Article

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Fusarium oxysporum Tolerance assay in Strawberry (*Fragaria x ananassa*) Varieties and Analysis of *FaPAL* Gene Expression in an *In vitro* System

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Abstract. Strawberry (*Fragaria x ananassa*) is one of the most commercially important fruits worldwide, and produces nutraceuticals flavonoids as pelargonidin, and other important antioxidants like quercetin and kaempferol. In Mexico, several strawberry varieties have been developed looking for more resistant plants to different pathogens like *Fusarium oxysporum*. Phenolics and flavonoids have been recognized as part of the defense mechanism of plants. These compounds arise from phenylalanine ammonia lyase (PAL) enzyme activity and strawberry contains several *FaPAL* genes; however, most publications don't specify which one is being analyzed, or they are used interchangeably. Although *FaPAL1*, *FaPAL2* and *FaPAL6* have been isolated, no expression differences nor analysis of their promoters have been done. In this work we use an *in vitro* system to analyze the putative *Fusarium oxysporum* tolerance of 'Camino Real' and 'Nikté' strawberry cultivars grown in Mexico. Phenotypical traits, phenolics and flavonoids from control and infected plants were analyzed. We also made a bioinformatic analysis of the *FaPAL* genes from complete and partial cDNAs, and genomics comparisons. The two *FaPAL* gene families were recognized. *Cis*-active elements were analyzed in the promotor regions of both *FaPAL* gene families and the specific expression of *FaPAL1* and *FaPAL2* genes was analyzed in comparison with defensive genes like *FaMBL1, FaWRKY1, FaCyf1, FaChi3,* and *FaPR1,* upon the application of chitosan and beta-aminobutyric acid (BABA) as elicitors. Specific responses were related to *FaEF1*-alfa and *FaGAPDH2* as optimal reference genes. We found that *FaPAL1* and *FaPAL2* responded strongly to chitosan, and BABA response suggest a downregulation of *FaPAL1*.
Keywords: Beta-aminobutyric acid; chitosan; *FaPAL* genes; flavonoids; *Fragaria x ananassa* Nikté.

Resumen. La fresa (*Fragaria x ananassa*) es una de las frutas de mayor importancia comercial a nivel mundial, y produce flavonoides nutracéuticos como la pelargonidina y otros importantes antioxidantes como la quercetina y el kaempferol. En México se han desarrollado diversas variedades de fresa buscando plantas más resistentes a diferentes patógenos como *Fusarium oxysporum*. Los fenólicos y flavonoides han sido reconocidos como parte del mecanismo de defensa de las plantas. Estos compuestos surgen de la actividad de la enzima fenilalanina amonio liasa (PAL) y la fresa contiene varios genes *FaPAL*; sin embargo, la mayoría de las publicaciones no especifican cuál se está analizando o se usan indistintamente. Aunque se han aislado *FaPAL1*, *FaPAL2* y *FaPAL6*, no se han realizado análisis de diferencias de expresión ni de sus promotores. En este trabajo utilizamos un sistema *in vitro* para analizar la supuesta tolerancia a *Fusarium oxysporum* de los cultivares de fresa 'Camino Real' y 'Nikté' cultivados en México. Se analizaron rasgos fenotípicos, fenólicos y flavonoides de plantas control e infectadas. También realizamos un análisis bioinformático de los genes *FaPAL* a partir de ADNc completos y parciales, y comparaciones genómicas. Se reconocieron las dos familias

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de genes *FaPAL*. Se analizaron elementos activos *cis* en las regiones promotoras de las dos familias de *FaPAL1* y se analizó la expresión específica de los genes *FaPAL1* y *FaPAL2* en comparación con genes defensivos como *FaMBL1*, *FaWRKY1*, *FaCyf1*, *FaChi3* y *FaPR1*, tras la aplicación de quitosano y ácido beta-aminobutírico (BABA) como inductores. Las respuestas específicas se relacionaron con *FaEF1α* y *FaGAPDH2* como genes de referencia óptimos. Encontramos que *FaPAL1* y *FaPAL2* respondieron fuertemente al quitosano, y la respuesta de BABA sugiere una regulación negativa de *FaPAL1*.
Palabras clave: Ácido beta-aminobutírico; quitosano; genes *FaPAL*; flavonoides; *Fragaria x ananassa* Nikté.

Introduction

Wild *Fragaria* (Rosaceae) species occur across the northern hemisphere and in southern South America and it looks like humans had consumed the fruits of wild *Fragaria* species for millennia. *Fragaria chiloensis* was domesticated in Chile by the Picunche and Mapuche people over 1,000 yr ago, and in Europe, *F. vesca* and *F. moschata* have been grown in gardens at least since the time of the Romans, at the 16th century. The modern cultivated strawberry, *Fragaria x ananassa* originated in the 18th century in Europe from hybridization between two species imported from North and South America, *F. virginiana* and *F. chiloensis* respectively [1]. *Fragaria vesca* is known as the woodland strawberry, it is an herbaceous perennial with a small genome (240 Mb) and 7 chromosomes ($2n = 2x = 14$), but *Fragaria* × *ananassa* arisen from two New World species and is octoploid ($2n = 8x = 56$). These species also hybridize naturally in northwestern North America, but there is no evidence that they were cultivated by the native Americans in this area [2]. The strawberry (*Fragaria x ananassa*) plant was introduced to Irapuato, Guanajuato, Mexico in 1852. However, it was until starting the 40's, that Guanajuato state positioned itself as the largest producer of strawberries in Mexico. Due to commercial factors during the 50's, Mexican strawberry producers were conditioned to acquire strawberry mother plants from USA like 'Camino Real', 'Festival', 'Sweet Charly', 'Camarosa', and 'Albion' varieties; resulting in a local profitability decrease of the crop [3].

