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*Article*

# FaNPR3 Members of the NPR1-like Gene Family Negatively Modulate Strawberry Fruit Resistance Against *Colletotrichum acutatum*

**Victoria Súnico [1†], José-Javier Higuera [1†], Francisco Amil-Ruiz [3], Isabel Arjona-Girona [4], Carlos J. López-Herrera [3], Juan Muñoz-Blanco [1], Ana María Maldonado-Alconada [2,*] and José L. Caballero [1*]**

[1] Grupo Biotecnología y Farmacognosia Vegetal (BIO-278), Departamento de Bioquímica y Biología Molecular, Campus de Rabanales, Edif. Severo Ochoa-C6, Universidad de Córdoba, UCO-CeiA3, 14071 Córdoba, Spain; b12susam@uco.es (V.S.); b92hisoj@uco.es (J.J.H.); bb1mublj@uco.es (J.M.B); bb1carej@uco.es (J.L.C);

[2] Grupo Bioquímica, Proteómica y Biología de Sistemas Vegetal y Agroforestal, Departamento de Bioquímica y Biología Molecular, Universidad de Córdoba, UCO-CeiA3, 14014 Córdoba, Spain; bb2maala@uco.es (A.M.M).

[3] Unidad de Bioinformática, Servicio Central de Apoyo a la Investigación (SCAI), Universidad de Córdoba, 14071 Córdoba, Spain; b72amruf@uco.es (F.A.R)

[4] Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible (CSIC), Alameda del Obispo s/n, 14004, Córdoba, Spain; isabelarjona@ias.csic.es (I.A.G); lherrera@ias.csic.es (C.J.L.H)

† These authors contributed equally to this work.

* Correspondence: bb1carej@uco.es (J.L.C); bb2maala@uco.es (A.M.M). Tel.: +34957218197 (J.L.C)

**Abstract:** Strawberry fruit is highly appreciated worldwide for its organoleptic and healthy properties. However, this plant is attacked by many pathogenic fungi, which significantly affect fruit production and quality at pre- and post-harvest stages, making chemical applications the most effective but undesirable strategy to control diseases, so far. Alternatively, genetic manipulation employing plant key genes involved in defense, such as members of the NPR-like gene family, has been successful in many crops to improve resistance. The identification and use of the endogenous counterpart genes in the plant of interest (as it is the case of strawberry) is desirable as it would increase the favorable outcome and requires prior knowledge of their defense related function. Using RNAi technology in strawberry, we show that transient silencing *of Fragaria ananassa* NPR3 members in fruit significantly reduces tissue damage after *Colletotrichum acutatum* infection whereas the ectopic expression of either *FaNPR3.1* or *FaNPR3.2* does not have an apparent effect. Furthermore, the ectopic expression of *FaNPR3.2* in *Arabidopsis thaliana* double mutant *npr3npr4* reverts the disease resistance phenotype to *Pseudomonas syringe* to wild type levels. Therefore, our results reveal that members of the strawberry FaNPR3 clade negatively regulate the defense response to pathogens, as do their *Arabidopsis* AtNPR3/AtNPR4 orthologs. Also, we provide evidence that FaNPR3 members act in strawberry (*F. ananassa*) as positive regulators of *WRKY* genes, *FaWRKY19* and *FaWRKY24*, and that in *Arabidopsis*, FaNPR3.2 negatively regulates its orthologous genes *AtNPR3*/*AtNPR4*. We report for the first time the functional characterization of FaNPR3 members in *F. ananassa* which provides a relevant molecular basis for the improvement of resistance in this species through new breeding technologies.

**Keywords:** *Fragaria ananassa;* strawberry defense; strawberry resistance; *Colletotrichum acutatum;* FaNPR3; *NPR*-like genes; AtNPR3; AtNPR4; FaWRKY19; FaWRKY24

## 1. Introduction

Strawberry (*Fragaria* spp.) represents a valuable and important food crop, being its fruit highly appreciated by consumers worldwide [1]. Beyond its nutritional interest and sensory attributes, such

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as texture, color, flavor and aroma, strawberry fruit provides substantial health benefits. This is due to the presence of bioactive compounds with a range of functions like control of blood glucose level, high antioxidant capacity and potential cancer prevention effects [2-5].

Strawberry is vulnerable to a wide diversity of pathogens, including fungi, which significantly affect fruit yield and quality, and *Colletotrichum acutatum*, is considered a serious fungal pathogen of this crop, causing severe losses in production [6-10]. The lack of pathogen-resistant cultivars is a major challenge for strawberry cultivation, making chemical applications the more widely used strategy to control diseases, endangering ecological and food security [11]. Indeed, classical breeding for resistance is an arduous task in strawberry due to the polygenic and quantitative inherited nature of this trait and to the complexity of the octoploid genome of commercial varieties (*Fragaria x ananassa*) [12-16]. Therefore, innovative solutions for a more sustainable strawberry production are highly in demand.

Although many environmentally friendly strategies have been applied in strawberry to overcome susceptibility to pathogens [17-22], the genetic modification approaches using key genes that control main defense pathways in plants has led to improve resistance in many crops [23-27] and the heterologous expression of defense genes from other species, including those encoding chitinases, beta-1,3-glucanases and thaumatin II, has been reported in strawberry to successfully enhance resistance [28-35]. More recently, new breeding strategies have emerged as important tools to accelerate crop improvement based on powerful biotechnological techniques such as CRISPR/Cas and novel concepts of cisgenesis and intragenesis-assisted by synthetic biology [36-40], which have provided a valuable and more ecological framework for strawberry disease management than the chemical agents used until now [41]. However, it requires the prior identification and characterization of the appropriate endogenous candidate genes [14,42].

Over the last two decades, studies on the genome structure and the strawberry-pathogen interactions have provided new molecular information on putative components of main defensive pathways in this crop [43-52]. Nevertheless, accurate defense pathways remain yet elusive in strawberry and key defense network elements are still largely unknown and need to be functionally validated. For this reason, our research focuses on delving into endogenous strawberry genes homologous to crucial components of the defense pathways known in other plants, with the aim to characterize and functionally validate valuable genetic elements to accelerate improvement of resistance in this crop.

In this sense, members of the nonexpressor of pathogenesis-related gene family (NPR gene family) have been described as important molecular elements in the immune responses in plants. Indeed, *Arabidopsis thaliana* (*Arabidopsis*) AtNPR1 has been identified as a master regulator of salicylic acid (SA)-mediated defense responses [53,54]. AtNPR family differentiates into clade I (NPR1 and NPR2), clade II (NPR3 and NPR4) and clade III (NPR5/BOP2 and NPR6/BOP1) where only members of clade I and II have been associated with defense. Thus, single mutants *npr1* and *npr2* show increased susceptibility to *Pseudomonas syringe* (*Pst*) pathogen infection whereas double mutant *npr3npr4* displays enhanced resistance [55]. AtNPR1 protein acts as "sensor" of SA and in response to SA-induced redox changes is released from an inactive oligomeric cytoplasmic state and translocated as a monomeric form to the nucleus where, in association with transcription factors like TGAs, activate defense-related genes [53,56-58]. On the other hand, AtNPR3 and AtNPR4 also interact with TGAs, but opposite to AtNPR1, after binding SA, negatively regulate the immune response. Thus, it has been proposed that AtNPR3/4 may act directly as transcriptional corepressors of defense-related genes [59] and also mediate the degradation of AtNPR1 and defense-related JAZs (Jasmonate-zim represor proteins), acting as adaptors for the ubiquitin ligase (CRL3) complex to enhance defense against pathogens [53,60,61].

The wide relevance of NPR family members in plant defense is evidenced by the fact that orthologs of all three AtNPR clades are conserved in most angiosperm species. Furthermore, the ectopic expression of AtNPR1 in many crop species, including strawberry, enhances broad-spectrum disease resistance, suggesting the existence of closely related defense response mechanisms among these species [62-68]. However, the molecular functions of AtNPR1 and its paralogs are still not fully

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understood and increased resistance using *Arabidopsis* AtNPR members often correlates with undesirable fitness costs [53,69-71], which highlights the importance of using functionally well-characterized endogenous NPR orthologs to engineer improved resistance in crops.

Recently, a comprehensive identification and phylogenic analysis of the strawberry NPR-like family has been published [49]. Accordingly, 6 NPR-like members are present in the diploid species (*Fragaria vesca*) and 23 members in cultivated species (*F. ananassa*), with each *F. vesca* member matching 3 to 5 homoalleles in the cultivated species and each clade headed by two *Arabidopsis* orthologs. Thus, the unique clade I member in *F. vesca*, FveNPR1, and its 5 homoalleles in *F. ananassa*, FaNPR1a-e, display high degree of identity among them and the *Arabidopsis* clade I members, AtNPR1 and AtNPR2. Similarly, the *F. vesca* clade II members, FveNPR31, FveNPR32, and FveNPR33, and their corresponding *F. ananassa* homoalleles FaNPR31a-c, FaNPR32a-g, and FaNPR33a-d, show high identity to *Arabidopsis* clade II members, AtNPR3 and AtNPR4. Finally, clade III FveNPR5 members in the diploid species and its *F. ananassa* 4 homoalleles, FaNPR5a-d, share high identity with *Arabidopsis* clade III members, AtNPR5 and AtNPR6. Based on these comparative molecular studies and on the transcriptomic profile of these genes in response to *Colletotrichum fructicola*, the potential orthologues of AtNPR1 and AtNPR3/4 were identified in strawberry.

The spite these studies, the molecular functions of strawberry NPR-like members in promoting plant survival against pathogens remains uncovered. Just a first approach to functional characterization of the *F. vesca* gene *FvNPR31* by its ectopic expression in a heterologous system such as wild-type *Arabidopsis* has been published [72]. In the present work, we describe for the first time the functional characterization in the cultivated species *F. ananassa* of members of clade II NPR-like gene family using RNAi technology and gene complementation studies in *Arabidopsis* double mutant *npr3npr4*. Our results show that members of the FaNPR3 clade negatively regulate the defense response to pathogens, as do their *Arabidopsis* AtNPR3/AtNPR4 orthologs.

## 2. Results

### *2.1. Expression Pattern of FaNPR Gene Family after Transient Silencing and Overexpression of the FaNPR3 Members in Strawberry Fruit*

To understand the biological role that the FaNPR3 family plays in strawberry defense, we use a transient expression-induced gene silencing and overexpressing approach in fruit. Thus, for silencing, one of the two halves of a fruit was infiltrated with *Agrobacterium tumefaciens* (agroinfiltrated) carrying the silencing construct (either pFRN::FaNPR3*all*.RNAi or pB7GWIWG2::FaNPR32.RNAi) and the opposite half of the same fruit with the control construct (either pFRN or pB7GWIWG2). Two days after agroinfitration (dai), the fruit was inoculated in both halves with *C. acutatum*, (see experimental design in Material and methods). Changes in the expression pattern of all *FaNPR* genes for every experimental condition were analysed by qRT-PCR.

