemibiotrophic agent of anthracnose disease, causes severe economic losses to strawberry (*F.* × *ananassa*) production all over the world. The heaviest economic losses are the result of fruit infections, which can occur on either immature fruits preharvest or mature fruits at harvest or in the postharvest storage stage. In all cases, however, anthracnose symptoms become apparent only during storage or shelf life, when fruit production reaches its highest value. This is because of the ability of *Colletotrichum* species to develop latent infections on immature fruits, becoming quiescent until fruit ripens (Prusky, 1996). On white fruits, *C. acutatum* becomes quiescent as melanized appressoria after 24 h of interaction (Guidarelli *et al*., 2011). Quiescence is a well-known phenomenon among fruit fungal pathogens. *Colletorichum acutatum* is one of the most common species causing quiescent infections, developing distinct appressoria which are melanized in the immature state of the fruits (Emmett and Parbery, 1975). Appressoria formation, while enabling penetration of the host, is key for fungus survival under unfavourable conditions (Adikaram *et al*., 2015). Therefore, the growth arrest as melanized appressoria is a hallmark of the dormant state of the fungus (Guidarelli *et al*., 2011). Only when fruit ripens to the red stage the pathogen restores its growth and symptoms become apparent within 3 days.

Guidarelli *et al.* (2011) found that a *RALF* gene, a homologue to *AtRALF33*, is up-regulated after 24 h of *C. acutatum* inoculation in red ripe strawberry fruits, where the pathogen is active, whereas no difference in the expression of this gene was found in inoculated white unripe fruits.

Here, we aimed to analyse the putative role as a susceptibility gene of *FaRALF33-like* gene in anthracnose development in strawberry after *C. acutatum* inoculation. To test whether RALF up-regulation also occurs in fungal infections other than *Colletotricum*, the *FaRALF-33-like* expression in white unripe and red ripe fruits inoculated with *C. acutatum*, *Botrytis cinerea*, or *Penicillium expansum* was analysed at 24 and 48 h post-inoculation (hpi). Thereafter, *Agrobacterium tumefaciens*-mediated transient transformation was used to silence *FaRALF-33-like* gene in red ripe strawberry fruits and, in parallel, to overexpress this gene in white unripe fruits, all inoculated with *C. acutatum*. Anthracnose symptoms of transfected white and red strawberries inoculated with *C. acutatum* were monitored following histological analysis of the infected tissues. The results indicate that this gene influences the appearance of disease symptoms in strawberry fruits, suggesting an important role for *FaRALF-33-like* gene in the different susceptibility of these fruits to the pathogen.

RESULTS

FaRALF-33-like expression was up-regulated in red ripe strawberry fruits at 24 h on *C. acutatum* or *B. cinerea* inoculation, whereas no difference was observed on *P. expansum* inoculation

The full-length cDNA of *FaRALF33-like* gene previously found to be up-regulated in 24 h *Colletotrichum acutatum*-inoculated red *Fragaria* × *ananassa* fruits was isolated by PCR using primers designed on the *FvRALF-33-like* (GenBank accession number: XM_011460413.1). *FaRALF-33-like* cDNA is 345 bp long and codes for a protein of 114 amino acids. Peptide sequence alignment with the homologous proteins *AtRALF33* and *FvRALF-33-like* highlights typical sequence motifs present in known RALF peptides, with an N-terminal signal peptide for cell secretion and the proteolytic cleavage site motif RRILA for the pro-peptide processing by the subtilisin-like protease and release of mature peptide (Supplementary Fig. S1). According to Campell and Turner (2017) RALF sequence classifications *FaRALF33-like* belongs to the clade I subfamily of RALF peptides, since its mature form contains the YISY, YYNC and CRC motifs typical of this clade (Supplementary Fig. S1).

The *FaRALF-33-like* gene is up-regulated 24 h post *C. acutatum* inoculation (hpi) in red ripe strawberries fruits, where the pathogen is active (Guidarelli *et al*., 2011). No difference in the expression of this gene was found in inoculated white fruits. Moreover, previous histological studies showed that at 16 hpi most conidia germinated on both white unripe and red ripe fruit stages, and only at 24 hpi, *C. acutatum* arrests its growth as a quiescent melanized appressoria in white strawberries, while it develops intercellular hyphae on red fruits (Guidarelli *et al*., 2011). In order to find out whether the up-regulation of *FaRALF-33-like* gene is specifically related to the susceptibility of ripe stages or if it also occurs in white unripe fruits at later times of infection or in fruits inoculated with other fungal pathogens, a gene expression analysis was performed in white and red fruits at 24 and 48 hpi with *C. acutatum, B. cinerea* or *P. expansum*. *FaRALF-33-like* gene was quantified by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Consistently with previous findings, a significant increase in the expression level of *FaRALF-33-like* gene was observed at 24 hpi in red ripe *C. acutatum*-inoculated fruits when compared to the mock-inoculated strawberries whereas no difference was detected in white unripe fruits (Fig. 1). This indicates that the expression of this gene is indeed specific to the red ripe stage of the