Later, those varieties shown a decrease in the productivity and quality of the fruit, mainly due to fungal problems by *Fusarium oxysporum* f. sp. *fragariae*, and by the strawberry virus complex (SVC) in Mexico. The various strawberry cultivars differed greatly in their adaptation to the specific regional and local environmental conditions of each location. Some cultivars were resistant to a particular pathogen in one region but very susceptible in other locations because of the different pathotypes [4]. For these reasons, new varieties were developed such as 'Buenavista', 'Cometa', 'Nikté' and 'Pakal', with the intention to adapt them to the prevailing local climate and looking for better tolerance to these pathogens [5,6]. 'Nikté' variety was selected because it was a short-day variety with large fruits, bright red external color, and red pulp. It shown high survival in acclimatization up to 91.9 % in greenhouse and 75 % shade, and a high accumulation of total biomass and partition of dry matter towards leaves, roots, and crown [7,8]. It also has high precocity, fruit quality, and a higher percentage of Brix degrees [9,10]. In addition, it shown tolerance to the two-spot spider, and the fruits were more tolerant to frost in winter [11]. *Fusarium oxysporum* f. sp. *fragariae* (*Fof*) wilt was first found in Australia in 1962 and then in Japan in 1969, while in Mexico it was found in the late 1980's and early 1990's [12,13]. There have been reported 14 species of fungi of which *Fusarium oxysporum* is the most frequent fungus in the central region of Mexico, it attacks from the early stages of the crop and causes losses greater than 50 %, and is the most common [14,15]. In a genotype selection study, it was observed that 'Nikté' variety showed a better tolerance to the local viral complex and to *Fof*, in comparison to other cultivars evaluated such as 'Camino Real' [9]. However, those results were controversial because reproducibility was not statistically significant.

Strawberry fruits are highly perishable and susceptible to biotic stress by pathogens in the field, and during postharvest storage resulting in severe crop losses [16,17]. Due to its commercial importance, most of the studies on defense responses on strawberries, have been done directly on harvested fruits or in plants grew in greenhouses or in the field, where many biotic and abiotic factors may be present at the same time triggering several and different plant responses. Strawberry resistance to pathogens has been reported to be mostly polygenic and quantitatively inherited; however, few strawberry genes directly involved in the defense molecular mechanism as well as their interaction with other metabolic genes are known [18]. This is important because strawberry is one of the most widely consumed berries and it's a good source of natural phenolic antioxidants [19].

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Fusarium oxysporum spp. can trigger several reactions on the plant during infection, by penetrating the epidermal cells of the plant roots. Plants responds with a rapid accumulation of compounds at the site of infection to stop the spread of the fungi and other pathogens [20]. Some of these compounds are phenolics which form physical barriers by polymerization into larger molecules such as lignins and proanthocyanidins [21]. These ones bind to polysaccharides of the cell wall forming a hard, crystalline structure, which helps stop the pathogen advance. Flavonoids have been also reported to limit the growth of the pathogens, participating in the inhibition of microbial pectinases, cellulases and xylanases, and in the chelation of metals necessary for enzymatic activity and mycelial growth [22]. In addition, they have a protective function due to their radical scavenging properties against oxidative stress caused by infection [23]. Multiple compounds are required for all these activities including hydroxybenzoic and hydroxycinnamic acids, the latter often produced as simple esters with glucose or hydroxy-4-carboxylic acids characterized by their hydroxylated aromatic rings [21]. Phenylalanine ammonia lyase (PAL) is the entry-point enzyme to the phenylpropanoid pathway resulting into different phenolic compounds, such as flavonoids like anthocyanins, and lignin among other important plant compounds [24]. Although PAL is not defined as a defensive enzyme, it has been recognized that phenylpropanoid pathway starting with PAL enzyme activity, becomes active after priming and once biotic or abiotic factors affects the strawberry plants [25].

In fungi, both vegetative and sporulating cells are capable of chitin synthesis, and its secretion occurs in a polarized mode. Chitin accumulation occurs at growth sites such as hyphal tips and cross-walls in filamentous fungi and emerging buds in yeasts. Chitin and chitosan accumulation mostly occurs in the cell wall's layers adjacent to the plasmalemma, where these glycans play a fundamental role in maintaining the cell wall's shape and integrity. Moreover, they provide protection against foreign materials (e.g., cell inhibitors) and environmental stressors to which fungi might be exposed. Owing to its positive charge, chitosan can retain anionic storage materials, such as polyphosphates, which are highly abundant in the Zygomycetes' cell wall [26]. Chitosan also exerts a role in some pathogenic fungi such as *Colletotrichum graminicola* and *Magnaporthe oryzae*. During infection by these species, the chitin deacetylase-catalyze the conversion of chitin into chitosan to preserve the appressorium from the hydrolysis by plant chitinases. It has been reported that *F. oxysporum* CFR 8 may produce extracellular chitin deacetylase in solid state fermentation [27]. On the other hand, an inhibitory effect of chitosan was also demonstrated with soil-borne phytopathogenic fungi including *Fusarium* wilt pathogens [28,29]. Chitosane is a mixture of chitin polymers of different length treated with acid to degrade the long chains, it has been used as elicitor to induce plant defenses [30]. Additionally, it has antifungal, antibacterial, antiviral, and antioxidant activity. Even though, it is still a mixture of chitin polymers of different length treated with acid to degrade the long chains [31]. The presence of amino groups and their proximity to hydroxyl groups in both structures, may result in charged molecules under certain conditions which can either bind to some proteins / receptors or destabilize the charge of specific regions on the cell wall or plasma membrane of plant cells, triggering defense responses.

BABA is a natural plant compound and it's known to induce plant defenses since time ago [32]. BABA levels are controlled by the plant's immune system and can induce plant defense mechanisms against many biotic and abiotic stresses caused by microorganisms and physical conditions [33-36]. In comparison to other elicitors, BABA has a chemically defined structure, and can be easily obtained in pure form. Recently, RNA-seq datasets of BABA - primed *Arabidopsis thaliana* and *Hordeum vulgare,* resulted in thousands of elicited genes [37]. The BABA production by the plant cells [32], may allow this small molecule to reach easier some targets and the response may be different from that one produced by chitosan.

Chitosan is a linear polysaccharide composed of randomly distributed units of β-(1–4)-linked D-glucosamine (deacetylated unit), and BABA is a b-aminobutyric acid, both structures are shown in Fig. 1.

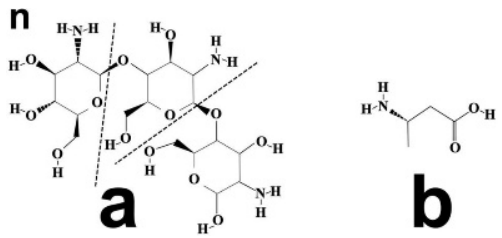

Fig. 1. Structure of chitosan **(a)**, and BABA **(b)**. Chitosan is a polymer of β-(1–4)-linked D-glucosamine repeated "n" times, in comparison to BABA structure.