Results in Figure 1 show the silencing effect of pFRN::FaNPR3*all*.RNAi agroinfiltration on the expression of all *FaNPR3* paralogous genes. Two days after agroinfiltration and throughout the time analyzed, a significant decrease on *FaNPR3.1* transcript accumulation in pFRN::FaNPR3*all*.RNAi samples was detected compared to that of their corresponding pFRN control ones (Figure 1A). The expression of *FaNPR3.1* decreased to a lower significant level at 5 dai, which corresponds to 3 days post inoculation with *C. acutatum* (3 dpi). Figures 1B,C, show that the expression patterns of genes *FaNPR3.2* and *FaNPR3.3*, respectively, were similar to that of *FaNPR3.1* and a significant reduction in transcript accumulation was detected for both genes after 2 dai, in fruit half agroinfiltrated with the silencing construct compared with that of its corresponding fruit half control. As for *FaNPR3.1*, a remarkable and significant decrease in transcript accumulation was observed around 5 dai in *FaNPR3.2* and *FaNPR3.3* genes. On the contrary, no significant differences in the expression level of other members of the *FaNPR* gene family such as *FaNPR1* and *FaNPR5* genes, were detected between fruit control and fruit silenced samples (data not shown). On the other hand, in fruit control samples, a relevant induction in the expression of *FaNPR3.2* was detected after *C. acutatum* inoculation which remained significantly high even after 5 dpi (Figure 1B), compared to that of genes *FaNPR3.1* and

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*FaNPR3.3* (Figures 1A,C), which indicates that *FaNPR3.2* gene members are preferentially activated in response to pathogen infection. Under these experimental conditions, similar patterns of gene silencing were detected for all the *FaNPR3* genes in fruit agroinfiltrated with the silencing construct regardless of whether or not it had been inoculated with *C. acutatum* (data not shown). All in all, these results reveal that, after fruit agroinfiltration, the pFRN::FaNPR3*all*.RNAi construct is successfully silencing specifically the three *FaNPR3* variants of the *NPR*-like gene family.

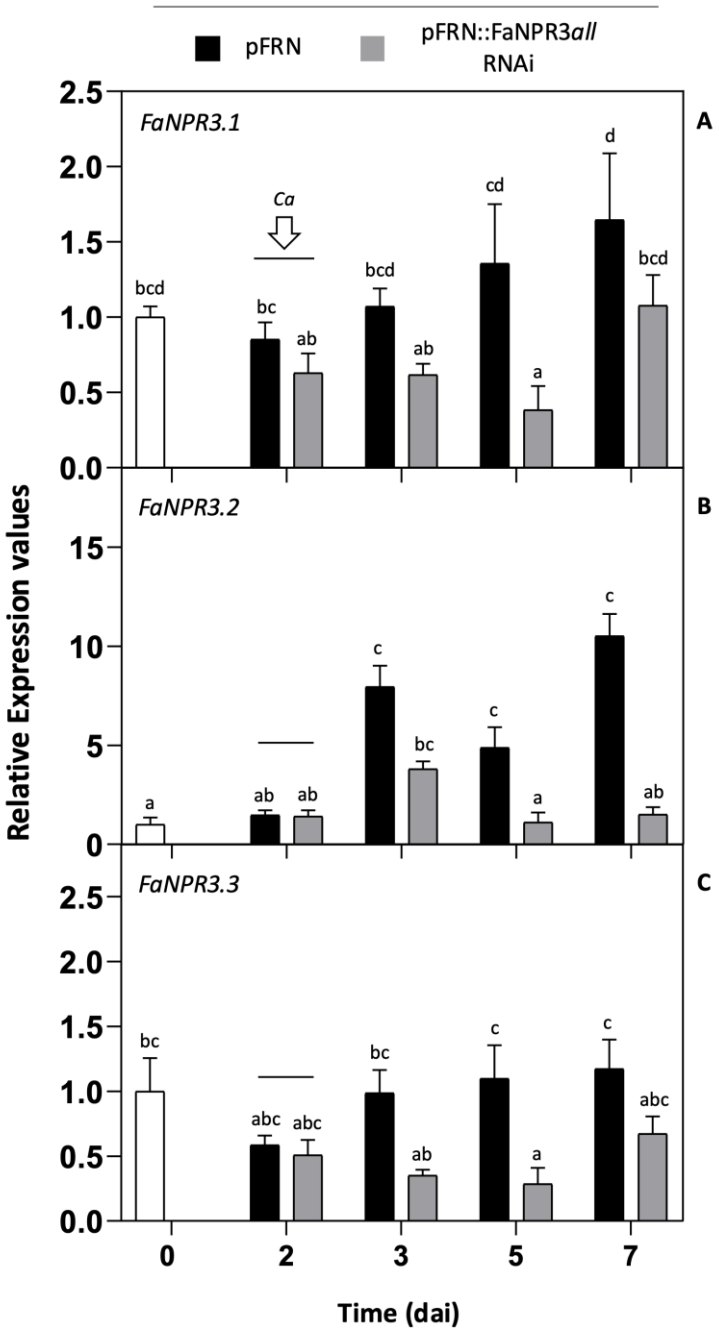


**Figure 1.** Gene expression pattern of *FaNPR3* genes in strawberry fruit after agroinfiltration with pFRN::FaNPR3*all*.RNAi construct. qRT-PCR analysis was accomplished in agroinfiltrated strawberry fruit before (0 and 2 days) and after (3, 5 and 7 days) *C. acutatum* inoculation (*Ca*). The time scale is shown as for agroinfiltration fruit samples (dai). For all the genes, arrow and horizontal bar indicate the time of *Ca* inoculation. Black and grey columns, pFRN (control) and pFRN::FaNPR3*all*.RNAi (silencing) agroinfiltrations, respectively. Data from all time points are referred to data in time cero,

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represented as 1 (white column). Bars, mean ± standard error. Note the different scales in the relative expression level axis. Statistical significance was determined by one-way ANOVA. Letters indicate significant differences ($p < 0.05$) as per HSD Tukey's post-hoc test.

Figure 2 shows the effect of the pB7GWIWG2::FaNPR32.RNAi construct on the expression of *FaNPR3* members after transient agroinfiltration of fruit. Thus, a significant reduction in transcript accumulation of *FaNPR3.2* was detected after 2 dai in the fruit half sample agroinfiltrated with this construct as compared to its corresponding opposite fruit half agroinfiltrated with the control vector pB7GWIWG2 (Figure 2B). This significant reduction was evident for up to 7 dai (5 dpi). However, no significant changes in transcript accumulation were detected for *FaNPR3.1* (Figures 2A) and *FaNPR3.3* (Figures 2C) genes at any time sampled when comparing the fruit half silenced with its corresponding fruit half control. Similarly to the results shown in Figure 1B, after *C. acutatum* inoculation, a relevant induction in the expression of *FaNPR3.2* was detected in fruit control samples (Figure 2B) whereas no significant changes in gene expression were detected for *FaNPR3.1* and *FaNPR3.3* (Figures 2A and C, respectively), which strongly support that *FaNPR3.2* is preferentially activated in response to pathogen infection. As aforementioned, under these experimental conditions, no relevant changes in transcript accumulation were found for any of the other gene members of the *NPR*-like family, *FaNPR1* and *FaNPR5*, and similar patterns of gene silencing were detected for all the *FaNPR3* genes when fruit agroinfiltrated was not inoculated with *C. acutatum* (data not shown). These results evidence that silencing of *FaNPR3.2* is mostly achieved in strawberry fruit after agroinfiltration with the pB7GWIWG2::FaNPR32.RNAi construct.

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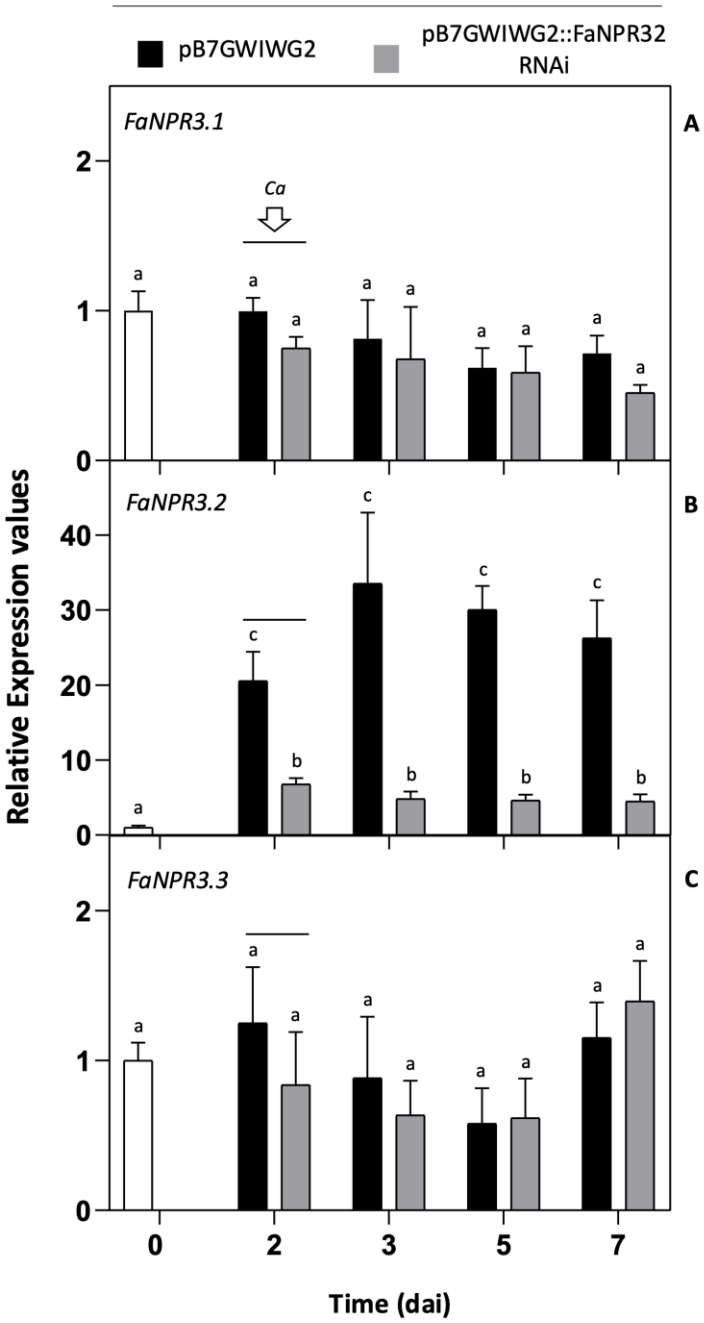


**Figure 2.** Gene expression pattern of *FaNPR3* genes in strawberry fruit after agroinfiltration with pB7GWIWG2::FaNPR32.RNAi. qRT-PCR analysis was accomplished in agroinfiltrated strawberry fruit before (0 and 2 days) and after (3, 5 and 7 days) *C. acutatum* inoculation (*Ca*). The time scale is shown as for agroinfiltration fruit samples (dai). Arrow indicates the time of *Ca* inoculation. Black and grey columns, pB7GWIWG2 and pB7GWIWG2::FaNPR32.RNAi agroinfiltrations, respectively. Data from all time points are referred to data in time cero, represented as 1 (white column). Bars, mean ± standard error. Note the different scales in the relative expression level axis. Statistical significance was determined by one-way ANOVA. Letters indicate significant differences ($p < 0.05$) as per HSD Tukey's post-hoc test.