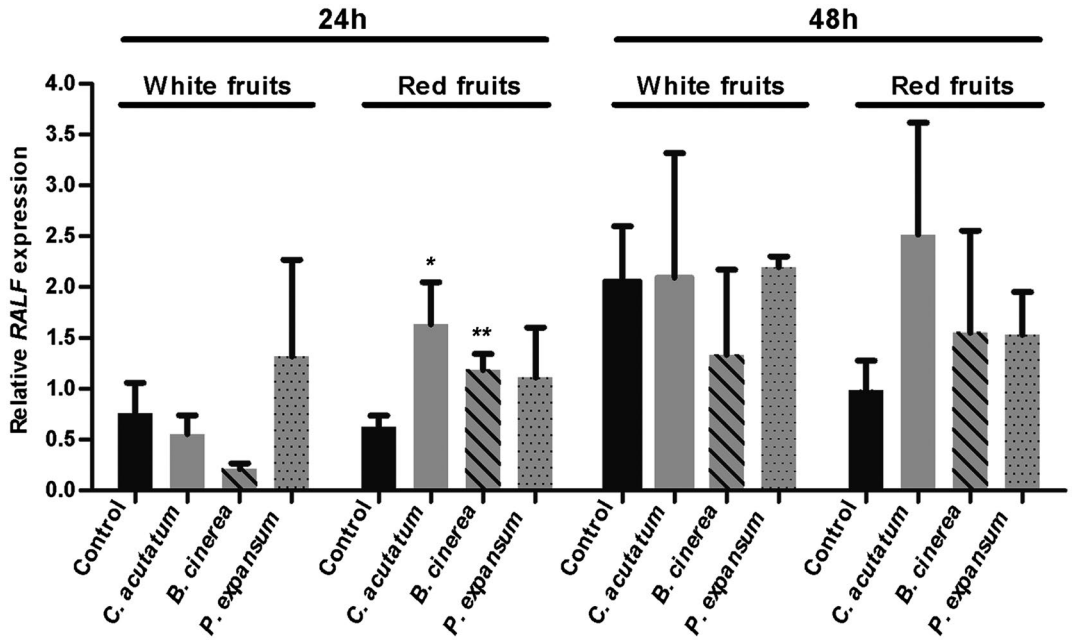

Fig. 1 Analysis of gene expression displays an increase in *FaRALF-33-like* gene expression in red ripe strawberry fruits at 24 h after inoculation with *Colletotrichum acutatum* or *Botrytis cinerea,* whereas no changes in *FaRALF-33-like* gene expression were observed after inoculation with *Penicillium expansum*. Expression of *FaRALF-33-like* gene transcripts at 24 h (left) or 48 h (right) after *C. acutatum* (grey bar), *B. cinerea* (striped bar) or *P. expansum* (dotted bar) inoculation in white and red fruits. Expression levels in mock-inoculated white and red fruits are displayed in black bars. Data were normalized to the transcript level of the housekeeping *elongation factor 1α* gene. The data are the means and SD of three biological replicates. The asterisks indicate significant difference compared with the control (Student's *t*-test: $*P < 0.05$, $**P < 0.01$).

strawberry fruits interacting with actively growing *C. acutatum* as subcuticular intercellular hyphae, thereby suggesting a possible involvement of this gene in the susceptibility of the red ripe stage. At 48 hpi *C. acutatum* did not alter the expression level of *FaRALF-33-like* gene in either red ripe fruits or white unripe fruits (Fig. 1).

When red ripe strawberry fruits were inoculated with *B. cinerea*, the causal agent of grey mould, *FaRALF-33-like* gene expression increased at 24 hpi, similarly to what was described for *C. acutatum*-inoculated fruits, whereas no difference was observed in the white unripe stage at either 24 hpi or 48 hpi (Fig. 1). This may indicate that *FaRALF-33-like* gene participates in the susceptibility of the red ripe fruits against fungal pathogens not only restricted to *C. acutatum.* In contrast, neither white nor red strawberry fruits increased *FaRALF-33-like* expression at 24 or 48 h after *P. expansum* inoculation (Fig. 1), the causal agent of blue mould.

Silencing of *FaRALF-33-like* gene in red ripe strawberries did not alter the susceptibility to *C. acutatum* but decreased the infection process after *C. acutatum* inoculation

To gain insight in the possible role of *FaRALF-33-like* gene in the different susceptibility to *C. acutatum* of unripe and ripe strawberry fruits, *Agrobacterium* transient transformation was used to transiently silence the expression of this gene in red fruits. Fruits were harvested six days post-agroinfiltration (pai) and inoculated with *C. acutatum* for 72 h before analysing the fruit response to the anthracnose disease both as visual analysis of symptoms and observation of the fungal infection structure through microscopy analysis. The following conditions were evaluated in order to appreciate the influence of altered *FaRALF-33-like* gene expression in fruit susceptibility: pK7:*FaRALF-33-like* fruits (agroinfiltrated with the silencing vector), pK7:00 (agroinfiltrated with empty vector, as control) and wild-type non-agroinfiltrated red strawberries. The silencing *FaRALF-33-like* expression in red fruits is expected to render these fruits more susceptible to *C. acutatum* infection. The expression of *FaRALF-33-like* gene was significantly induced in both wild-type and control infiltrated strawberries (pK7:00) at 72 hpi with *C. acutatum*. The time (72 hpi) was chosen in order to observe the symptoms before sampling for gene expression analysis. Here, *RALF* gene expression increased by about 5- and 4-fold, respectively. In contrast, in pK7:*FaRALF-33-like* transfected strawberries the induction of *FaRALF-33-like* gene expression was almost abolished (Fig. 2A), indicating that the RNA interference mechanism induced by the expression of RALF self-complementary hairpin RNA silenced this gene.

As regards the fruit phenotype, wild-type and pK7:00 control agroinfiltrated red strawberries at 72 hpi showed typical anthracnose symptoms with dark and sunken lesions on the fruit

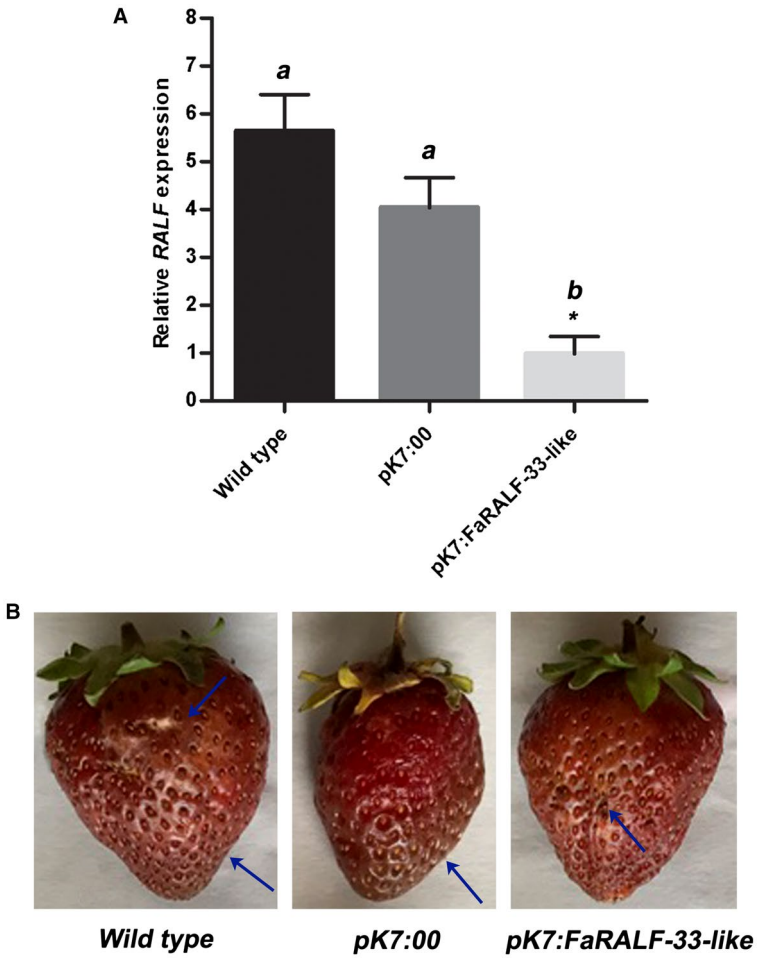

Fig. 2 Silencing of *FaRALF-33-like* gene renders red ripe strawberries less susceptible to *Colletotrichum acutatum*. (A) The transcript levels of *FaRALF-33-like* gene in wild-type red strawberries were compared with those of mock-silenced red fruits (pK7:00) and *FaRALF-33-like*-silenced red fruits (pK7:*FaRALF-33-like*), all inoculated for 72 h with *C. acutatum*. Amplification of *RALF* gene untranslated region was normalized to the transcript level of the housekeeping *elongation factor 1α* gene. The data are the means and SD of three biological replicates. The letters indicate significant difference (Student's *t*-test) compared with wild-type or mock-treated fruits. The asterisks indicate $P < 0.05$. (B) Disease symptom analysis in wild-type infected red fruits (left), mock-silenced red fruits (pK7:00) (middle) and *FaRALF-33-like*-silenced red fruits (pK7:*FaRALF-33-like*) (right), all infected for 72 h with *C. acutatum*. One representative infected red fruit of each condition (wild-type, pK7:00, pK7:*FaRALF-33-like*) is shown. Arrows indicate anthracnose symptoms.

surface (Fig. 2B). Symptom development was very similar in the pK7:*FaRALF-33-like*-silenced fruits, indicating that other mechanisms besides *RALF* gene induction are involved in disease susceptibility to *C. acutatum* in red ripe strawberries fruits.