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In this work, we used an *in vitro* system to compare under controlled and axenic conditions, the putative difference in tolerance of strawberry (*Fragaria x ananassa* cv.'Nikte' and 'Camino Real') plantlets to *Fusarium oxysporum* f. sp. *fragariae* [9]. Also, we made a bioinformatic analysis of the *FaPAL* genes and their promotor regions and compared their gene expression after chitosan and BABA applications, in the *in vitro* system. This with the intention to associate these genes with responses to biotic factors.

Experimental

Biological material

Axenic *in vitro* strawberry plants (*Fragaria* × *ananassa* cv. 'Camino Real' and 'Nikté') were obtained from the strawberry breeding program, conducted by INIFAP and CINVESTAV [9]. The strain of *Fusarium oxysporum* f. sp. *fragariae* (*Fof*) was obtained from Dr. Alba Jofre lab at the Irapuato Unit of CINVESTAV-IPN.

In vitro propagation of strawberry plantlets

Strawberry plants were micropropagated in Murashige and Skoog (MS) medium supplemented with 0.5 mg/mL benzyl amino purine (BAP), 30 g/L sucrose, and 8 g/L agar [38]. The explants including the crown, petiole segments and stipules, were sown directly in the culture medium at the minimum depth necessary to keep them in vertical position. Explants were cultivated at 25 °C in a growth chamber provided with white light fluorescent lamps F96T8/TL850 PLUS Phillips LONG LIFE of 59 W (Correlated Color Temperature 5000 K), for 3-4 weeks with a photoperiod of 16 h light / 8 h dark. Shoots were planted for rooting in half strength MS medium, supplemented with 20 g/L sucrose and 8 g/L agar, and cultivated under same conditions. For the micropropagation process, the shoots were subcultured again to MS medium and kept at 10 ºC under same photoperiod. Photosynthetic photon flux density (PPFD) was measured in µmol/m^2/s, using a PAR meter (LI-COR, Inc LI-190R Quantum Sensor) and its distribution according to wavelength in the visible spectrum was also measured with a MK350S Premium model spectrophotometer.

Preparation of the fungal solution

Fof was reproduced and maintained on plates with solid PDA medium in dark conditions at 28 °C for 2 weeks. Once the mycelium developed, a piece of agar with mycelium was taken and inoculated in liquid PDB medium in 125 mL flasks. It was kept stirring at a temperature of 28 °C for one week. Final solution was adjusted to a concentration of $1x10^6$ conidia/mL of PDB medium to inoculate the strawberry plants.

Inoculation of plants with the fungus

Under sterile conditions, 10 strawberry plants of each cultivar were taken, the roots were cut transversely from each plant until approximately 1 cm of root length was left, and only the tips were immersed in the previously prepared inoculation solution. Subsequently, the strawberry seedlings infected with the *Fof* fungus were placed into 50 mL Falcon tubes, on an inverted "V" shape plastic mesh bridge. Each tube contained approximately 10 mL of 50 % liquid MS medium, without sucrose. The tubes were kept at room temperature with a photoperiod of 16 h light and 8 h dark for 4 weeks. As control, strawberry plants were used, performing the same dissection procedure, but immersing the tips of the roots in sterile PDB medium and transferring them to Falcon tubes with the same medium and under the same conditions mentioned above.

Assessment of disease severity

To evaluate the degree of disease progression and tolerance to *Fof* by each cultivar, both the infected and control seedlings, plants were evaluated weekly with the severity scale for root, crown and leaves as described, with some modifications as shown in Table 1 [39].

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Table 1. The severity scale of the disease caused by *Fof* in leaves, crowns, and roots of strawberry plants, of the cultivars 'Nikté' and 'Camino Real' [39].

Scale	Percentage of damage	Leaves damage description
1	0 %	Healthy plants with all green leaves
2	<25 %	Beginning of wilting symptoms on stems
3	>25 %, <50 %	Pronounced wilting symptoms, limp and some dead leaves
4	>50 %, <75 %	Most leaves wilted/dead, small plants
5	>75 %	Withered leaves/dead plant
Scale	**Percentage of damage**	**Leaves damage description**
1	0 %	Healthy crowns, without necrosis or brown discoloration
2	<25 %	Half of the crown green and the other half with brownish necrosis
3	>25 %, <50 %	Green part of the crown becomes dull, increasing area with necrosis in brown color.
4	>50 %, <75 %	Less than 30 % of the crown is slightly dull green, and most with brownish necrosis.
5	>75 %	All crown tissue with brown discoloration necrosis
Scale	**Percentage of damage**	**Leaves damage description**
1	0 %	Healthy opalescent and shiny roots
2	<25 %	Dull roots with slight brownish necrosis at the origin
3	>25 %, <50 %	Roots with slight brownish necrosis in most of the tissue
4	>50 %, <75 %	Roots with brown necrosis except at the tips
5	>75 %	All root tissue with dark brown necrosis

Quantification of disease severity by weight

Tolerance to the disease caused by *Fof* was quantified by plant weight [15]. Previously infected and non-infected plants were cut by half to divide the plant into the upper part including trefoils and petioles and the lower part including roots and crown. Fresh weight of both parts was independently taken, then they were subjected to a drying process in an oven at 80 °C for 24 h and weighed again to obtain the dry weight.

Determination of phenolic compounds and flavonoids in strawberry plants (*Fragaria x ananassa*) cv. 'Nikte'

A total of 432 plants cv. 'Nikte' were used, 216 corresponded to the control treatment and 216 to the *Fof* treatment. After different incubation times with the fungus (0, 1, 3 and 24 h) the strawberry plants were also divided by half, the upper part included trefoils and petioles, and the lower part included roots and crown. Samples of at least 400 mg of both tissue parts were weighed in triplicate, wraped in aluminum foil, frozen with liquid nitrogen (N_2), and stored at -80 °C until use. Samples of 400 mg frozen tissue were crushed in a mortar with liquid nitrogen (N_2) until very small and uniform particles were formed to perform the