The effect of ectopic overexpression of *FaNPR3.1* and *FaNPR3.2* genes in strawberry fruit on the transcriptional pattern of all strawberry NPR-like gene family members was also analyzed using the pK7WG2::FaNPR31.OE and pB7WG2::FaNPR32.OE constructs for transient fruit expression,

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respectively, which carry the inserted genes under the control of the strong promoter CaMV35S. In our experimental conditions, the transient overexpression of either *FaNPR3.1* or *FaNPR3.2* variants (see Supplementary Figure 1) in strawberry fruit did not significantly alter the transcript accumulation level of any other endogenous member of the *FaNPR* gene family (data not shown).

### *2.2. The Silencing of FaNPR3 Genes in Strawberry Fruit Reduced Fruit Tissue Damage after C. acutatum Inoculation*

According to the above results, the evaluation of fruit tissue damage, and the comparative study of susceptibility to *C. acutatum* between the two opposite halves of the same fruit (one agroinfiltrated with either the silencing or the overexpression construct and the other with the corresponding empty vector as control) was accomplished after 6 dai. Overall, no relevant visual differences were observed in external tissue damage of opposite halves of the same fruit, in fruit samples silenced with the pFRN::FaNPR3*all*.RNAi or pB7GWIWG2::FaNPR32.RNAi silencing construct (upper panels, Figures 3A and 4A, respectively) and fruit samples where *FaNPR3.1* or *FaNPR3*.2 was overexpressed (upper panels, Figures 3B and 4B, respectively). However, a relevant reduction in internal tissue damage was clear within fruit half agroinfiltrated with the silencing construct versus fruit half agroinfiltrated with control vector in both pFRN::FaNPR3*all*.RNAi (Figure 3A, lower panel) and pB7GWIWG2::FaNPR32.RNAi (Figure 4A, lower panel) silenced fruit samples. When genes *FaNPR3.1* and *FaNPR3.2* were ectopically overexpressed in strawberry fruit (lower panels of Figures 3B and 4B, respectively), no relevant visual difference in internal tissue damage was observed within fruit half agroinfiltrated with the overexpression construct compared to opposite half agroinfiltrated with the control vector.

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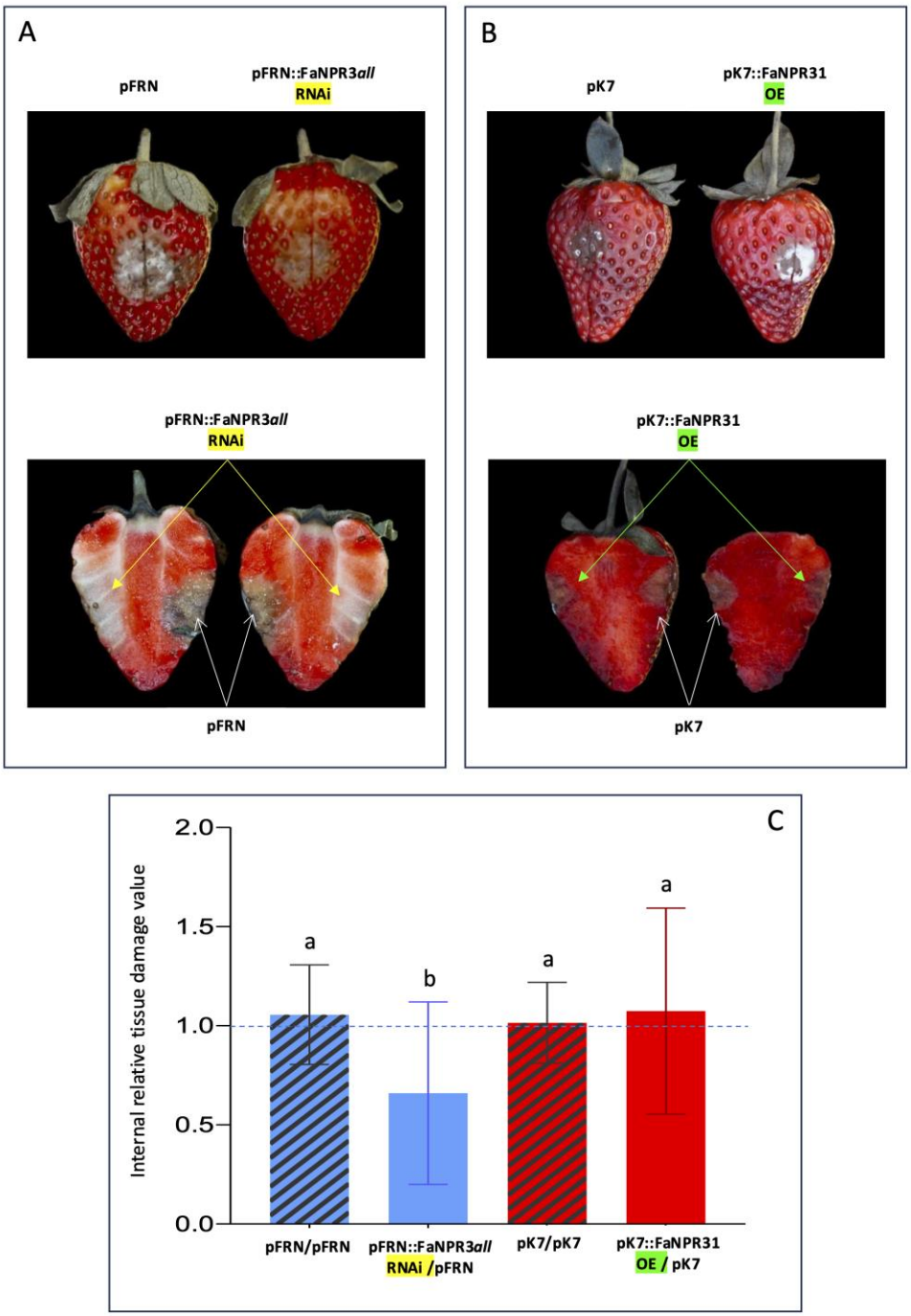


**Figure 3.** Silencing effect of pFRN::FaNPR3*all*.RNAi and ectopic overexpression of *FaNPR3.1* in strawberry fruit after *C. acutatum* infection. (**A**) and (**B**) upper panels, external surface disease symptoms on the two agroinfiltrated opposite halves of the same fruit, after silencing and overexpression, respectively. (**A**) and (**B**) lower panels, internal tissue damage of the same fruit shown in the corresponding upper panels. pFRN::FaNPR3*all*.RNAi and pFRN, silencing construct and its corresponding empty vector, as control. pK7::FaNPR31.OE and pK7, overexpression construct and its corresponding empty vector, as control. A relevant fruit is shown for each condition, as an example. (**C**) Statistically analysis of internal tissue damage ratio of the two opposite halves of the same fruit, according to the 1 to 5 severity scale; striped and plain blue bars, pFRN/pFRN and pFRN/pFRN::FaNPR31-RNAi agroinfiltrated values, respectively; striped and plain red bars, pK7/pK7 and pK7/pK7::FaNPR31-OE agroinfiltrated values, respectively. Data correspond to mean ± SD. Within each bar, means with different letters are significantly different by LSD test at $p < 0.05$. A ratio value of 1, indicate no differences between opposite halves of the same fruit.

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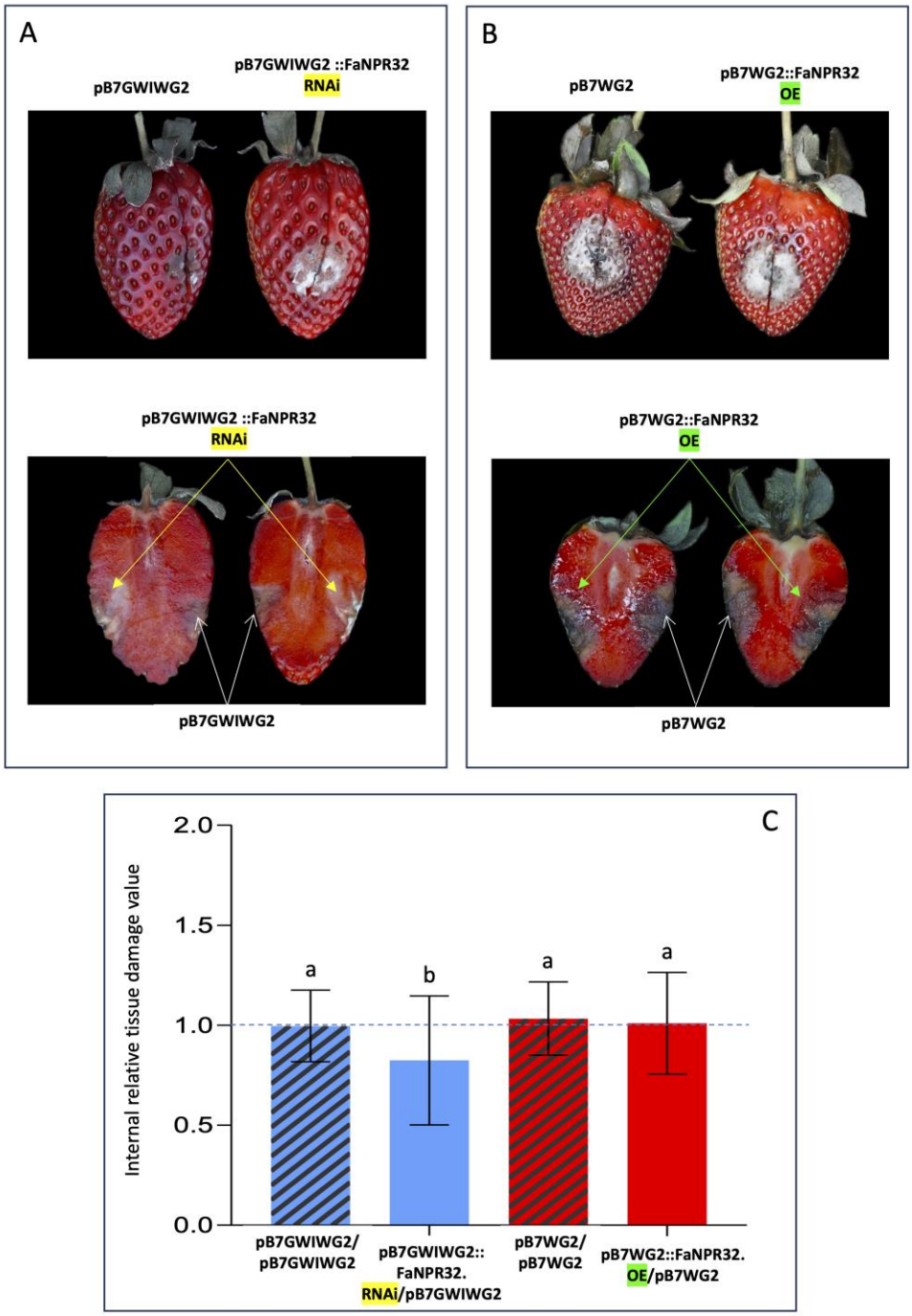