The histological analysis of 48 hpi red fruits revealed the presence of penetrated hyphae underneath the fruit surface in wild-type and mock-silenced red fruits. Similar penetrated hyphae were apparent also in *FaRALF-33-like*-silenced red fruits (Fig. 3A, B and C) although here tissue colonization by *Colletotrichum* hyphae appeared less deep than in control fruits. To evaluate the fungal growth, the expression of the housekeeping gene *β-tubulin*, specific to *C. acutatum* (Brown *et al.*, 2008), was analysed by qRT-PCR. In *FaRALF-33-like*-silenced red fruits the fungus grows similarly to wild-type red fruits but less than in mock-silenced red fruits (Fig. 3D).

Overexpression of *FaRALF-33-like* gene in white unripe strawberries led to increased susceptibility of fruits to *C. acutatum*

The overexpression of *FaRALF-33-like* was induced in white unripe strawberries by infiltration for 6 days with *Agrobacterium* carrying the plasmid 35S:*FaRALF-33-like*. Overexpression in these strawberries was evaluated by qRT-PCR at 3 dpi (days post-inoculation) with *C. acutatum.* As mentioned above, the time (72 h) was chosen in order to observe the symptoms before sampling for gene expression analysis. The expression of *FaRALF-33-like* gene increased 50-fold with respect to the control white fruit (infected wild-type or infected 35S:00) (Fig. 4A), indicating that overexpression was efficient and that agroinfiltration itself did not alter *FaRALF-33-like* expression. Seventy-two hours after

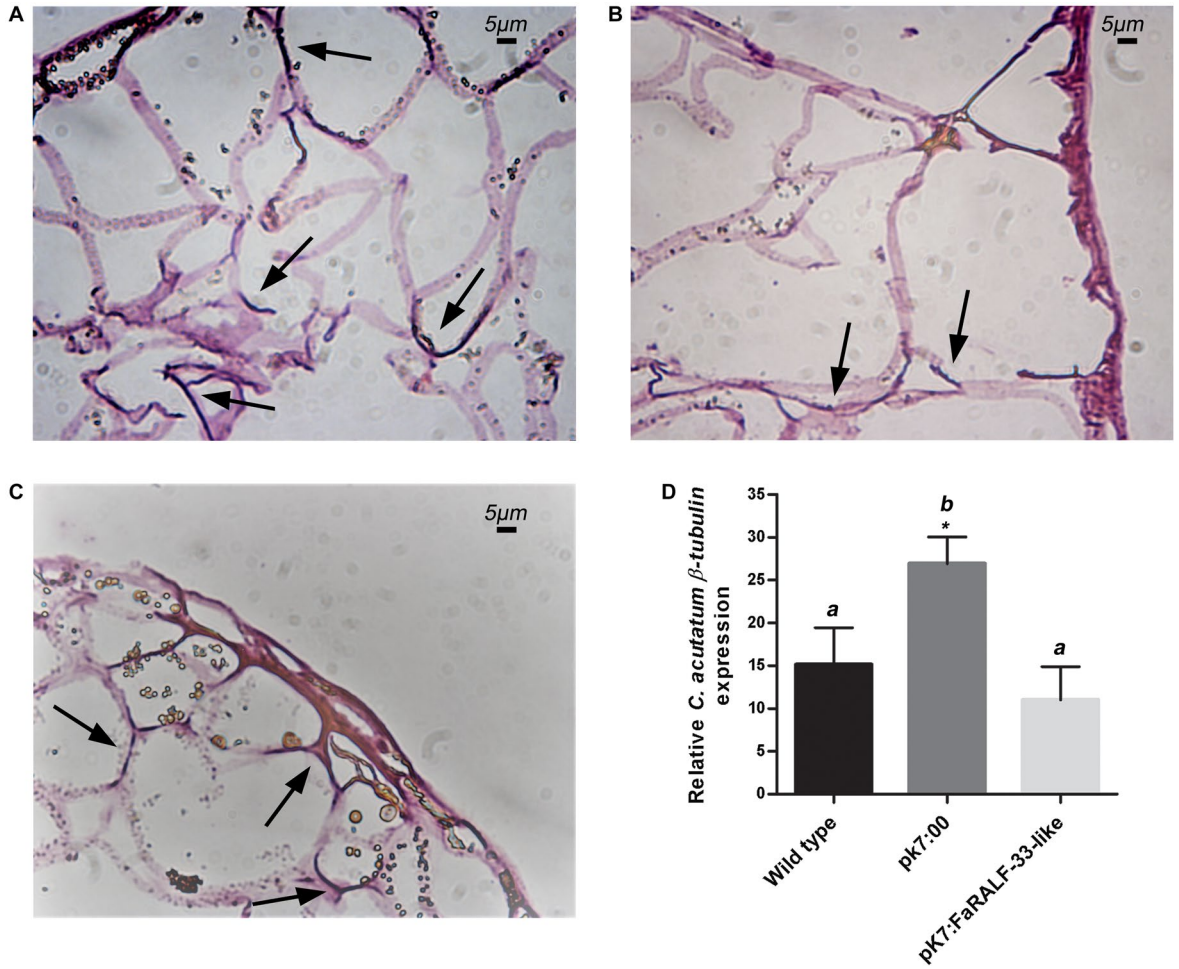

Fig. 3 Histological analysis of *FaRALF-33-like*-silenced 48 h *Colletotrichum acutatum*-infected red fruits displays only superficial infection which correlates with *C. acutatum* similar growth in wild-type red fruits and in *FaRALF-33-like*-silenced fruits. Optical microscopy of wild-type red fruits (A), mock-silenced red fruits (B) and *FaRALF-33-like* silenced red fruits (C). Tissue slices were stained with haematoxylin and eosin. Inter-intracellular hyphae are indicated with arrows. Bar: 5 μm. (D) *Colletotrichum acutatum β-tubulin* expression in wild-type red strawberries (black bar) was compared with those of mock-silenced red fruits (pK7:00, dark grey) and *FaRALF-33-like*-silenced red fruits (pK7:*FaRALF-33-like,* light grey). The data are the means and SD of three biological replicates. The letters indicate significant difference (Student's *t*-test) compared with wild-type or mock-treated fruits. The asterisks indicate $P < 0.05$.