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corresponding tests. Samples of 100 mg were placed in a 30 mL glass bottle with a lid, and 2 mL of the extraction buffer [Methanol (Karal) acidified with 0.05 % trifluoroacetic acid (Sigma-Aldrich), and water: acetone (40:60) (v/v) acidified with 0.05 % trifluoroacetic acid, (30:70 v/v)] were added. They were kept in the dark and constantly agitated in a rotomix shaker at 70 rpm for 2 h at room temperature. They were subsequently centrifuged at 17,000 x *g* for 10 minutes at 4 ºC. The supernatants were concentrated in a rotary evaporator (BÜCHI 461, Germany) at 37 ºC for 6 min. The entire procedure was under dark conditions, and the containers and materials were covered with aluminum foil to avoid photooxidation of the compounds. The acid hydrolysis of the extracts was carried out under dark conditions as described [40]. For each mL of obtained extract, 2 mL of 2 N HCl (Karal) was added, subsequently it was boiled for 24 min on a heating plate. Samples were incubated on ice for 15 min, centrifuged at 17,000 x *g* at 4 °C for 20 min, and the supernatant was recovered. From this supernatant, compounds were extracted with 1 mL ethyl acetate (Karal) added to the sample, vortexed for 15 s and the supernatant was recovered with a micropipette repeating this procedure four times. All extractions were recovered and combined, then samples were concentrated in the rotary evaporator at 37 °C for 5 min and the compounds were recovered with 2 mL of pure methanol (Karal). From this extract, total phenolic and flavonoids compounds were quantified by spectrophotometry, using the Multiskan Go spectrometer (Thermo Scientific, USA) and 96-well polystyrene Nunc plates (Sigma, USA). The quantification was carried out by triplicate and calibration curves were used to determine the content of each compound. Quantification of total phenolic compounds was carried out using the established procedure, to 100 µL of the hydrolyzed extract of each sample, 500 µL of Folin Ciocalteau reagent 1 N (Sigma-Aldrich) was added, then mixture was shaken for 5 min and 400 µL of 7.5 % sodium carbonate (w/w) was added (Karal) [41]. It was vortexed for 10 s and finally incubated for 90 min in dark conditions at room temperature. Different known concentrations of gallic acid (Sigma) from a stock solution of 1 mg/mL diluted in pure methanol (Karal), were used as standard to make the calibration curve. Spectrophotometry was carried out at 765 nm respect to the blank (sterile distilled water replacing the Folin Ciocalteau reagent and pure methanol replacing the standard). The amount of total phenolic compounds was expressed as mg of gallic acid/100 g of fresh weight. Flavonoid quantification was performed by the aluminum chloride colorimetric method by triplicate [42]. To 500 µL of the hydrolyzed extract of each sample, 460 µL of pure methanol (Karal), 20 µL of 10 % aluminum chloride (Fluka) and 20 µL of 7.5 % potassium acetate (Karal) were added and vortexed for 15 s. Subsequently, it was incubated in dark conditions for 45 min at room temperature. Finally, spectrophotometry was done with 200 µL of the initial mixture placed in a 96-well polystyrene plate at 450 nm with respect to the blank (sterile distilled water replacing quercetin solution and 10 % aluminum chloride). Different known concentrations of quercetin (Sigma) were used as a standard, from a stock solution of 1 mg/mL dissolved in pure methanol (Karal). Fig. S1 shows the standard curves of gallic acid and quercetin.

Statistical analysis

Data from the disease severity evaluation, as well as the concentration of phenolic and flavonoids compounds with the control treatments and those infected with *Fof*, were analyzed using ANOVA for multifactorial design and a Tukey's multiple of means comparison test with the RStudio statistical software version 1.2.5033.

Identification of strawberry PAL protein sequences

The *FaPAL* genes and amino acid sequences were downloaded from the Plant Comparative Genomics portal of the Department of Energy's Joint Genome Institute, Phytozome (https://phytozome-next.jgi.doe.gov/), using the *Fragaria* x *ananassa PAL6* complete cds (GenBank: HM641823.1) as query sequence. PAL structural domains were downloaded from the Pafm database of the InterPro platform (https://www.ebi.ac.uk/interpro/search/text/). The software MEGA11 was used to analyze the PAL amino acid sequences and corroborate the active site in PAL sequences. The obtained complete *FaPAL* sequences were compared with the whole genome of *Fragaria x ananassa* Camarosa Genome v1.0.a2 (Re-annotation of v1.0.a1), and *Fragaria x ananassa* Royal Royce Genome v1.0 (Genome Database for Rosaceae, GDR) [43].

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Gene structure, domains identification and conserved motifs

Exon-intron distribution from DNA sequences and the coding domain sequences (CDS) were determined using .gff file and strawberry PAL gene IDs. Conserved domains present in FaPAL protein sequences were identified using the Conserved Domain Search Service (CD Search) site of The National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Conserved motifs were predicted using the Multiple Em for Motif Elicitation (MEME) tool of the MEME Suite software. Domains, motifs and CDSs were visualized by TBtools software [44].

Promoter elements

Identification of *cis*-regulatory elements was performed using the plant *cis*-acting regulatory element database, PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [45], the 2,000 bp upstream sequence gene putative promoter and the genome annotation file of each gene was used. Mapping was visualized by TBtools software [44].

Treatments of strawberry plantlets with elicitors

A chitosan stock solution was prepared dissolving 100 mg of low molecular weight chitosan (Sigma 448869, Chitosan - low molecular weight) in 10 mL 1 M glacial acetic acid (Karal 1006), pH was adjusted to 5.8 with 0.1 N KOH, sterilized in autoclave and kept at 4 °C until use. Diluted acetic acid in distilled and deionized water (DDW) at pH 5.8 was used as control for chitosan induction. A stock of BABA (Sigma 757454, (S)-3-aminobutyric acid) 10 mg/mL in DDW was sterilized by filtration and kept at 4 °C until use. Three complete strawberry plants var. 'Nikté' of 6 weeks old (approximately 300 mg of fresh tissue), were removed from the MS culture medium, and gently washed with sterile DDW. Then plants were immersed in the fresh prepared elicitor solutions of different concentrations (0, 0.1, 1.0, and 10 mg/mL) and kept at 25 °C, to establish the optimal chitosan and BABA concentration, samples were observed each 24 h until 5 days to check the elicitors effect (Fig. S2). Similar criteria as those used in Table 1 were applied [39]. All experiments were done by triplicate.