**Figure 4.** Silencing effect of pB7GWIWG2::FaNPR32.RNAi and ectopic overexpression of *FaNPR3.2* in strawberry fruit after *C. acutatum* infection. (**A**) and (**B**) upper panels, external surface disease symptoms on the two agroinfiltrated opposite halves of the same fruit, after silencing and overexpression, respectively. (**A**) and (**B**) lower panels, internal tissue damage, of the same fruit shown in the corresponding upper panels. pB7GWIWG2::FaNPR32.RNAi and pB7GWIWG2, silencing construct and its corresponding empty vector, as control. pB7WG2::FaNPR32.OE and pB7WG2, overexpression construct and its corresponding empty vector, as control. A relevant fruit is shown for each condition, as an example. (**C**) Statistically analysis of internal tissue damage ratio of the two opposite halves of the same fruit, according to the 1 to 5 severity scale; striped and plain blue bars, pB7GWIWG2/pB7GWIWG2 and pB7GWIWG2/pB7GWIWG2::FaNPR32-RNAi agroinfiltrated values, respectively; striped and plain red bars, pB7WG2/pB7WG2 and pB7WG2/pB7WG2::FaNPR32-OE agroinfiltrated values, respectively. Data correspond to mean ± SD. Within each bar, means with

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different letters are significantly different by LSD test at $p < 0.05$. A ratio value of 1, indicate no differences between opposite halves of the same fruit.

A statistical analysis of the internal fruit tissue damage was conducted either in silenced and overexpressed fruit halves and compared to their corresponding control halves. The tissue damage value obtained by normalizing fruit halves transformed with the silencing construct with respect to the corresponding opposite fruit halves transformed with the empty vector were significantly reduced in pFRN::FaNPR3*all*.RNAi and pB7GWIWG2::FaNPR32.RNAi silenced samples (mean value of 0.6603, and 0.8242, respectively) compared to those obtained when both fruit halves were agroinfiltrated with control constructs (mean value of 1.0556, and 0.9958, respectively) (Figures 3C and 4C, left). However, no significant differences were found in FaNPR3.1 and *FaNPR3.2* overexpressed samples (mean value of 1.0737, and 1.0097, respectively) compared to their corresponding control samples (mean of 1.0152, and 1.0327, respectively) (Figures 3C and 4C, right). For external fruit tissue damage, all the ratio values show no significant differences either for silenced or overexpressed samples (data not shown). These results unravel a positive correlation between the silencing of members of the *FaNPR3* gene family and an increase in fruit resistance to *C. acutatum* infection.

*2.3. Analysis of Defense Related Genes in Strawberry Fruit Silenced in FaNPR3 Genes*

To gain insights into the strawberry defense network associated with members of the FaNPR3 clade, we analyzed the expression profile of several strawberry genes already known to respond to *C. acutatum* infection such as *FaPR1*-1(*AtPR1* ortholog), *FaPR2*-1 (*AtPR2* ortholog), *FaPR5.2* (AT4G11650 ortholog), *FaWRKY19* (previously reported as *FaWRKY33-2; AtWRKY25/33/26* ortholog), *FaWRKY24* (previously reported as *FaWRKY1*; *AtWRKY75* ortholog), *FaWRKY41* (previously reported as *FaWRKY70-1; AtWRKY54/70* ortholog), and *FaWRKY60* (previously reported as *FaWRKY70-2; AtWRKY54/70* ortholog). The expression level of *FaWRKY19* and *FaWRKY24* genes increased in fruit control sample two days after agroinfiltration with either pFRN or pB7GWIWG2) and remained significantly high upon *C. acutatum* inoculation (Figure 5A and B). However, a significantly reduction in transcript accumulation was detected for both genes, *FaWRKY19* and *FaWRKY24*, in the corresponding opposite fruit half agroinfiltrated with the silencing constructs pFRN::FaNPR3*all*.RNAi (Figure 5A) and pB7GWIWG2::FaNPR32.RNAi (Figure 5B). Similar patterns of down-regulation were detected for *FaWRKY19* and *FaWRKY24* genes when the agroinfiltrated fruit was not inoculated with *C. acutatum* (data not shown). For *FaPR1-1*, *FaPR2-1*, *FaPR5-2*, *FaWRKY41*, and *FaWRKY60* genes, no significant difference was found in their transcript accumulation patterns when comparing fruit agroinfiltrated with the silencing construct (either pFRN::FaNPR3*all*.RNAi or pB7GWIWG2::FaNPR32.RNAi) *vs* its corresponding control construct (data not shown).

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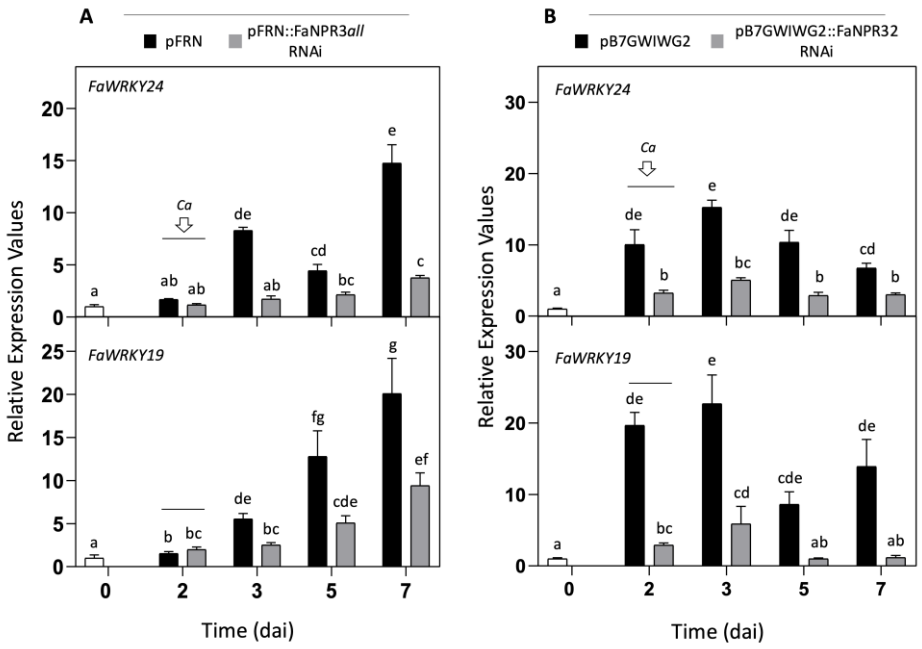


**Figure 5.** Silencing effect of *FaNPR3* members on the expression of *FaWRKY19* and *FaWRKY24* genes in strawberry fruit after *C. acutatum* infection. (**A**) Silencing effect of pFRN::FaNPR3*all*.RNAi. (**B**) Silencing effect pB7GWIWG2::FaNPR32.RNAi. Black columns, the expression of *FaWRKY19* and *FaWRKY24* genes in half fruit agroinfiltrated with pFRN (**A**) or pB7GWIWG2 (**B**) control vectors. Grey columns, the expression of *FaWRKY19* and *FaWRKY24* genes in half fruit agroinfiltrated with pFRN::FaNPR3*all*.RNAi (**A**) or pB7GWIWG2::FaNPR32.RNAi (**B**). qRT-PCR analysis was accomplished in agroinfiltrated strawberry fruit before (0 and 2 days) and after (3, 5 and 7 days) *C. acutatum* inoculation (*Ca*). The time scale is shown as for agroinfiltration fruit samples (dai). Arrow indicates the time of *Ca* inoculation. Data from all time points are referred to data in time cero, represented as 1 (white column). Bars, mean ± standard error. Note the different scales in the relative expression level axis. Statistical significance was determined by one-way ANOVA. Letters indicate significant differences ($p < 0.05$) as per HSD Tukey's post-hoc test.

These results strongly evidence that the expression of genes *FaWRKY19* and *FaWRKY24* is positively modulated in fruit by FaNPR3 members of the strawberry NPR3-like gene family.

### *2.4. The Strawberry FaNPR3.2 Gene Complements the Arabidopsis Atnpr3npr4 Double Mutant Disease Resistance Phenotype*

To get a deeper insight into the function of the strawberry FaNPR3 family, in parallel to the silencing of the *FaNPR3.1* and *FaNPR3.2* in strawberry, a heterologous complementation analysis has been conducted in *Arabidopsis* plants with *FaNPR3.2*.

Therefore, *Arabidopsis* transgenic lines were generated by transforming WT and *npr3npr4* double mutant plants with pB7WG2::FaNPR32-OE, the DNA cassette driving the expression of the strawberry *FaNPR3.2* under the control of a strong promoter. Homozygous plants from each line were selected to perform functional complementation tests by characterizing the expression of the transgene and their disease resistance phenotype upon inoculation with virulent *Pseudomonas syringae* pathovar tomato strain DC3000 (*Pst*).

The *FaNPR3.2* transcript accumulates abundantly in the leaves of the overexpressing lines *npr3npr4*::FaNPR3.2 and even at higher levels in WT::FaNPR3.2 (Figure 6A). Interestingly, as shown in Figure 6B, the disease symptoms development at 3, 5 and 7 dpi on the leaves of *npr3npr4*::FaNPR3.2

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and WT::FaNPR3.2 overexpressing transgenic plants was similar to that of WT plants, showing chlorotic symptoms and necrotic lesions, in contrast to the asymptomatic *npr3npr4* double mutant plants.