C. acutatum inoculation, wild-type white fruits did not show anthracnose symptoms, and 35S:00 agroinfiltrated control fruits showed light anthracnose symptoms, probably as consequence of agroinfiltration stress (Fig. 4B). On the other hand, symptom development was apparent in 35S:*FaRALF-33-like* white fruits, which were rotten.

The histological analysis of 48 h-infected unripe tissues showed that *C. acutatum* internal hyphae were distinguishable in the superficial layer of epidermal cells of all the three types of fruits (Fig. 5A, B and C); however, in white *FaRALF-33-like*-overexpressing fruits, a higher percentage of penetration events, with deeper internal hyphae than in wild-type and mock-overexpressing white fruits, were distinguishable. Additionally, the fungus grew more in *FaRALF-33-like*-overexpressing white fruits compared to wild-type and mock-overexpressing fruits (Fig. 5D), suggesting a higher susceptibility of these fruits to *C. acutatum*.

Silencing or overexpression of *FaRALF-33-like* gene led to a different pattern expression of plant defence genes in strawberry fruits

To gain insight into the different susceptibility to *C. acutatum* in *FaRALF-33-like*-silencing or overexpressing fruits, the expression of genes associated with plant defence was evaluated. They consist of a *Chitinase* gene, a gene encoding for a PR-10 (pathogenesis-related protein 10) named *Fra a 1E,* a polygalacturonase-inhibiting protein *PGIP* gene and genes encoding for two transcription factors of the WRKY family, *FaWRKY51* and *FaWRKY42.* These two *WRKY* genes are homologous to

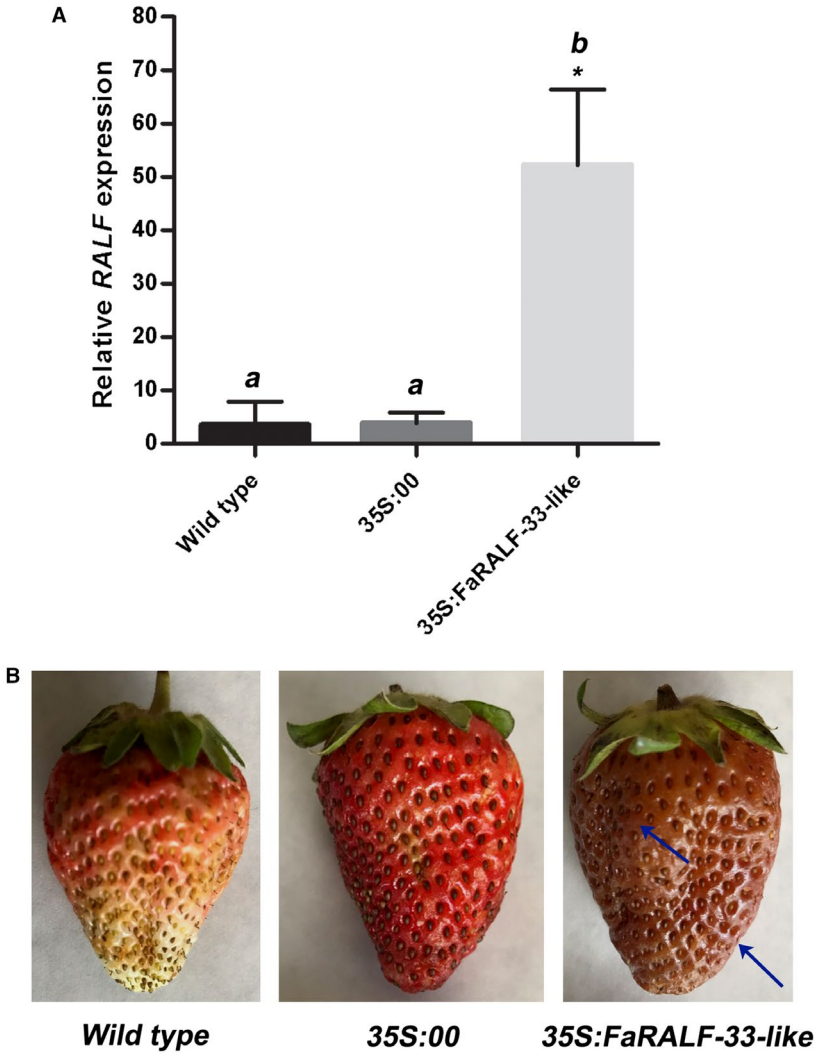

Fig. 4 Overexpression of *FaRALF-33-like* gene renders white unripe strawberries more susceptible to *Colletotrichum acutatum*. (A) The transcript levels of *FaRALF-33-like* gene in wild-type white strawberries were compared with those of mock-overexpressing white fruits (35S:00) and *FaRALF-33-like*-overexpressing white fruits (35S:*FaRALF-33-like*), all inoculated for 72 h with *C. acutatum*. The *FaRALF-33-like* primers used were mapped in the *RALF* untranslated region. Data were normalized to the transcript level of the housekeeping *elongation factor 1α* gene. The data are the means and SD of three biological replicates. The letters indicate significant difference (Student's *t*-test) compared with wild-type or mock-treated fruits. The asterisks indicate $P < 0.05$. (B) Disease symptom analysis in wild-type white fruits (left), mock-overexpressing white fruits (35S:00) (middle) and *FaRALF-33-like*-overexpressing white fruits (35S:*FaRALF-33-like*) (right), all infected for 72 h with *C. acutatum*. One representative infected red fruit of each condition (wild type, 35S:00, 35S:*FaRALF-33-like*) is shown. Arrows indicate anthracnose symptoms.

A. thaliana AtWRKY 46 and *AtWRKY 33* and here they are named *FaWRKY51* and *FaWRKY42*, respectively, accordingly to the nomenclature proposed by Wei *et al.* (2016). These genes all have a consolidated function in plant immune response to fungal pathogens (Amil-Ruiz *et al.*, 2011; De Lorenzo *et al.*, 2001; Jain and Kumar, 2015; Pandey and Somssich, 2009; Sharma *et al.*, 2011) and in our previous microarray analysis were found to be differently regulated by *C. acutatum* inoculation at 24 hpi in white and red fruits (Guidarelli *et al.*, 2011). In particular, in the microarray analysis, *Chitinase* and *FaWRKY42* were found to be up-regulated in both white and red fruit upon *C. acutatum* infection, whereas *Fra a E1* and *FaWRKY51* were found to be up-regulated only in white fruits. Here, in *FaRALF-33-like*-silenced red fruits the levels of transcripts of *Chitinase* were unaltered with respect to wild-type fruits, whereas *Fra a 1E* and *FaWRKY42* levels remained similar to the mock-silenced red fruits control, but lower than the wild-type, suggesting that agroinfiltration (and not *RALF* gene silencing) was determining a decrease in expression. On the other hand, *PGIP* and *FaWRKY51* gene expression was decreased (Fig. 6A) with respect to both controls (wild-type and mock-silenced fruits), as specific response associated with *RALF* gene silencing.