Primers design for gene expression assays

The sequences of the references and target genes were taken from previously published documents, the National Center for Biotechnology Information (NCBI) or respective genome sites as follows: *FaEf1α*, *FaGADPH2* and *FaWRKY1* [46], *FaPR1* [25], *FaCyf1* [47], *FaChi3* (https://www.ncbi.nlm.nih.gov/nuccore/AF134347.1/), and *FaMBL1* [48]. Sequences were obtained from the Rosaceae Database (https://www.rosaceae.org/species/fragaria/all), the two Phytozome sites (https://phytozome-next.jgi.doe.gov/info/Fxananassa_v1_0_a1, https://phytozome-next.jgi.doe.gov/info/FxananassaRoyalRoyce_v1_0 and NCBI (https://www.ncbi.nlm.nih.gov/data-hub/genome/GCA_019022445.1/) [49]. Primers were designed using the Primer3 program (https://primer3.ut.ee), to rule out secondary structures, the Beacon Designer Full Version (PREMIER Biosoft) and UNAFold (http://www.unafold.org) programs were used. Finally, the NCBI BLAST tool was used to discard nonspecific hybridizations. Primers for *FaPAL1* and *FaPAL2* were specifically designed using the complete cDNAs to distinguish between them. Used primers are listed in Table 2. The efficiency of each pair of oligos was determined using curves generated according to the equation $E = 10^{-1/slope}$ of 5 triplicate cDNA dilutions (250 ng/µL, 50 ng/µL, 10 ng/µL, 2 ng/ µL and 0.4 ng/µL).

Table 2. Sequences of the used primers to amplify specific sequences.

Gene	NCBI acc. number	Annealing temperature (Tm, ºC)	Sequence (forward/reverse) Orientation 5' to 3'	Amplicon (pb)	Ref.
FaEF1α	BK009992.1	57	TGGATTTGAGGGTGACAACATGA GTATACATCCTGAAGTGGTAGACGGAGG	145	[46]
FaGAPDH2	AF421145	60	CCCAAGTAAGGATGCCCCCATGTTCG TTGGCAAGGGGAGCAAGACAGTTGGTAG	117	[46]

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Gene	NCBI acc. number	Annealing temperature (Tm, ºC)	Sequence (forward/reverse) Orientation 5' to 3'	Amplicon (pb)	Ref.
FaPR1	AB462752.1	60	ACATGGGATGCCAATCTAGC CCACAGGTTCACAGCAGATG	150	[25]
FaMBL1	KF962716.1	57	AAACCAACACGGCCAATAAG GTCTGTCGGGTAGTCGAAGC	116	[48]
FaWRKY1	EU727547	60	ACAGCAGTAAGATTAGGGATGAAGAAGGGAG GCTTCTTCACATTGCAACCCTGATGCGTG	196	[46]
FaCyf1	AJ845186	60	GCCAAGTTCAACATGCTACTC TGCTCCACCTCCATCTGAT	116	[47]
FaChi3	AF134347.1	60	ACCAAGTTCAGCTCGCAGAT TCCTAATGGCCTTGAAGTGG	177	[25]
FaPAL1	KX450226.1	57	CTTCCTCAAGATTGCTGCTT AATTCCTCCCTCACAAACCT	159	This work
FaPAL2	KX450227.1	57	CATTACTCCCTGCTTGCCTCTCC CCTGAGCTGATACCGACTTGTTCG	171	This work

RNA extraction

Modification of a previously reported protocol was used [50]. Briefly, strawberry plants control and treated with chitosan or BABA solutions, were shortly dried onto a sterile paper towel. Then were immediately grounded in a sterile mortar with liquid nitrogen until fine powder was obtained. Three mL of extraction buffer (200 mM Tris-Cl, pH 8.5; 300 mM LiCl; 1.5 % lithium dodecyl sulfate; 10 mM EDTA; 1 % sodium deoxycholate; 2 % polyvinylpyrrolidone; 1 % Tergitol NP-40 and 1 % β-mercaptoethanol) were added, and grinding was continued until paste was formed. It was transferred to a 2 mL Eppendorf tube and kept on ice until thawed. Tubes were centrifuged for 10 min at 15,000 x *g* at 4 °C. One mL of the supernatant was transferred to other tube with 1 mL 5.8 M potassium acetate in acetic acid solution (37.67 g potassium acetate in 11.32 mL glacial acetic acid), mixed by inversion and centrifuged for 30 min at same previous conditions. One mL of the supernatant was transferred to other tube with 1 mL isopropanol, mixed and stored at -20 °C overnight. Tubes were centrifuged 20 min at 15,000 x *g* at 4 °C. Supernatants were decanted, pellets were washed once with 70 % ethanol and air dried for 10 min. Then 25 µL of a mixture containing 0.5 µL of DNase I (Invitrogen 18068015 DNase I, Amplification Grade), 2.5 µL of 10X DNase buffer, and 22 µL of DDW were added and incubated at 25 ºC for 15 min. Extraction buffer (250 µL) was added and mixed by vortex. Same volume of 5.8 M potassium acetate was added and mixed by inversion. Samples were centrifuged 20 min, at 15,000 x *g* at 4 °C. Supernatant was recovered into a 2 mL Eppendorf tube, gently mixed with 1.5 mL ethanol, and placed at -70 ºC for 1 hour. Tubes were centrifuged 20 min at 15,000 x *g* at 4 ºC, supernatant was discarded and 500 µL washing buffer (10 mM Tris-HCL, pH 7.5; 0.5 mM EDTA; 50 mM NaCl; 50 % ethanol) were added. Tubes were inverted several times until pellets were detached, then 10 µL silica 1 g/mL at pH 2 (Sigma S5631, Silicon dioxide) were added, and mixed by inversion until homogeneity [51]. Tubes were centrifuged at 500 x *g* for 10 sec at room temperature, liquid was discarded and pellet washed once. Centrifuged again 1 min at 15,000 x *g* at 4 °C, liquid was discarded, and pellet was dried upside down in laminar flow hood for 20 min. DDW (80 µL) was added and heated 5 min at 68 °C. Centrifuged for 1 min at 15,000 x *g* at 4 °C and nucleic acids were recovered by taking 70 µL from the upper phase and transferred to new tubes. RNA quality was checked using a spectrophotometer NanoDrop 2000.