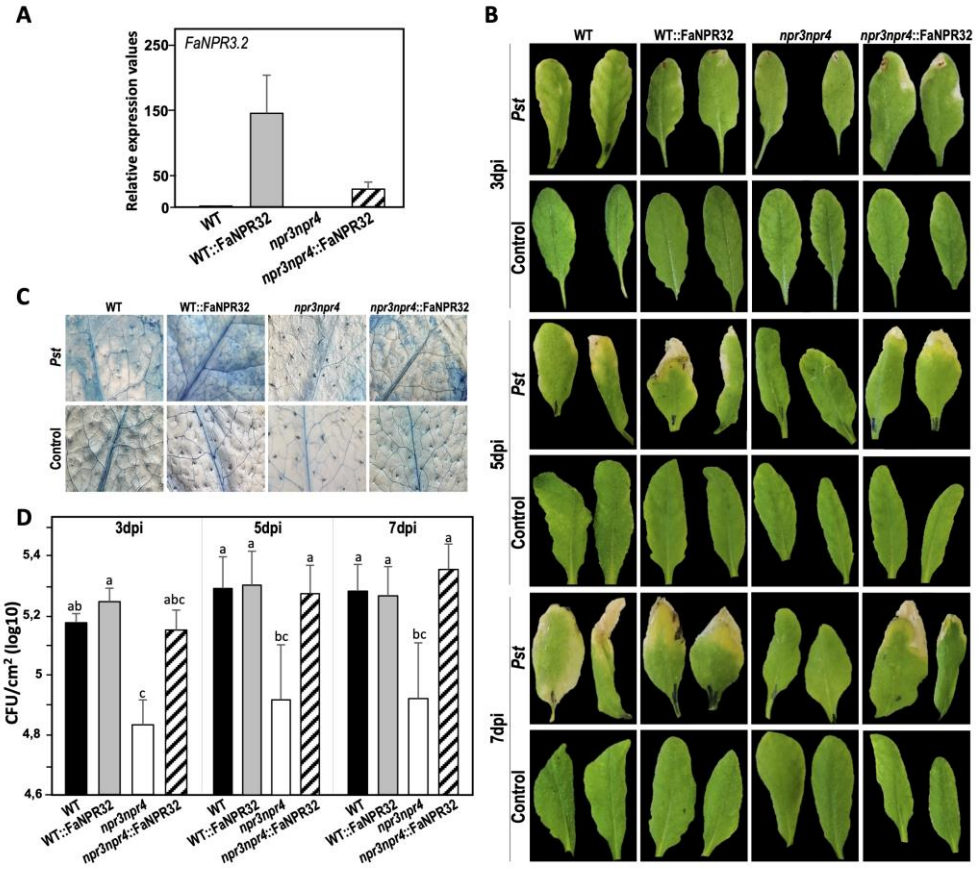


**Figure 6.** Characterization of *Arabidopsis* lines**.** (**A**) *FaNPR3.2* expression in *Arabidopsis* WT, *npr3npr4* mutant and *FaNPR3.2* overexpressing lines *npr3npr4*::FaNPR32 and WT::FaNPR32 (black, grey, white and striped bars, respectively). Transcript accumulation was monitored by qRT-PCR in non-infected plants as described in the Materials and methods. Expression levels were normalized with respect to the internal control *ACTIN2* and displayed relative to the threshold value of the wild type (no expression of *FaNPR3.2*) that was given a value of 1 for convenience. Note that no amplification of *FaNPR3.2* strawberry gene is detected in neither WT nor double mutant *npr3npr4* using the specific primers (Supplementary Table S1). Bars represent the mean levels of transcript quantified from three independent biological experiments (±SD). (**B**-**D**) Disease resistance phenotype of *Arabidopsis* lines

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upon *Pst* inoculation (**B**) Symptoms development on leaves 3, 5, and 7 days post inoculation (dpi) ($10^6$ CFU $mL^{-1}$); (**C**) Trypan blue staining for the detection of cell death 1 dpi ($10^5$ CFU $mL^{-1}$); (**D**) In planta bacterial growth monitored 3, 5 and 7 day post inoculation ($10^6$ CFU $mL^{-1}$). CFU, colony-forming units. Statistically significant differences are labelled (one-way ANOVA, Tukey's multiple comparisons test, $P < 0.05$). *Pst* were pressure infiltrated into fully expanded mature leaves from 4-5 weeks old *Arabidopsis* plants. As control, leaves were infiltrated with 10mM $MgCl_2$. The whole experiment was performed three times with similar results.

Next, we analyzed the *Pst*-induced cell death phenotype by trypan blue staining and found that plants overexpressing *FaNPR3.2* in the WT and *npr3npr4* mutant genetic background showed noticeably enhanced cell death compared to WT and double mutant *npr3npr4*, respectively (Figure 6C). Indeed, our results show that the presence of endogenous AtNPR3 and AtNPR4 in *Arabidopsis* together with the strawberry FaNPR3.2 results in cell death spreading across the entire infected leaves of WT::FaNPR3.2 plants, whereas it was more restricted in *npr3npr4*::FaNPR3.2 leaves. In addition, bacterial growth of virulent *Pst* was significantly restrained in the absence of AtNPR3 and AtNPR4 (double mutant *npr3npr4*) at all times tested (3, 5 and 7 dpi), while overexpression of *FaNPR3.2* reverted the WT disease phenotype in *npr3npr4* plants, displaying similar bacterial density than WT plants (Figure 6D).

Thus, *Arabidopsis npr3npr4*::FaNPR3.2 plants reverts the *Pst*-increased resistance phenotype of the double mutant *npr3npr4* to wild type levels, in terms of disease symptoms development and bacterial growth.

### *2.5. Changes in the expression profile of defence-related genes in Arabidopsis*

To shed light on the molecular mechanisms underlying FaNPR3.2 complementation of *Arabidopsis npr3npr4* double mutant, the expression profile of the typical SA defense pathway marker genes *PR1*, *PR2*, and *PR5*, was analyzed both at basal levels and after infection with *Pst* and compared among all the *Arabidopsis* lines. The results show that transcript accumulation of this set of genes was barely detectable in naïve non infected WT plants (Figure 7A), and it increased following infection (Figure 7B), indicative of the activation of the defense response. However, in enhanced resistant *npr3npr4* mutant plants the basal transcript level for these genes far exceeded that of WT, in uninfected conditions, and was further strongly induced after *Pst* infection. Curiously, *Arabidopsis* plants overexpressing *FaNPR3.2*, which reverts the WT disease phenotype, displayed remarkably high basal levels of *PR*s transcript and these marker genes were even further induced upon *Pst* challenge (Figure 7B).

Ours results reflect a clear uncoupling between the resistance phenotype and the induction of these defense-related markers in *Arabidopsis* plants overexpressing the strawberry *FaNPR3.2* gene.

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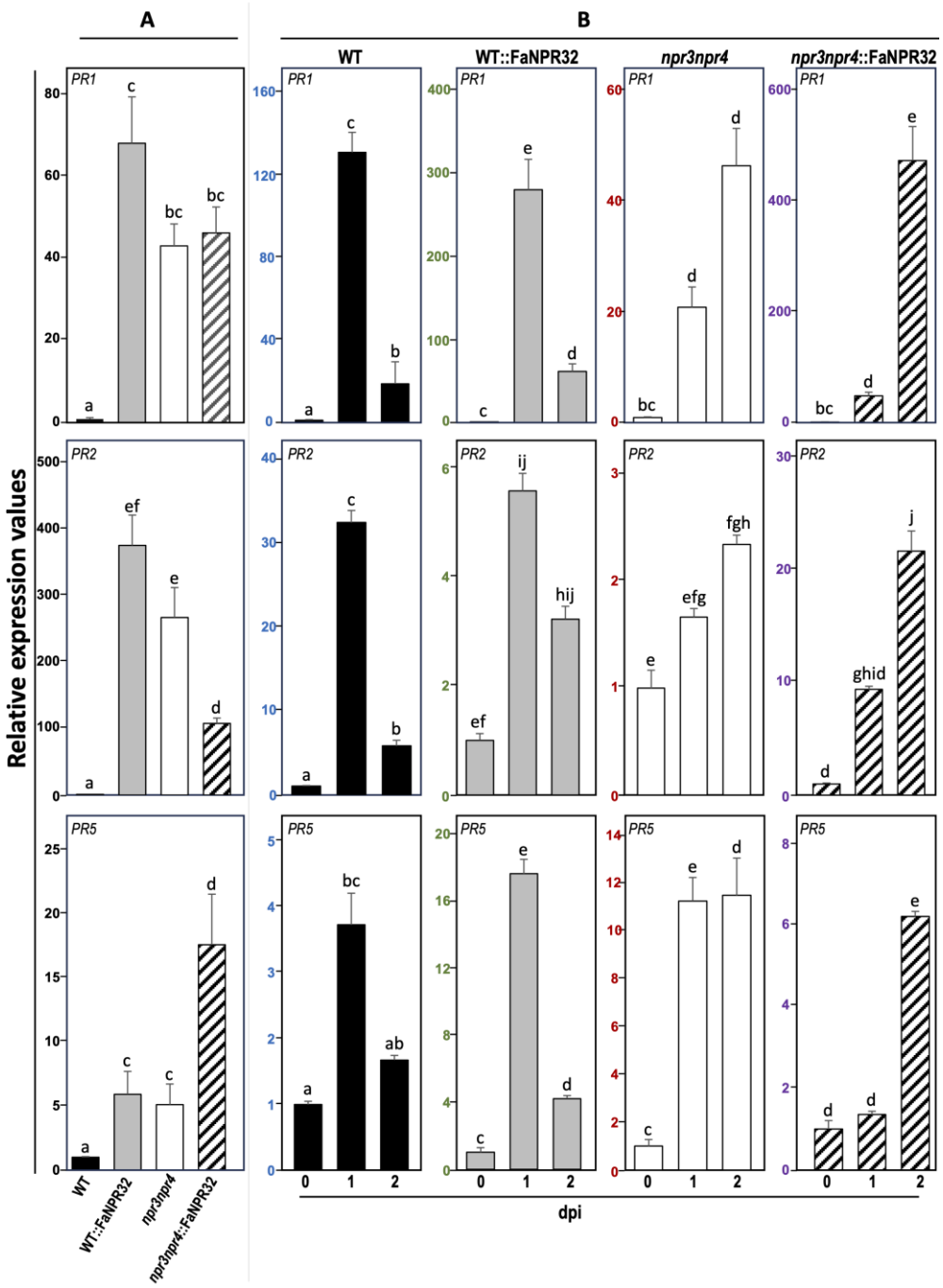


**Figure 7.** Expression of the defense-related genes *PR1*, *PR2*, and *PR5* in *Arabidopsis* lines. Relative expression level was monitored by qRT-PCR in control (**A**) and infected plants 1 and 2 days post inoculation (dpi) with *Pst* ($10^6$ CFU $mL^{-1}$) (**B**), in WT, WT::FaNPR32, double mutant *npr3npr4*, and *npr3npr4*::FaNPR32 (black, grey, white and striped bars, respectively). Expression levels were normalized with respect to the internal control *ACTIN2* and displayed relative to the expression in mock-treated wild-type samples (A) or to the expression in mock-treated samples of each line (B) that were given a value of 1. Bars, mean ± standard error. Note the different scales in the relative expression level axis. Statistical significance was determined by one-way ANOVA. Letters indicate significant differences ($p < 0.05$) as per HSD Tukey's post-hoc test.

We next explored whether the enhanced basal level of these classic defense markers observed in lines overexpressing *FaNPR3.2* was related with an impaired expression profile of any of the endogenous members of the *NPR* gene family in *Arabidopsis*, *AtNPR1*, *AtNPR3* and *AtNPR4*. Thus,

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the *NPR1* basal transcript level in uninfected plants was similar in WT, *npr3npr4* mutant and their corresponding overexpressing FaNPR3.2 lines (Figure 8A), and also upon *Pst* infection, the At*NPR1* gene expression remained unaltered in all lines tested (Figure 8B). As expected, no *AtNPR3* and *AtNPR4* transcript was detected in *npr3npr4* and *npr3npr4*::FaNPR3.2 lines. Interestingly, the basal transcript level of both endogenous *AtNPR3* and *AtNPR4* was significantly reduced in WT::FaNPR3.2 compared to that of WT plants. (Figure 8A). In addition, a significantly stronger induction of *AtNPR3* and *AtNPR4* genes was observed in WT::FaNPR3.2 overexpressing lines compared to that of WT lines (Figure 8B).