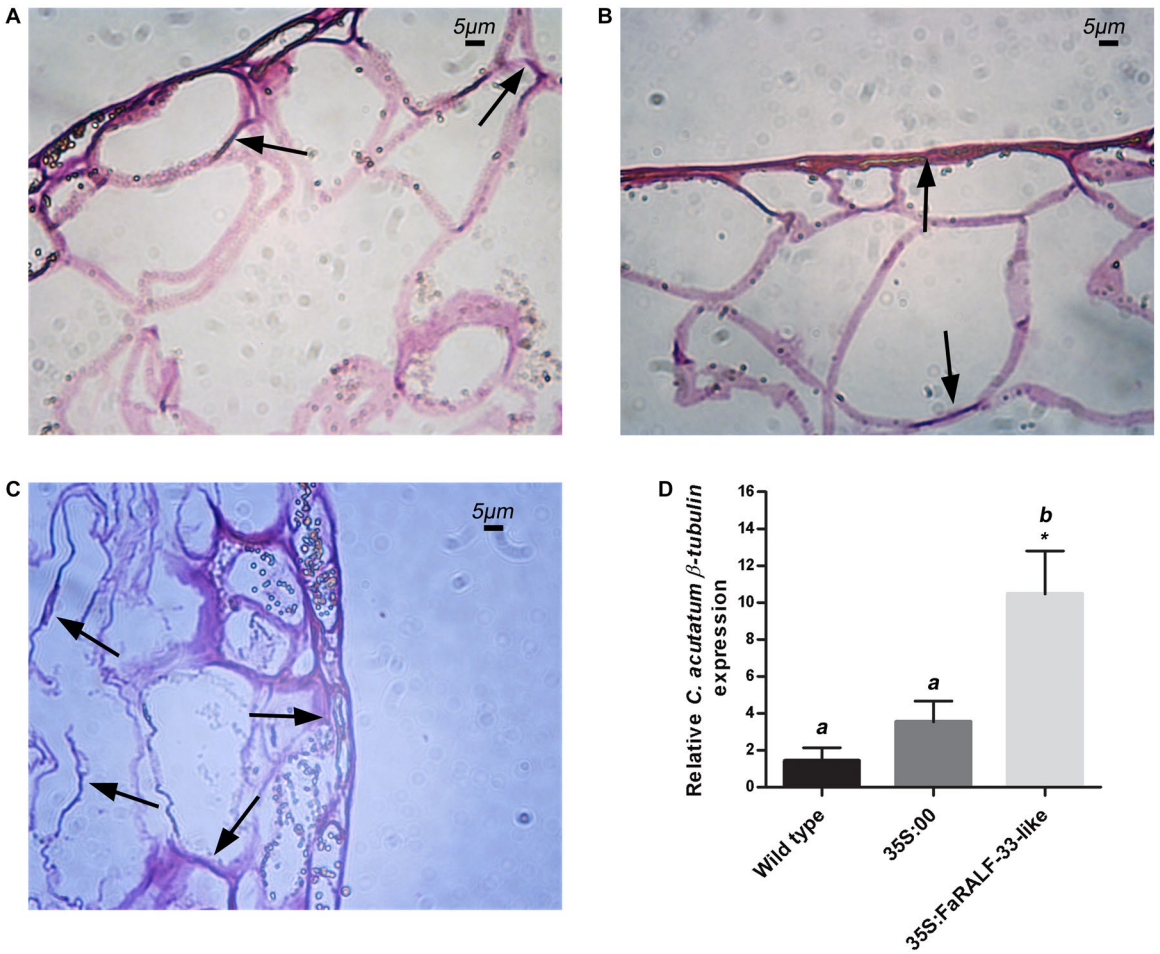

Fig. 5 Histological analysis of *FaRALF-33-like* overexpressing 48 h *Colletotrichum acutatum*-infected white strawberry fruits shows a high percentage of penetration events which correlates with *C. acutatum* increased growth in *FaRALF-33-like*-overexpressing fruits. Optical microscopy of wild-type white fruits (A), mock-overexpressing white fruits (B) and *FaRALF-33-like* overexpressing white fruits (C). Tissue slices were stained with haematoxylin and eosin. Inter-intracellular hyphae are indicated. Bar: 5 μm. (D) *Colletotrichum acutatum β-tubulin* expression in wild-type white strawberries (black bar) was compared with those of mock-overexpressing white fruits (35S:00, dark grey), and *FaRALF-33-like*-overexpressing white fruits (35S:*FaRALF-33-like,* light grey). The data are the means and SD of three biological replicates. The letters indicate significant difference (Student's *t*-test) compared with wild-type or mock-treated fruits. The asterisks indicate $P < 0.05$.

When *FaRALF-33-like* was overexpressed in white fruits, the expression of *Chitinase*, *Fra a 1E* and *FaWRKY51* increased compared to wild-type or mock-overexpressing white fruits, whereas *PGIP* and *FaWRKY42* did not display any significant differences compared to the mock-overexpressing control. This could suggest that the white fruits that become more susceptible to *C. acutatum* by RALF gene overexpression counteract pathogen colonization by increasing the expression of key defence genes.

DISCUSSION

Fruits undergo a transcriptional reprogramming as they ripen. This different expression profile induces substantial metabolic and structural changes, prompting increased susceptibility to many fungal infections (Gapper *et al.*, 2014; Guidarelli *et al.*, 2014; Sanchez-Sevilla *et al.*, 2017). *Colletotrichum* spp. are pathogens associated with quiescent infections leading to ripe rots during storage and transport of fruits. In order to reduce fruit losses due to postharvest rotting it is important to elucidate the mechanisms underlying the ontogenic variation of fruit susceptibility during ripening.