cDNA synthesis and qRT-PCR assay

For cDNA synthesis, 150 ng RNA were placed in PCR tubes, and a mixture containing 0.2 µL DNase I, 0.5 µL 10X DNase buffer and DDW up to 3 µL were added. Tubes were incubated at 25 ºC for 15 min, added with 5.0 µL 0.25 mM EDTA and heated for 10 min at 65 ºC. The cDNA synthesis was carried on in a Veriti 96-Well Thermal Cycler (Applied Biosystems, 4375786), using the SuperScript® III First-Strand Synthesis System for RT-PCR kit (Invitrogen 18080051). Briefly, a mixture of 1 µL 50 µM oligo(dT)$_{20}$, 2 µL 10 mM dNTP mix and 2 µL of DDW were added. Tubes were heated at 65 ºC for 5 min and placed on ice for 1 min. A

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mixture containing 2 µL 10X RT buffer, 4 µL 25 mM $MgCl_2$, 2 µL 0.1 M DTT, 1 µL RNaseOUT (40 U/µL) and 1 µL SuperScript III RT (200 U/µL) were added. Samples were heated at 50 °C for 60 min, followed by 5 min at 85 °C. One µL RNase H was added and incubated at 37 °C for 20 min. The obtained cDNA was measured and adjusted to 100 ng/µL with DDW, samples were stored at -80 ºC until use. For qPCR, each reaction mix had 4 µL cDNA, 0.25 µL respective 10 µM oligonucleotides, 0.75 µL DEPC treated water, and 5 µL SYBR Green PCR Master Mix (Applied Biosystems, 4309155). qPCR reactions were carried out in 96-well plates (Thermo Scientific 96-well Piko PCR Plates, clear) on the PikoReal Real Time PCR System (Thermo Scientific, N11471). Thermal cycling conditions included initial denaturation at 94 °C for 10 min; followed by 40 amplification/denaturation cycles at 94 °C for 15 sec and 60 sec at the specific temperature for each oligo alignment. To determine the amplicon specificity, analysis of the dissociation curve was performed from 60 ºC to 95 °C, increasing 0.2 °C / sec. Four reactions were performed for each gene by triplicate, with respective controls reactions.

Calculations

Gene expression analyzes of control, chitosan and BABA-treated strawberry plantlets were performed using the comparative −ΔΔCt method, where the expression of target genes was normalized to the endogenous reference genes in comparison with untreated controls [52]. To assess the relative amounts of each of the amplified products, Ct values were normalized using the arithmetic mean of the reference *FaEf1α* and *FaGADPH2* genes. Data are shown as mean ± standard error and were statistically evaluated with ANOVA, followed by a LSD test, using the RStudio program (RStudio Team 2020). RStudio, PBC, Boston, MA (http://www.rstudio.com/).

Results and discussion

Plants propagation

Shoots from strawberry explants (4 to 6 shoots / explant) cultivated in micropropagation medium were incubated during the first week after transfer to fresh medium. Then, shoots were cultured in rooting medium for two weeks until roots reached 2-4 cm length.

In vitro tolerance evaluation of *Fragaria x ananassa* 'Nikté' and 'Camino Real' cultivars to *Fof* infection

Each seedling propagated under sterile conditions was infected at the roots according to the procedure described in Materials and Methods and mounted on the plastic mesh bridge inside the Falcon tube containing MS medium under sterile conditions. Representative images of the disease severity scales are shown in Fig. 2.

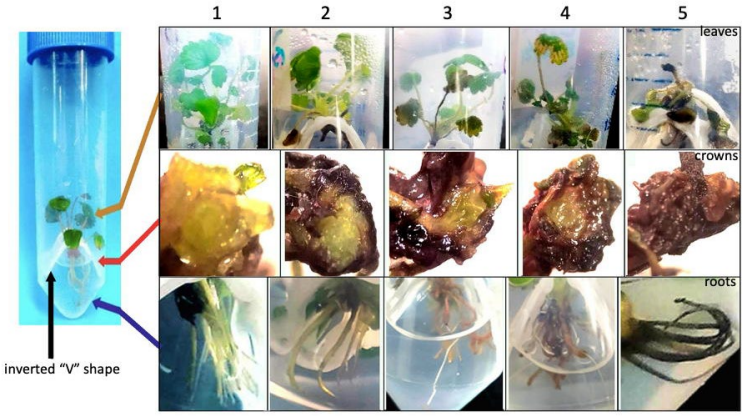

Fig. 2. Representative images of the severity scale of the disease and the main symptoms in leaves, crowns, and roots of strawberry plants in the *in vitro* system. Numbers indicate the degree of severity of the damage according to Table 1. Note the inverted "V" shape plastic mesh bridge at the bottom of the tube to hold the plantlet.

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Table S1 shows the values assigned to the *Fof* tolerance levels in 'Nikté' and 'Camino Real' strawberry plants, according to the infection severity scales, described in Table 1. The average damage level on leaves of 'Nikté' plants was 2.67 ± 1.21 while in 'Camino Real' cultivar was 2.7 ± 1.12, representing less than 25 % damage in plants infected by *Fof* in both cultivars, in comparison to the control where plants didn't show any visual damage, keeping level 1 throughout the experiment. The 'Camino Real' crown tissue had less damage than 'Nikté' with a value of 3.07 ± 1.11 and 2.87 ± 1.07 on average respectively, representing less than 50 % on average of the damaged area. In roots of infected plants of both cultivars, it was observed that less than 50 % of the tissue was damaged by the fungus, the level of damage shown by 'Nikté' was 3.47 ± 1.11 and 3.9 ± 0.92 by 'Camino Real', while control plants remained healthy with level 1 (Table S1). A significant difference was found between control and *Fof* treatments in each of these plant organs, but no statistical differences were found between cultivars.

To evaluate more precisely the level of infection severity, fresh and dry weight of tissues from 'Nikté' and 'Camino Real' cultivars were determined. Table 3 shows the obtained values in leaves and roots of strawberry plants and their comparison between *Fof* infected and control plants. Fresh weights of control leaves from 'Nikté' and 'Camino Real' cultivars were 129.31 ± 34.02 mg and 133.21 ± 32.15 mg respectively. Those were higher than the weight of infected leaves 'Nikté' which had 102.45 ± 40.49 mg, that is 20.77 % less than control plants, and 'Camino Real' had 93.91 mg corresponding to a 29.50 % decrease in comparison to the control. Similarly, dry weight of control leaves of both cultivars was 13.84 ± 4.45 mg for 'Nikté' and 14.90 ± 4.45 mg for 'Camino Real'. Both higher compared to the dry weight from infected plants where Nikté' had 11.17 ± 3.92 mg representing a 19.29 % decrease and 'Camino Real' had 10.81 ± 3.87 mg corresponding to a 27.44 % decrease in comparison to the control. Fresh and dry weight of infected tissues decreased significantly ($p<0.05$) compared to control plants.