The results shown in Figure 7A and Figure 8A evidence that in the FaNPR3.2 overexpressing lines a positive correlation exists between the enhanced basal level of *PR*s and the reduced basal level of *AtNPR3* and *AtNPR4*.

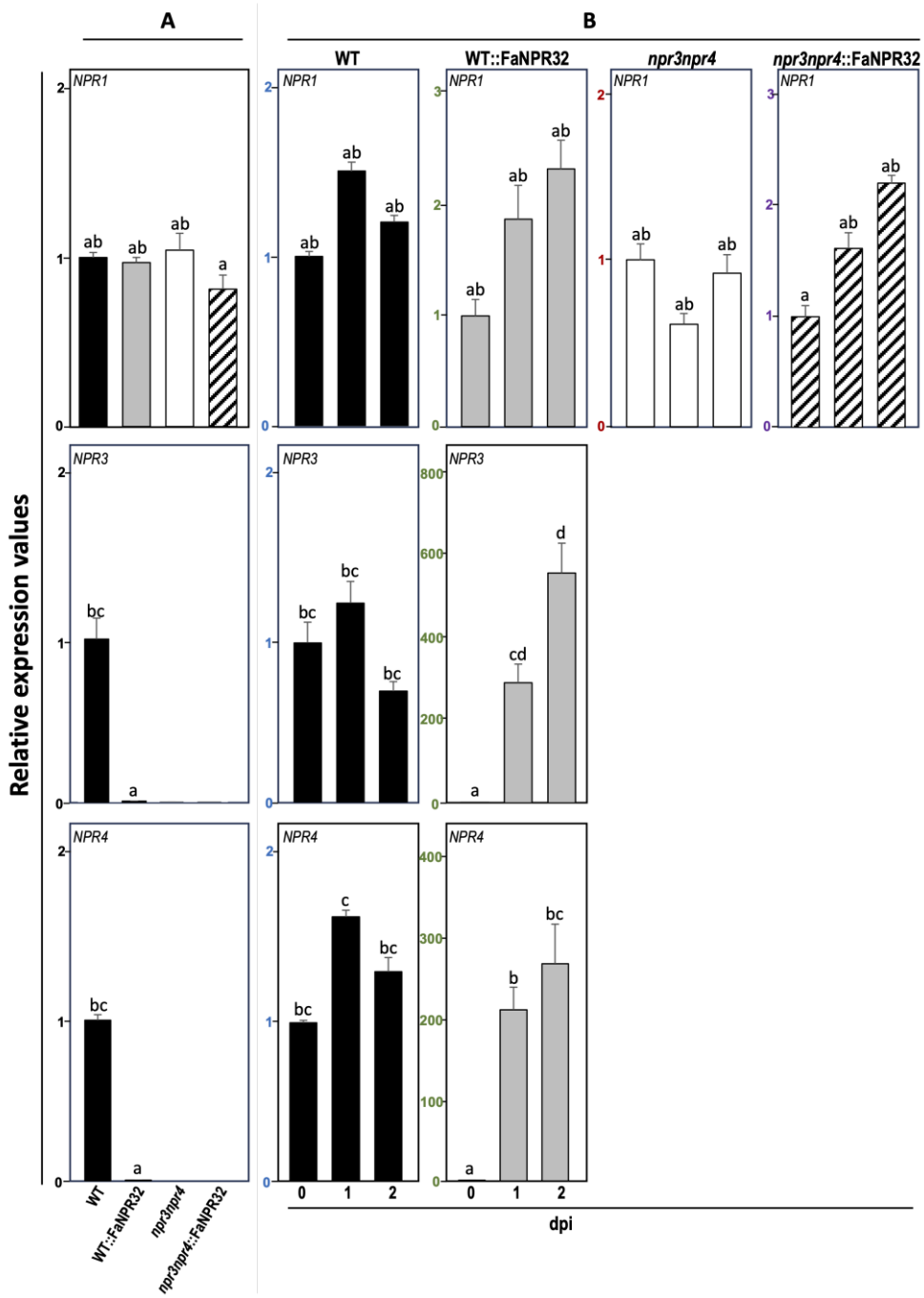

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**Figure 8.** Expression of the endogenous *AtNPR1*, *AtNPR3* and *AtNPR4* genes in *Arabidopsis*. Relative expression level was monitored as described in Figure 7. Note that the absence of transcript for *AtNPR3* and *AtNPR4* in the overexpressing lines in the double mutant *npr3npr4* background proves that *Arabidopsis* primers do not amplify strawberry orthologous genes.

## 3. Discussion

### *3.1. Members of the FaNPR3 Clade Negatively Modulate Strawberry Fruit Resistance Against Colletotrichum acutatum*

This report primarily focuses on understanding the molecular role that *FaNPR3* members of the strawberry *NPR*-like gene family play in the defense response against pathogens. Thus, we have been quite successful in transiently silencing all members of *FaNPR3* clade II in strawberry fruit using *A. tumefaciens* carrying the silencing construct pFRN::FaNPR3*all*-RNAi (Figure 1). This construct contains a DNA sequence that was previously predicted to promote highly effective siRNAs matching all *FaNPR3* genes in strawberry (*F. ananassa*) [40]. Furthermore, we have also transiently silenced predominantly *FaNPR3.2* homeologs in strawberry fruit (Figure 2) using the pB7GWIWG2::FaNPR32-RNAi silencing construct, highly predicted to produce siRNAs targeting essentially *FaNPR3.2* alleles. Transient reduction of either all *FaNPR3.1*, *FaNPR3.2*, and *FaNPR3.3* transcripts or mainly *FaNPR3.2* transcripts, led to a clearly noticeable decrease in disease symptoms and significantly reduced internal strawberry fruit tissue damage after *C. acutatum* inoculation (Figures 3C and 4C, left). On the contrary, transient overexpression of either *FaNPR3.1* or *FaNPR3.2* genes did not significantly affect fruit susceptibility to *C. acutatum* (Figures 3C and 4C, right). According to previously published results and consequently with the agroinfiltration methodology used, no clear difference in the external surface damage was detected between silenced (or overexpressed) and control fruit samples [48].

Interestingly, the reduction of the internal fruit tissue damage was more relevant after the silencing of all members of the *FaNPR3* gene family than only *FaNPR3.2*, evidencing that the FaNPR3 paralogs negatively modulate strawberry fruit defense against *C. acutatum*. Supporting this idea, it has also been recently shown that ectopic expression of the *FvNPRL-1* (the homolog of *FaNPR3.1* in *F. vesca*) in wild-type *Arabidopsis* led to plants suppressing resistance to *Pst*, suggesting that FvNPRL-1 could probably function as a negative regulator of the SA-mediated defense in this heterologous system [72]. All in all, these results are consistent with a role of the strawberry *FaNPR3* gene family similar to that of their *Arabidopsis* orthologs, AtNPR3/4, as negative regulators of plant defense [59].

### *3.2. FaNPR3.2 Negatively Modulates Resistance in Arabidopsis*

Analysis of gene knockout mutants constitutes the priority and direct approach in revealing and clarifying gene function. However, so far there are no known strawberry varieties with *FaNPR3* knockout genes. Thus, to deepen the knowledge of FaNPR3 members have in the defense response, a heterologous expression system approach was used. We have previously used this procedure to successfully carry out functional studies of strawberry defense-related genes in *Arabidopsis* [44]. Accordingly, *Arabidopsis npr3npr4* double mutant plants, lacking FaNPR3.2 orthologues, that display enhanced resistance against virulent *Pst* [55] were complemented with its strawberry counterpart. Convincingly, the enhanced disease resistance of *Arabidopsis* double mutant *npr3npr4* to virulent *Pst* is fully complemented by overexpression of *FaNPR3.2*, as the resistance phenotype in the *Arabidopsis npr3npr4*::FaNPR3.2 plants, in terms of disease symptoms development and bacterial growth, resembles that of the wild type plants (Figure 6 A, B and D). Similarly, ectopic expression of this strawberry gene *FaNPR3.2* in the WT background resulted in plants (WT::FaNPR3.2) that exhibit no appreciable differences in the resistance phenotype compared with WT plants. These results strongly support the aforementioned role of the strawberry FaNPR3.2 protein, similar to that proposed in *Arabidopsis* for NPR3/NPR4 in negatively regulating resistance [59]. This is not surprising given the high amino acid sequence similarity and the existence of well conserved motifs between the FaNPR3.2 protein and its *Arabidopsis* orthologs [49,72,73].

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Curiously, overexpression of FaNPR3.2 in the *Arabidopsis* WT and *npr3npr4* background results in increased cell death in response to *Pst* infection compared to WT and the double mutant *npr3npr4* plants, especially when coexisting with *Arabidopsis* proteins AtNPR3 and AtNPR4 (Figure 6C). However, it does not appear to have an appreciable effect on restricting bacterial growth (Figure 6D). In this sense, our results agree with an additional role for FaNPR3.2 as positive regulator of cell death in *Arabidopsis* throughout the defense response to *Pst*, as it has been reported for AtNPR3/4. Consequently, the double mutant *npr3npr4* suppresses cell death in response to avirulent pathogen infections [74].

### *3.3. Silencing of FaNPR3 Members in Strawberry Fruit Downregulates FaWRKY19 and FaWRKY24 Gene Expression*

In an attempt to expand our understanding of potential molecular players within the strawberry regulatory defense network downstream FaNPR3, we have monitored the molecular signature of several strawberry SA- and JA- responsive genes known to be up-regulated after *C. acutatum* infection [42]. Only transcript accumulation of the *WRKY* genes *FaWRKY19* and *FaWRKY24* [50] was found to be significantly reduced in fruit after agroinfiltration with both *FaNPR3.1* and *FaNPR3.2* silencing constructs (Figure 4), and no relevant change was detected in the expression level for the remaining SA- and JA-responsive strawberry genes analyzed (data not shown).