During a microarray analysis of 24 h *C. acutatum*-inoculated unripe and ripe strawberry fruits a gene encoding for a RALF peptide was found to be up-regulated only in red ripe susceptible stages (Guidarelli *et al.*, 2011). In different plant species, RALF peptides are encoded by a gene family of different size (37 in *A. thaliana*, 33 in *Malus domestica*, 4 in *Vitis vinifera* and 9 in *Fragaria vesca*). Depending on the sequence

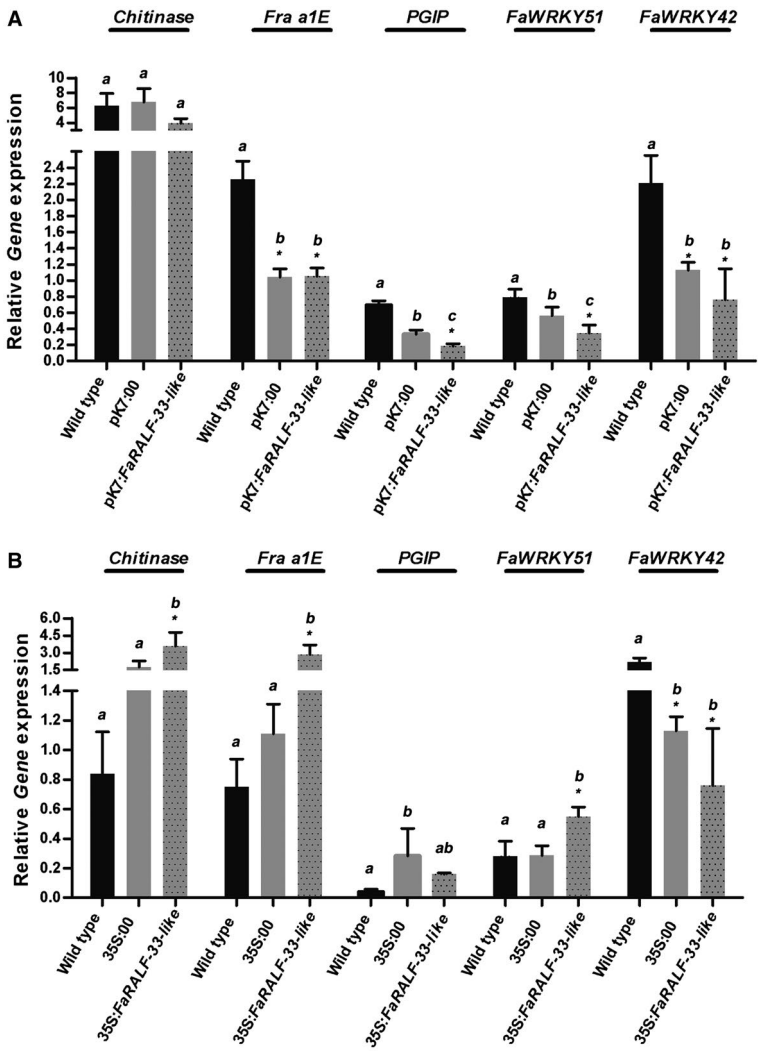

Fig. 6 Silencing (A) or overexpression (B) of *FaRALF-33-like* gene triggers a different defence-related gene expression pattern. (A) The transcript levels of *Chitinase*, *Fra a 1E, PGIP, FaWRKY51* and *FaWRKY42* genes in wild-type red strawberries were compared with those of mock-silenced red fruits (pK7:00) and *FaRALF-33-like*-silenced red fruits (pK7:*FaRALF-33-like*), all inoculated for 72 h with *Colletotrichum acutatum.* (B) The transcript levels of *Chitinase*, *Fra a 1E, PGIP, FaWRKY51* and *FaWRKY42* genes in wild-type white strawberries were compared with those of mock-overexpressing white fruits (35S:00) and *FaRALF-33-like*-overexpressing white fruits (pK7:*FaRALF-33-like*), all inoculated for 72 h with *C. acutatum.* Data were normalized to the transcript level of the housekeeping *elongation factor 1α* gene. The data are the means and SD of three biological replicates. The letters indicate significant difference (Student's *t*-test) compared with wild-type or mock-treated fruits. The asterisks indicate $P < 0.05$.

similarity and recognition of typical distinctive motifs in primary sequences, RALF peptides have been divided into four main clades (Campbell and Turner, 2017). The *Fragaria* × *ananassa* gene up-regulated in red fruit on *C. acutatum* interaction shows high similarity with *AtRALF33* and *F. vesca FvRALF-33-like* (GenBank accession number : XM_011460413.1). Given the presence of a number of aminoacidic hallmark motifs and of the typical RRILA aminoacidic recognition sequence for subtilisin-like peptidase processing of the mature peptide, RALF33 homologues were classified as clade I members. Subtilisin processing was shown to be necessary for RALF peptide activity (Srivastava *et al.*, 2009). Similarly, the aminoacidic motif YISY found at 5–10 amino acid position in RALF mature peptide was shown to be crucial for binding to RALF receptor and downstream signalling (Pearce *et al.*, 2010).

We have shown that silencing *FaRALF-33*-like expression in 72 h *C. acutatum*-inoculated red fruits (Fig. 2) can lead to a delay in fruit colonization by the fungal pathogen, appreciable by histological analysis of the infected tissues showing less penetrated infective hyphae than in wild-type fruit (Fig. 3 and Supplementary Fig. S4) and a decrease in the expression of the plant defence genes *PGIP* and *FaWRKY51* (Fig. 6A). In contrast,

72 h *C. acutatum*-inoculated white unripe fruits overexpressing the *FaRALF-33-like* gene decreased the ontogenic resistance of these fruits, leading to the appearance of disease symptoms (Fig. 4) and deep penetrated subcuticular hyphae (Fig. 5 and Supplementary Fig. S7), which are generally absent in white unripe fruits. The different response of agroinfiltrated strawberry fruits to *C. acutatum* supports the hypothesis that *FaRALF-33-like* plays an important role in the susceptibility of fruits against the fungal pathogen *C. acutatum*.

Ambient pH is a key player in fungi growth and development (Penalva and Arst, 2004). Intriguingly, fungal pathogens have the ability to adjust the extracellular pH as a way to facilitate infection by regulating a plethora of virulence factors (Alkan *et al*., 2013; Jennings, 1989). Secretion of organic acids leads to acidification, a feature of necrotrophic pathogens (Cessna *et al*., 2000). In contrast, hemibiotrophs induce extracellular alkalinization during the early biotrophic stages of infection (Prusky and Yakoby, 2003). Several fruit-infecting species, including *Colletotrichum* spp. (Alkan *et al*., 2013; Prusky *et al*., 2001) and the root-infecting fungus *Fusarium oxysporum*, have been shown to induce host alkalinization by releasing ammonia. In order to alkalinize the environment surrounding the plant tissue, through ammonia secretion, fungi must develop a significant hyphal biomass (Fernandes *et al*., 2017). However, due to the low number of hyphae in the early stages of infection, fungal pathogens have adapted pH regulatory mechanisms present in the hosts. *Fusarium oxysporum* displays a functional homologue of the plant RALF peptides and uses it to enhance its own infectious potential by mimicking plant RALF-induced alkalinization (Masachis *et al*., 2016). A plausible explanation for the susceptibility role of RALF against *C. acutatum* in red ripe strawberry fruits is the fact that its expression favours fungal pathogenicity by increasing the extracellular pH in the first stages of strawberry fruit colonization, after breaking the quiescence state. Consistently, we observed that *C. acutatum*-inoculated red ripe fruits showed an increased expression of *FaRALF-33-like* compared to the mock-inoculated fruits at 24 hpi (Fig. 1). In contrast, the expression level of 48 h *C. acutatum*-inoculated red ripe fruits is similar to the mock-inoculated fruits. *Colletotrichum acutatum*, which is probably not able to secrete ammonia at early times post-infection, would induce RALF expression in susceptible red ripe fruits favouring its pathogenicity. Thus, our results reinforce the hypothesis that fungi not only encode homologues of RALF in their genome (Masachis *et al*., 2016), but may also hijack the host RALF pathway by prompting RALF expression in plants, as previously shown by Dobón *et al.* (2015). Further research is needed to elucidate the way in which *C. acutatum* could induce RALF expression in the host. However, silencing RALF in red ripe fruits while decreasing anthracnose symptoms could not prevent disease onset. Many other pathways, different from RALF signalling, may be involved in red ripe strawberry fruit susceptibility.