Table 3. Quantitative evaluation of tolerance to *Fusarium oxysporum* spp. of the cultivars 'Nikté' and 'Camino Real' by fresh and dry weight.

Tissue	Weight (g)	'Nikté'		'Camino Real'	
		Control	*Fusarium*	Control	*Fusarium*
Leaves	fw	129.31 ± 34.02 a	102.45 ± 40.49 b	133.21 ± 32.15 a	93.91 ± 40.81 b
Leaves	dw	13.84 ± 4.45 a	11.17 ± 3.92 b	14.90 ± 4.41 a	10.81 ± 3.87 b
Roots	fw	47.78 ± 16.23 a	30.38 ± 13.06 b	50.57 ± 22.34 a	34.24 ± 16.35 b
Roots	dw	4.62 ± 1.42 a	3.07 ± 1.15 b	4.08 ± 1.66 a	3.10 ± 0.95 b

Values represent the average of three biological replicates with ten technical replicates and different letters denote significant differences between treatments (control and *Fof*) according to the Tukey statistical test (ANOVA-Tukey) $p<0.05$.

Results in Tables S1 and Table 3 show no statistically significant differences ($p<0.05$) according to the Tukey statistical test (ANOVA-Tukey), in the *Fof* tolerance between the two 'Camino Real' and 'Nikté' varieties in the *in vitro* system. For this reason, we used just the 'Nikté' variety further on.

Our data agree with those already reported where tolerance to the disease was evaluated and damage between 25 and 50 % of root damage was observed in both cultivars [53]. Crown results differs slightly from our data since they report damage between 25 % and 50 % in 'Nikté,' and from 50 % to more than 75 % in 'Camino Real'.

Quantification of phenolic and flavonoid compounds in the strawberry plantlets (*F. x ananassa* cv. 'Nikté') in response to *Fof* infection

In our study, leaves and roots showed an increase in the concentration of total phenolic and flavonoid compounds in plants infected by the pathogen. Fig. 3 shows the concentrations of phenolics and flavonoids in leaves and root tissues of control and infected plants at different times, respectively. In the roots, we observed 69.5 ± 10.54, 59.92 ± 7.46, 49.67 ± 13.08 and 55.92 ± 20.46 mg in infected plants at 0, 1, 3, and 24 h post-

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inoculation times; while in the control they were 55.53 ± 9.91, 29.84 ± 12.26, 40.12 ± 12.47 and 47.71 ± 24.94 mg respectively. So, increases in total phenolic compounds in roots of infected plants were 20.1, 50.2, 19.22 and 14.37 % respectively. However, increase was statistically significant ($p<0.05$) only for 0 and 1 h. On the other hand, phenolics in leaf tissues of infected plants at same times were 118.73 ± 39.90, 98.73 ± 18.22, 79.76 ± 22.93 and 65.61. ± 16.10 mg while in control were 91.30 ± 25.72, 70.02 ± 21.88, 57.56 ± 24.33 and 50.22 ± 17 mg which corresponds to an increase of 23.1, 29.07, 27.83 and 23.45 % respectively in the concentration of phenolic compounds in the infected leaves. However, it was significant ($p<0.05$) only during the 1 h.

For flavonoids, a statistically significant increase ($p<0.05$) was observed in the roots of infected plants at all evaluated times, obtaining an average of 3.23 ± 0.55, 2.65 ± 0.31, 1.87 ± 0.60 and 6.07 ± 3.31 mg respectively, compared to control plants having 2.00 ± 0.55, 1.12 ± 0.62, 1.26 ± 0.53 and 2.32 ± 1.42 mg. An increase in flavonoids in infected plants was seen since time 0 h, decreasing during 1 and 3 h and increasing again at 24 h with 6.07 mg. Infected leaves increased the flavonoid's accumulation during all evaluated times, but it was significant ($p<0.05$) only during 1, 3 and 24 h. The highest concentration of flavonoids was at 0 h with 4.68 ± 0.96 mg, decreasing at 1 h with 2.14 ± 0.68 mg and increasing again at 3 h (3.60 mg) and 24 h (3.54 mg). Control plants shown lower accumulation of flavonoids (3.32 ± 0.62, 1.61 ± 0.68, 1.67 ± 1.12 and 2.13 ± 0.92 mg) respectively.

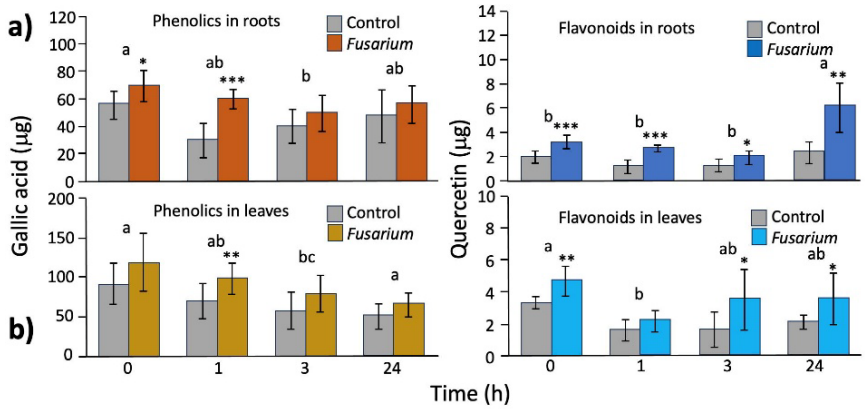

Fig. 3. Effect of post-inoculation times of *Fof*, on the total concentration of phenolic (mg gallic acid/100 g of fresh weight) and flavonoid (mg quercetin /100 g of fresh weight) compounds. **(a)** phenolics and flavonoids in roots, **(b)** phenolics and flavonoids in leaves, of control and infected (*Fragaria x ananassa* cv. 'Nikte') plants. Values are the average of three biological replicates with three technical replicates ± SD. *Denotes significant difference $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. Different letters denote significant differences between the post-inoculation times (0, 1, 3 and 24 h) according to the Tukey's statistical test ($p< 0.05$, $n= 9$).