Unexpectedly, and in contrast to the transcriptional co-repressor role described for its *Arabidopsis* NPR3/4 orthologs [59], our results reveal that in strawberry fruit members of the FaNPR3 clade positively regulate *FaWRKY19* and *FaWRKY24* genes. Very intriguingly, the concomitant decrease in transcript accumulation of both genes following silencing of *FaNPR3* members correlates with the decrease in tissue damage observed in fruit after *C. acutatum* inoculation. This result is consistent with our previous report that silencing of the *FaWRKY24* gene (previously reported as *FaWRKY1*) in strawberry fruit increases the resistance of this tissue to *C. acutatum* infection [44]. In fact, *WRKY* genes are well known to modulate defense responses either positively or negatively [75] and in *F. ananassa*, down-regulation of other members of the *WRKY* family such as *FaWRKY29* or *FaWRKY64* has also been correlated with enhanced resistance against pathogens [52]. Interestingly, the strawberry FaWRKY19 protein shows high degree of amino acid similarity with *Arabidopsis* AtWRKY25/33/26 proteins [50] and WRKY25/33 orthologs have also been correlated with plant increased resistance. AtWRKY33 protein has been described as a key component of the defense related pathway in plants which exhibits a complex and contradictory functional role. Indeed, silencing of *AtWRKY33* gene led to plants with increased susceptibility to necrotrophic fungal pathogens [76] but also, the ectopic expression of this gene caused enhanced susceptibility of plants to the bacterial pathogen *Pst* [77], suggesting that this AtWRKY33 protein can act either positively or negatively on plant defense depending on the pathogen lifestyle [78]. Consistent with our results in strawberry, down regulation of *WRKY33* orthologs in rice led to enhanced resistance to *Xanthomonas oryzae* [79]. Similarly, AtWRKY25-overexpressing plants display increased bacterial growth and enhanced disease symptoms, while silencing of *AtWRKY25* reduced disease symptoms after *Pst* infection [80]. These reports highlight that different plant species can assemble slightly different molecular mechanisms in response to pathogens, and that components of the plant defense network can exhibit a versatile way of acting depending on the lifestyle of the attacker encountered [78,81].

In strawberry, it remains to be further studied whether FaNPR3 members control downstream defense-response components differently than their orthologs in *Arabidopsis* and/or whether this protein acts as a molecular switch when the type of pathogen differs. Curiously, based on the structural differences between strawberry NPR-like proteins and their orthologs in *Arabidopsis*, it appears that functional divergence may occur in strawberry NPRs orthologs [49].

### *3.4. Resistance to Pseudomonas syringae in Arabidopsis Plants Overexpressing FaNPR3.2 is Uncoupled from PRs Gene Expression*

Our results showing the recovery of *npr3npr4* mutant plants resistance phenotype to wild type levels, in terms of disease development, cell death and bacterial growth, by ectopic expression of

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*FaNPR3.2* (Figure 6), strongly support that FaNPR3.2 and AtNPR3/AtNPR4 proteins have similar functions in plant defense. To further extend our knowledge about the role of FaNPR3.2, we monitored the expression pattern of the classical *PR* markers (a hallmark of the SA signaling pathway) [55] in *Arabidopsis* plants overexpressing *FaNPR3.2* and compared them with those in WT and *npr3npr4* double mutant plants.

Paradoxically, our data reflect the uncoupling between *PR*s transcript accumulation and the resistance phenotype. Indeed, overexpression of *FaNPR3.2* in both *npr3npr4* and WT genetic backgrounds, results in higher constitutive expression levels for this set of *PR* genes, that are further induced upon *Pst* infection (Figure 7). Interestingly, the uncoupling between SA-dependent markers expression profiles and the resistance phenotype has been reported previously by our group when complementing the *Arabidopsis* mutant *wrky75* by overexpressing strawberry FaWRKY1 [44]. Furthermore, uncoupling has been previously reported by Zeier et al., 2004 [82], who evidenced that systemic acquire resistance can be executed independently from *PR*s markers under specific external conditions.

At this point, explaining the uncoupling mechanism driven by *FaNPR3.2* would be speculative. However, our results show that *AtNPR1* transcript level in absence of infection is similar in WT, *npr3npr4* mutant and overexpressing FaNPR3.2 plants (Figure 8A, upper panel) and the level of *AtNPR1* induction in *Pst*-challenged plants does not differ significantly among all the *Arabidopsis* lines (Figure 8B, upper panel). In contrast, as expected, *AtNPR3* and *AtNPR4* transcripts are absent in *npr3npr4*::FaNPR3.2 lines (Figure 8A, middle and lower panels), but interestingly, the transcript basal level of these two *Arabidopsis* genes is reduced in WT::FaNPR3.2 compared to that of wild-type plants (Figure 8B, middle and lower panels). This last data is very revealing since the *FaNPR3.2* overexpression in *Arabidopsis* correlates with a decrease in the levels of the two well-known negative coregulators of *PR* expression [59] which could explain the high constitutive level of *PR*s observed in these plants (Figure 7). Also, AtNPR3/4 have been described as Cullin 3 RING ubiquitin ligases adaptors mediating NPR1 degradation [60]. Thus, the reduced basal level of NPR3/4 in the WT::FaNPR32 overexpressing background is consistent with the reduced degradation of the positive defense regulator AtNPR1, which also could contribute to the higher *PR*s basal level detected in those plants.

Interestingly, in response to *Pst* infection, *AtNPR3* and *AtNPR4* transcripts levels are significantly induced and restored to wild-type levels in *FaNPR3.2* overexpressing plants (Figure 8B). This result evidence that the repression that FaNPR3.2 exerts on *AtNPR3* and *AtNPR4* genes is abolished in response to the pathogen, which suggests that FaNPR3.2 may be sensitive to redox changes and/or to variations in SA levels related to the plant response to infection, like its *Arabidopsis* orthologs. Besides, it is known that under those conditions, the master regulator NPR1 is not the only substrate for NPR3/4- mediated degradation [53]. NPR3/4 proteins can as well bind and act as adaptors to other key regulatory proteins that mediate immune responses, such as the JA transcriptional repressor JAZ1, target them towards the Cullin 3 RING ubiquitin ligase mediated degradation pathway [60]. In fact, AtNPR3/4- mediated SA-dependent degradation of JAZs makes it possible to tailor an efficient specific defense response against biotrophic pathogens without compromising resistance to necrotrophic pathogens due to activation of the JA-signaling pathway [61]. Our data on the role of FaNPR3.2 in *Arabidopsis* are reminiscent of that of AtNPR3/4 in the crosstalk between the SA and JA signaling pathways to prioritize one over the other and thus adjust the response against necrotrophic or biotrophic pathogens as necessary [83]. Although more research is needed to clarify this aspect, the results presented here support a dual role for FaNPR3.2 in *Arabidopsis* as negative regulators of AtNPR3 and *AtNPR4* genes and the defense response, and modulators of the extent of the immune response after infection.

On the other hand, our results are not in concordance with those described by Shu et al. [72], reporting that the ectopic expression of *FvNPRL-1* in *Arabidopsis* wild type seedlings suppressed SA mediated *PR1* expression. To further investigate this discrepancy, we grew *Arabidopsis* WT, *npr3npr4* double mutant, and their corresponding FaNPR3.2 overexpressing lines on MS plates with or without SA and monitored the *PRs* expression pattern in those seedlings. As shown in Supplementary Figure

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1, all seedling lines analyzed, displayed a similar *PR1*, *PR2* and *PR5* expression pattern in response to SA treatment profile to that observed previously in plants upon *Pst* infection. These results strengthen the positive correlation observed in plants between *FaNPR3.2* overexpression and the higher constitutive basal and *Pst* induced levels of *PRs* and differ notably from those reported by Shu et al. 2018. Although intriguing, this inconsistency can be attributed to the specific NPR3 alleles overexpressed, *FvNPR3.1*, from *F. vesca*, *vs FaNPR3.2*, from *F. ananassa*. Indeed, the putative hinge region (LENRV) in the SA- binding core is conserved in FvNPR3.1 protein and in all members in strawberry FaNPR3.1 and FaNPR3.3 clade but not in FaNPR3.2 (FENRV) (Supplementary Figure 3) [49,72]. Also, this region is conserved in AtNPR1 (LENRV) but differs in AtNPR3 and AtNPR4 (LEKRV). It has been described that punctual amino acid differences in this region conduct to slightly differences in SA-binding capacity of AtNPR4 and its interaction with AtNPR1 [73,84].

The plant immune system is a complex mechanism of highly connected molecular network whose components can exhibit diverse functions to refine a successful plant defense against different pathogens. Unraveling the interaction of FaNPR3 members and downstream components of the different defense related pathways in strawberry represents a challenge and an exciting task that will help to engineer broad-spectrum disease resistance in this important crop.

## 4. Materials and Methods

### *4.1. Biological Material, Growing Conditions, and Pathogen and Elicitor Treatments*

Strawberry fruit (*Fragaria* × *ananassa* cv. Primoris) was harvested and grown under field conditions at the Experimental Farm "El Cebollar", IFAPA (Huelva, Spain). Fruits were collected with pedicel (about ten centimeters long) at an early red stage with a degree of pigmentation of about 25% as described [85], sterilized with commercial bleach (1:60 v/v), and kept individually with the pedicel immersed in sterile MS medium (0.25% Murashige Skoog) supplemented with 0,4% (w/v) sucrose, throughout the whole assay period (7 days). The MS medium was replaced every two days to minimize the effect of water stress. Fruits were kept at 25 $^{0}$C, with a photoperiod of 16h light/8h dark.

*Colletotrichum acutatum* strain CECT 20240 was used for fruit inoculation experiments. *C. acutatum* was grown at 20°C with 16/8 photoperiod, on strawberry agar (500 g/L liquefied strawberry berries and 1.5 % bacteriological agar) to improve the infectivity of the pathogen. For pathogen inoculations, starting conidia suspensions ($10^5$ conidia/mL) were prepared by diluting a stock of conidia previously obtained by scraping the surface of mycelia grown for 4 weeks, in sterile distilled water (0.03% Tween-80), filtering it through glass wool, and counting cells in a Neubauer Chamber.

The *Arabidopsis thaliana* plants used in this study belong to in the Columbia (Col-0) ecotype. Wild-type control plants (WT) (Nottingham *Arabidopsis* Stock Centre, N1093), *npr3npr4* double knockout mutant plants (provided by X. Dong, Duke University, Durham, USA), and the transgenic lines generated in this work were germinated and grown under controlled conditions (22°C, 50% humidity, 9/15 h photoperiod at a light intensity of 125 mol $m^{-2}$ $s^{-1}$) as previously described [44].

The *Arabidopsis* infection experiments were performed with 3- to 4-week-old plants using *Pseudomonas syringae* pv. tomato virulent strain DC3000 (*Pst*) provided by Dr. Antonio Molina (CBGP-UPM, Spain).

For the transient ectopic expression and silencing experiments in strawberry fruit, *Agrobacterium tumefaciens* strain AGL0 was used. For the stable transformation of *Arabidopsis* plants, the *A. tumefaciens* strain GV3101 was used.

For the salicylic acid (SA) treatment, *Arabidopsis* seeds were sterilized for 15 minutes (70% ethanol, 0,05% Triton), and then grown on solid Murashige-Skoog (MS) supplemented with 1% sucrose and 0,4% phytagel, under the controlled conditions mentioned above. Twelve-day-old *Arabidopsis* seedlings were transferred to equivalent medium containing 200 µM SA or a MS medium without SA (control) and incubated for 24h as described by Shu et al., 2018 [72].