On the other hand, in white unripe fruits that do not display an increase in RALF expression after *C. acutatum* inoculation (Fig. 1), RALF overexpression seems to stimulate fungal growth in a stage when it is normally quiescent and render fruits susceptible to infection (Fig. 4).

FERONIA was identified as the RALF receptor in plants (Haruta *et al*., 2014). Interestingly, fungal RALF seems to signal through FERONIA as well (Masachis *et al*., 2016). Besides its role in plant growth and development, signalling through FERONIA has also been implicated in negative regulation of plant defence (Masachis *et al*., 2016). *Feronia* knockout mutants express increased levels of several immunity response marker genes (Masachis *et al*., 2016), and are more resistant to some bacterial and fungal pathogens (Kessler *et al*., 2010; Masachis *et al*., 2016). Thus, the enhanced susceptibility to *C. acutatum* found in white unripe fruits transfected with RALF may also be explained by the increased signalling through FERONIA leading to a decreased defence response against pathogens. This occurs despite the increase in transcript levels of *Chitinase*, *Fra a 1E* and *FaWRKY51*, displayed by 35S:*FaRALF-33-like* white fruits (Fig. 6B), which could be due to the immune response of white fruit counteracting infection.

Whilst *C. acutatum* prompts alkalinization to induce extensive gene expression and colonization (Prusky and Yakoby, 2003), *P. expansum* and *B. cinerea* are known to induce tissue acidification to cope effectively with the host environment (Manteau *et al*, 2003; Prusky *et al*., 2004). Consistently, we observed no difference in RALF expression in *P. expansum*-inoculated red or white fruits at either 24 or 48 hpi (Fig. 1). Unexpectedly, *B. cinerea*-inoculated red fruits displayed an increased expression of RALF in red ripe fruits at 24 hpi (Fig. 1), similar to what we observed in *C. acutatum*-inoculated fruits.

Fungal pathogens have evolved multiple ways to cope efficiently with host defences. Early findings showed that in filamentous fungi the external pH is sensed and transmitted intracellularly by phenylalanine ammonia-lyase (PAL) leading to the proteolytic cleavage of the transcription factor PacC (Peñalva and Arst, 2002). The active form of PacC regulates pH-responsive gene expression, including those with similar functions but differential pH-expression patterns (Tilburn *et al*., 1995). Despite the need for an acidic environment, *B. cinerea* may activate the FERONIA pathway through RALF and consequently inhibit immune responses, rendering fruits more susceptible to colonization and infection.

Despite being classified as acidifiers and alkalinizers, depending on their ability to either decrease or increase the pH of the surrounding host tissue, fungal pathogens regulate the environmental pH depending on carbon availability (Bi *et al*., 2016). Carbon deprivation induces extracellular accumulation of ammonia and alkalinization, whereas carbon excess triggers acidification by releasing gluconic acid (Bi *et al*., 2016). Therefore, it is also possible that *B. cinerea* in that stage of infection and host niche

(24 hpi, red ripe strawberry fruits) (Fig. 1) senses low levels of carbon, thus increasing the tissue pH by inducing RALF expression.

In summary, our results indicate that *FaRALF-33-like* gene expression plays a key role in fruit immune responses to fungal pathogens. Future research is required to elucidate the host mechanisms governing RALF expression in disease and the possible crosstalk between fungi and hosts in inducing the expression of this gene.

EXPERIMENTAL PROCEDURES

Fungal material

Colletotrichum acutatum was isolated from strawberry fruits of different strawberry cultivars showing severe anthracnose symptoms. Monoconidial cultures were identified by morphological analysis and sequencing of ribosomal DNA internal transcribed spacer (ITS) regions. Isolate maya-3 was grown on potato dextrose agar (Sigma, St. Louis, MO, USA) at 20 °C for 10 days. Conidial suspensions of pathogen were prepared by washing the colonies with 5 mL of sterile distilled water containing 0.05% (v/v) Tween-80, quantified with a haemocytometer, and diluted to a concentration of about 10^6 conidia/mL for the infection trials. Infections with *B. cinerea* B05.10 and with *P. expansum* (CRIOF collection) were similarly performed.

Plant material

Fragaria × *ananassa* 'Alba' plants, cultivated in pots in a glasshouse, were used for all experiments. Standard growing conditions were maintained at 20 °C with a 16 h photoperiod.

For the analysis of susceptibility, fruits (three replicates of 20 fruits each) at unripe (20 days after flowering) or ripe (30 days after flowering) stage were inoculated with *C. acutatum* by dipping for 1 min in a suspension of 10^6 conidia/mL or distilled water (mock control), and stored at 20 °C and 70% relative humidity (RH) and observed every 24 h. After 3 days, the degree of susceptibility of strawberry fruits to the pathogen was scored as the percentage of infected fruits. The fruit surface was then excised with a clean scalpel and immediately frozen in liquid nitrogen and transferred to −80 °C until use. Total RNA was prepared as described by Lopez-Gomez and Gomez-Lim (1992) with minor modifications.

qRT-PCR

For qRT-PCR experiments, first-strand cDNA was synthesized from 1 μg of total RNA in a volume of 20 μL with oligo-$d(T)_{17}$ and Superscript III (Invitrogen Life Technology, Carlsbad, CA, USA), following the manufacturer's instructions. The cDNA concentration in the RT mix was quantified using a ND-1000 UV spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and 1 μg of cDNA was used for qRT-PCR experiments, employing an MX3000 thermal cycler (Stratagene, La Jolla, CA, USA) and Platinum Sybr-Green Kit (Invitrogen Life Technology), according to the manufacturer's instructions. The *elongation factor 1α* gene, having constitutive expression, was used to normalize raw data and to calculate relative transcript levels. The primer sequences used in qRT-PCR are displayed in Table S1.

Plasmid construction, *Agrobacterium* transformation and plant transformation

The full-length cDNA sequence for the *FaRALF-33-like* gene was amplified from the cDNA pool of *C. acutatum* 24 h inoculated red strawberry fruit using the primers forward 5′-ATGGCAAAGTCCTCTTCCATT-3′ and reverse 5′-TCAACTACGGCAGCGAGTGAT-3′. The translated peptide sequence was aligned with the *A. thaliana* RALF cDNAs using ClustalW software (https://www.ebi.ac.uk/Tools/msa/clustalw2/). The pK7GWIWG2(II) RNAi silencing and pK7WG2 overexpression vectors, described in Karimi *et al.* (2002) [VIB Department of Plant Systems Biology, Ghent University, Belgium (http://gateway.psb.ugent.be)], were used as destination plasmids for inducing silencing or overexpression in agroinfiltrated fruits. For this, the same pENTR/D-TOPO (Invitrogen Life Technologies) construct containing the full-length sequence of the *FaRALF33-like* cDNA, amplified with forward primer 5′-CACCATGGCAAAGTCCTCTTCCATT-3′ and reverse primer 5′-TCAACTACGGCAGCGAGTGAT-3′, was used in GATEWAY cloning (Invitrogen Life Technologies) (Supplementary Fig. S8). The plasmid constructs were checked by PCR [CaMV 35S promoter forward primer and attB2 reverse primer (5′-ACCACTTTGTACAAGAAA-3′)], by digestion using restriction enzymes and by DNA sequencing (Supplementary Fig. S9). The resulting plasmids (pK7:*FaRALF-33-like* for silencing, 35S:*FaRALF-33-like* for overexpression) were introduced into *A. tumefaciens* strain EHA105 using the freeze–thaw shock method (Holsters *et al.*, 1978). The *A. tumefaciens* EHA105 containing pK7:*RALF33* or 35S:*RALF33* were grown at 28 °C in Luria–Bertani (LB) medium with appropriate antibiotics. When the culture reached an optical density at 600 nm (OD_{600}) of about 0.8, *Agrobacterium* cells were harvested and resuspended in a modified MacConkey (MMA) medium [Murashige and Skoog salts, 10 mM 2-(*N*-morpholino) ethanesulphonic acid (MES), pH 5.6, 20 g/L sucrose and 200 μM acetosyringone], according to Spolaore *et al.* (2001). After 1 h of incubation at 22 °C, the *Agrobacterium* suspension was injected into fruits still attached to the plant using a sterile syringe. After 6 days fruits were harvested and inoculated with *C. acutatum* conidia for 3 days, as described above. Tissues from the surface of the whole fruit were collected and RNA was isolated, as previously described. qRT-PCR was used to evaluate the *FaRALF33-like* transcript level, as described above.

FaRALF-33-like silencing and overexpression

For silencing, 44 red strawberry fruits at about 27 days after anthesis (3 days before the 'red' stage) were agroinfiltrated with pK7:*FaRALF-33-like* for 6 days. They were harvested and

inoculated with *C. acutatum*, as described above. Twenty four of these fruits were collected 48 h after inoculation for histological analysis of the fungal infection, as described by Guidarelli *et al.* (2011). The other 20 fruits were phenotypically observed after 3 days of inoculation for anthracnose symptom evaluation and used for *FaRALF-33-like* gene expression analysis to confirm gene silencing. For this, RNA was isolated and qRT-PCR was performed, as described above. Similarly, for overexpression, 44 white fruits at about 17 days after anthesis (3 days before the 'white' stage) were agroinfiltrated with plasmid 35S:*FaRALF-33-like* for silencing for 6 days. They were harvested and inoculated with *C. acutatum*, as described above. The fruits were collected 48 h after inoculation for histological analysis of the fungal infection, as described by Guidarelli *et al.* (2011). As controls for silencing and overexpression, the same number (44) of white and red fruits were agroinfiltrated with pK7:00 (the empty silencing vector) and 35S:00 (the empty overexpression vector) plasmids or left in the wild-type condition. Phenotype observation, gene expression and histological analysis were performed as described above.

Statistics

Results were analysed for statistical significance (defined as $P < 0.05$ and indicated by asterisks in figures) by performing unpaired, two-sided Student's *t*-test with GraphPad Prism 7 Data Analysis Software (GraphPad Software, Inc., La Jolla, CA, USA). Mean and standard deviation (SD) values were calculated from at least three biologically and technically independent experiments.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1 FaRALF33-like protein sequence. (A) Sequence alignment between *Fragaria* × *ananassa* FaRALF33-like named protein, *Arabidopsis thaliana* RALF33 (Locus AEE83653) and *Fragaria vesca* subsp. *vesca* XP_011458715.1 PREDICTED: protein RALF-like 33. Proteolytic cleavage site (RRILA) for processing by subtilisin-like serine protease (S1P) (Srivastava *et al.*, 2009) is highlighted by a grey box. The YISY motif, crucial for receptor binding (Pearce *et al.*, 2010), is underlined. The four conserved cysteine residues for disulphide bond formation are indicated by vertical bars. (B) Schematic representation of FaRALF33-like sequence, signal peptides until residues 25 (dark grey), the variable region until RRILA processing site (light grey) and the mature peptide (black).

Fig. S2 Histological analysis of wild-type red fruits. Tissue slices were stained with haematoxylin and eosin. Inter-intracellular hyphae are indicated. Bar: 5 μm.

Fig. S3 Histological analysis of mock-silenced red fruits. Tissue slices were stained with haematoxylin and eosin. Inter-intracellular hyphae are indicated. Bar: 5 μm.

Fig. S4 Histological analysis of *FaRALF-33-like*-silenced fruits. Tissue slices were stained with haematoxylin and eosin. Inter-intracellular hyphae are indicated. Bar: 5 μm.

Fig. S5 Histological analysis of wild-type white fruits. Tissue slices were stained with haematoxylin and eosin. Inter-intracellular hyphae are indicated. Bar: 5 μm.

Fig. S6 Histological analysis of mock-overexpressing white fruits. Tissue slices were stained with haematoxylin and eosin. Inter-intracellular hyphae are indicated. Bar: 5 μm.

Fig. S7 Histological analysis of *FaRALF-33-like*-overexpressing fruits. Tissue slices were stained with haematoxylin and eosin. Inter-intracellular hyphae are indicated. Bar: 5 μm.

Fig. S8 (A) pK7GWIWG2(II):RALF silencing construct, referred to in the text as pk7:*FaRALF-33-like*. (B) pK7WG2:RALF overexpressing construct, referred to in the text as 35S:*FaRALF-33-like*. The size of the attB and attR sites, and RALF_CDS and ccdB_CDS are indicated on the right. Primers used for cloning are indicated in violet and the sequences displayed on the right.

Fig. S9 PCR displaying proper plasmid construction. (A) pK7GWIWG(II):RALF, referred to as pK7:*FaRALF-33-like* in the text. (B) pK7WG2:RALF, referred to as 35S:*FaRALF-33-like* in the text.

Table S1 Oligonucleotide primers used for qRT-PCR.