### *4.2. Plasmid Construction for Silencing and Overexpressing FaNPR3 Genes*

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For silencing, binary plasmids pFRN (courtesy of Dr. Marten Denekamp, Department of Molecular Cell Biology, University of Utrecht (Netherlands) and pB7GWIWG2.0 vector (VIGS-Plant Systems Biology, Belgium) were used. Standard Invitrogen protocols were used for the cloning steps using gateway technology. For the silencing of all members of *FaNPR3.1*, *-3.2*, and *-3.3* the 407 bp DNA candidate sequence described in Súnico et al., 2022 [40] was cloned into pCR8/GW/TOPO (Invitrogen) and subsequently transferred to pFRN destination vector to obtain the RNAi silencing construct pFRN::FaNPR3*all*.RNAi. For the silencing of *FaNPR3.2* members, a 535 bp DNA candidate sequence (Supplementary Figure 2) from the 5'UTR region of the *FvNPR3.2* gene was amplified by PCR and cloned into pDONR221 (InvitrogenTM), and subsequently transferred to pB7GWIWG2.0 destination vector to obtain the RNAi silencing construct pB7GWIWG2::FaNPR32.RNAi. Candidate sequences were selected and amplified from *Fragaria x ananassa* genome using www.invivogen.com/sirnawizard/design.php and http://sirna.wi.mit.edu as previously described [40]. The presence of the sense and antisense orientation of the candidate DNA fragments spaced by the CHS intron was confirmed by sequencing. For the ectopic expression of FaNPR3.1, a 1.764 kb cDNA fragment (Supplementary Figure 3) carrying the complete FaNPR3.1 ORF sequence was cloned into pENTR/D/TOPO (InvitrogenTM) and then transferred to pK7WG2.0 destination vector [86]. For the ectopic expression of FaNPR3.2, a 1.785 kb cDNA fragment (Supplementary Figure 4) carrying a complete FaNPR3.2 ORF sequence was cloned into pDONR221 (InvitrogenTM) and then transferred to pB7WG2 destination vector [86]. Both final constructs, pK7WG2::FaNPR31.OE and pB7WG2::FaNPR32.OE, respectively, carry the inserted genes under the control of the CaMV35S promoter. All cDNA fragments were obtained from *Fragaria ananassa* using total RNA as template. Primer information is provided in Supplementary Table 1. All DNA inserts were sequenced prior further manipulations.

All constructs were introduced into the *A. tumefaciens* strains indicated above. For the transient ectopic expression and silencing experiments in strawberry fruit, *A. tumefaciens* strain AGL0 carrying the pK7WG2::FaNPR31.OE, pB7WG2::FaNPR32.OE, pFRN::FaNPR31*all*.RNAi, and pB7GWIWG2::FaNPR32.RNAi construct, respectively, and their corresponding empty vectors was used. For the stable transformation of *Arabidopsis* plants, the *A. tumefaciens* strain GV3101 carrying the pB7WG2::FaNPR32.OE construct was used [87].

### *4.3. Agroinfiltration of Strawberry Fruit and Experimental Design*

To analyze the effect of silencing of *FaNPR3* genes on the defense response to *C. acutatum* inoculations, we confronted a query silencing situation to its non-silenced control within the same fruit, thus avoiding the existing variability among the strawberry fruits used in the assay. Thus, one half of the fruit was infiltrated with *A. tumefaciens* (agroinfiltration) carrying the silencing/overexpression construct and the opposite half with A. tumefaciens carrying the corresponding empty vector, as a control. Then, we compared the defence response to *C. acutatum* inoculations between halves of the same fruit. The complete and detailed protocol of this agroinfiltration procedure has been previously described in Higuera et al, 2019 [48]. Briefly, for each condition and gene, a set of 144 strawberry fruits were agroinfiltrated and two days later, 120 of them were inoculated with *C. acutatum* in both halves, leaving the remaining 24 fruits to analyze the silencing of each gene under no infection conditions. From the 120 inoculated fruits, 24 of them were used for gene expression studies and the 96 remaining fruits were used for tissue damage evaluation and statistical purposes. According to Higuera et al, 2019 [48], the whole assay was repeated twice during strawberry fruiting season for two years. Samples were collected from each of the two halves of three fruits at different times for 7 days after agroinfiltration (7 dai), were quickly frozen with liquid nitrogen and kept at -80ºC, to be used later for the gene expression analysis by RT-qPCR. Likewise, every year, fruit tissue damage was evaluated at 6 dai, which corresponds to 4 days post inoculation with *C. acutatum* (4 dpi), both in 48 infected fruits, previously agroinfiltrated with the silencing construct in one half of the fruit and the empty vector in the opposite half, and in 48 infected fruits where both halves were agroinfiltrated with the empty vector, as a control for statistical purposes [48]. The effect of ectopic expression of *FaNPR3.1* and *FaNPR3.2* genes in the response of

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strawberry fruits to *C. acutatum* inoculation was evaluated following an identical protocol and experimental design but monitoring tissue damage evaluation after fruit agroinfiltration with *A. tumefaciens* strains bearing the constructs pK7WG2::FaNPR31.OE and pB7WG2::FaNPR32.OE, respectively, and their corresponding empty vectors.

### *4.4. Stable Transformation of Arabidopsis Plants*

*Arabidopsis* wild-type and *npr3npr4* mutant plants were transformed with the *A. tumefaciens* GV301 strain containing both, the construct pB7WG2::FaNPR32.OE and its corresponding empty vector using the floral dip procedure [88]. Seeds from the transformed plants (T1), were harvested, sown, and selected based on their resistance to BASTA herbicide (ammonium glufosinate, Bayer CropScience, Ireland) by spraying 2-week-old plants 3 times every 48 hours with a solution containing 1.2% BASTA, 0.05% L-77 silwet (Phytotechnology Laboratory, USA). The resulting progeny (T2) segregating 3:1 (BASTA resistant: susceptible) was selected as carrying a single insertion and homozygous lines (T3) were obtained from at least 10 individuals for each construct and confirmed for homozygosis in the offspring for expression of the transgene by RT-PCR using the specific primers (Supplementary Table S1) and selected for further analyses.

### *4.5. Arabidopsis Infection Assay with Pseudomonas syringae*

*Pst* growth, plant inoculation and in planta bacterial growth analyses were performed as described previously with $10^6$ CFU $mL^{-1}$ in 10 mM $MgCl_2$ and were pressure infiltrated into leaves [89]. For monitoring cell death, 12 leaves per genotype and treatment were collected 24 h after inoculation in control and *Pst* infected leaves and stained using Tripan Blue as previously described previously [90]. All the experiments were repeated at least three times using ten plants per experiment and treatment.

### *4.6. RNA Extraction and Real-Time qPCR*

Total RNA from frozen independent halves of agroinfiltrated strawberry fruits was extracted with the Maxwell ® 16 LEV Plant RNA kit (Promega, Madison, WI, USA), according to the instructions provided by the manufacturer. *Arabidopsis* total RNA was extracted using 50 mg leaf sample from 12 days old seedlings (4 biological replicates each consisting of seedlings grown in the same plate) or 2-3 weeks old soil grown plants (4 biological replicates each consisting of leaves from 3 plants), using the Invitrap Spin Plant RNA MiniKit (Invitek Molecular, Germany) and contaminating genomic DNA was removed by DNase I (Invitrogen) treatment. Purified RNA was quantified spectrophotometrically on NanoDrop 1000 (Thermo Scientific) and RNA integrity (RIN) was verified using the Agilent 2100 Bioanalyser (Agilent Technologies, Germany). Reverse transcription (RT) was carried out using 250 ng and 2 µg of purified total RNA as template from strawberry and *Arabidopsis* samples respectively with an RIN value ≥ 8 following manufacturer instructions [iScript cDNA synthesis kit (Bio-Rad)], then 50 ng cDNA were used for RT-qPCRs using SsoAdvancedTM SYBR ® Green Supermix, MyIQ v1.004 and iCycler v3.1 real-time PCR systems (Bio-Rad) and specific primers for each of the gene tested with similar PCR efficiencies. Three technical replicates in the same run and three to four biological replicates in different runs were performed, as described in Encinas-Villarejo et al. 2009 [44]. All primers for qRT-PCR used in this study are listed in Supplementary Table S1.

Relative expression values were determined by the $2^{-\Delta\Delta Ct}$ method [91], using as internal standards the housekeeping genes actine 1 (*FaACT1*) and elongation factor 1$\alpha$ (*FaEF1a*) for strawberry [92], and *ACTIN2* for *Arabidopsis* [44]. One-way ANOVA followed by HSD Tukey's testing was computed using R functions "lm", "anova" and "TukeyHSD".

### *4.7. Assessment and Statistical Analysis after Pathogen Infection*

For both the silencing and the ectopic expression experiments in strawberry, a phenotypic evaluation was performed to determine fruit tissue damage after 4 days post inoculation (4 dpi) with

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*C. acutatum*, corresponding to 6 days after agroinfiltration (6 dai). For statistical purposes, fruits with both halves agroinfiltrated with the empty vector were used as control. Per year, a sample of 48 half fruits for silencing/overexpression, and their corresponding 48 control half fruits, were evaluated for internal damage, according to a scale of 1 to 5 (1 , 0% damage, asymptomatic tissues; 2 , up to 10% damage, weak injury; 3, between 10-25% damage, moderate injury; 4, between 25 and 50% damage, severe injury; 5, more than 50% damage, severely affected fruit), as described in Higuera et al. (2019) [48]. Therefore, the internal damage ratio was calculated by dividing the tissue damage value of the fruit half where the gene was silenced/overexpressed by the tissue damage value corresponding to the opposite half of the same fruit agroinfiltrated with the empty vector. Means and SE were calculated by Fisher's LSD ($\alpha$ = 0.05) and Statistix software (v9.0). A ratio value of 1 indicates no difference between the two halves of the same fruit.

For *Arabidopsis*, the statistical analysis of the bacterial growth data was performed as in Encinas-Villarejo et al. 2009 [44].

## 5. Conclusions

We have shown that FaNPR3 members in strawberry act as negative regulators of resistance to *C. acutatum* and as positive regulators of the *FaWRKY19* and *FaWRKY24* defense genes. Furthermore, our results in *Arabidopsis* agree with this role of FaNPR3.2 as negative regulator of defense responses and, in addition, evidence a positive effect of this strawberry gene on the induction of the classic *PR* resistant marker genes in this heterologous system. The novel results presented in this work highlight strawberry FaNPR3 members as promising candidates for new environmentally friendly breeding technology strategies to accelerate strawberry resistance improvement while minimizing fitness costs.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1; Figure S2, Figure S3, Figure S4; Table S1.

**Author Contributions:** V.S and J.J.H have contributed equally to this work, carrying out the experimental work, analyzed data, interpretation of results, and writing the draft; F.A.R, has contributed to the strawberry experimental work and statistical analysis of gene expression; I.A.G. and C.J.L.H., have contributed to strawberry susceptibility assays and statistical analysis of fruit tissue damage against *C. acutatum*; J.M.B. contributed to supervise experimental design, discussion and draft writing; J.L.C and A.M.M are both corresponding authors, have designed methods and experiments, interpreted the results, supervised the work, wrote original draft, and reviewed and edited the final version; J.L.C, was responsible for funding acquisition. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

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