Variation in the concentration of different secondary metabolites, including phenolic and flavonoids compounds, due to pathogen attack, may be due to the species, the cultivar, the type of tissue or the infection time. Therefore, in our study, the concentrations of these compounds were compared at different times post-inoculation to know when a significant variation in the concentrations was observed. It was found that one of the most significant variations over time was the decrease in phenolic compounds in tissue leaves of *Fof* infected plants, starting at time 0 h where the higher number of phenolics was obtained (118.73 ± 39.90 mg) and ending with 65.61 ± 16.10 mg at 24 h post inoculation. The other variation occurred in the infected root tissue where the number of flavonoids was shown from time 0 h (3.23 ± 0.55 mg) decreasing during the times of 1 h (2.65 ± 0.31 mg) and 3 h (1.87 ± 0.60 mg) and having a sudden accumulation of flavonoids at 24 h (6.07 ± 3.31 mg).

It has been reported that infection with *Fusarium oxysporum* in some crops as alfalfa [54], onion [55], flax [56], carnation [57] and bean cultivation [58] among others, induces an increase in phenolic compounds and flavonoids, as a defense mechanism in response to the infection. It has previously been reported that the concentration of phenolic compounds can vary according to the type of tissue. It was shown that, in bean crops, the hypocotyl of the plant accumulates 2 times more phenolic compounds and up to 5 times more flavonoids compared to the root during infection by *Fusarium oxysporum* f. sp. *phaseoli* [58]. Therefore, we analyzed

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whether the effect of the pathogen *Fof* on the concentrations of total phenolic compounds in the strawberry *Fragaria x ananassa* Duch. cv. 'Nikté' was different in the leaves and the roots. At all evaluated times (0, 1, 3, and 24 h), plant leaves *Fof* infected shown more phenolic compounds (118.73, 98.73, 79.76 and 65.61 mg respectively), compared to the roots (91.30, 70.02, 57.56, 50.22 mg respectively) (Fig. 3); a statistically significant difference was at 0 and 1 h post inoculation. In our study, the highest concentration of phenolic compounds was seen at 0 h in both controls (91.30 mg in leaves and 55.53 mg in roots), and in the *Fof* treatment (118.73 mg in leaves and 69.50 mg in root), decreasing later. An increase was found in strawberry fruits at 24 h intensifying at 48 h post inoculation [59]. In our study at the first 24 h, the root increases were 20.1, 50.2, 19.22 and 14.37 % respectively; but statistically significant during 0 and 1 h, while in the leaves an increase of 23.1, 29.07, 27.83 and 23.45 % respectively was observed, but significant at 1 h.

It has also been reported that pathogen infection can cause intense synthesis and accumulation of flavonoid compounds in strawberry fruits [60]. The interaction of *Botrytis cinerea* with strawberry, causes a response of defense genes starting 24 hours after inoculation [61]. In our work, flavonoid concentrations were evaluated in roots and leaves during the first 24 h (Fig. 3), and a statistically significant increase was observed in *Fof* infected plants during all evaluated times (0, 1, 3 and 24 h). This increase in flavonoids was observed in both leaves and roots from 0 h (3.23 mg), decreasing at 1 h (2.65 mg) and 3 h (1.87 mg) post inoculation; having the highest flavonoids accumulation in the roots at 24 h (6.07 mg). This is consistent with previous reports where some genes of the phenylpropanoid pathway were strongly induced at 12 h after *Fusarium oxysporum* infection, with a maximum expression at 48 hours in flax cell suspension [62]. Also, an increase in phenolic compounds levels and flavonoids starting at 24 h, with a maximum at 48 and 96 h was observed after inoculation with *Fusarium oxysporum* f. sp. dianthi in carnation [57]. Similarly, flavanols and flavonols compounds increased in strawberry fruits because of an infection by *Colletotrichum nymphaeae*, but in the stolons most of these compounds decreased [63]. We conclude that in our *in vitro* system, strawberry plants respond to *Fof* infection synthesizing phenolics and flavonoid compounds as a defense mechanism at the first hours of infection.

These results shown that phenylpropanoid pathway was active upon *Fof* infection and this pathway starts with the phenylalanine ammonia lyase (PAL) enzyme activity. This enzyme has a key role in all plants since it initiates the phenylpropanoid pathway that generates vital phenolics and flavonoids, among other key compounds like lignin. However, *Fragaria x ananassa* contains several *FaPAL* genes but most publications don't specify which *FaPAL* gene is being analyzed, or they are used interchangeably [64,65,66]. Although some *FaPAL* genes have been isolated, no expression differences nor analysis of their promoters have been done. Currently, it is impossible to distinguish the specific enzyme activity from the different FaPAL enzymes expressed from the different *FaPAL* genes. So, to understand how a particular metabolic pathway works, it is necessary to know which genes are involved in it and which stimuli each one responds to. With the intention to identify which *FaPAL* gene (s) was(were) involved in the response to biotic factors like *Fof* infection, we made a bioinformatic analysis of the studied *FaPAL* genes.

Identification of strawberry PAL genes and protein sequences

There were eight *FaPAL* genes sequences found at NCBI as follows:

Fragaria x *ananassa* phenylalanine ammonia-lyase 1 (PAL1) mRNA, complete cds GenBank: KX450226.1

Fragaria x *ananassa* phenylalanine ammonia-lyase 2 (PAL2) mRNA, complete cds GenBank: KX450227.1

Fragaria x ananassa phenylalanine ammonia lyase 6 (PAL6) mRNA, complete cds GenBank: HM641823.1

Fragaria x *ananassa* FaPAL1 mRNA for phenylalanine ammonia-lyase, partial cds GenBank: AB360390.1

Fragaria x *ananassa* FaPAL2 mRNA for phenylalanine ammonia-lyase, partial cds GenBank: AB360391.1

Fragaria x *ananassa* FaPAL3 mRNA for phenylalanine ammonia-lyase, partial cds GenBank: AB360392.1

Fragaria x *ananassa* FaPAL4 mRNA for phenylalanine ammonia-lyase, partial cds GenBank: AB360393.1

Fragaria x *ananassa* FaPAL5 mRNA for phenylalanine ammonia-lyase, partial cds GenBank: AB360394.1

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Aligning of all FaPAL protein sequences show that FaPAL1 and FaPAL6 are almost identical, and FaPAL2 have several differences. Even partial sequences show similar differences between FaPAL1, FaPAL6 and FaPAL2. Complete FaPAL amino acid sequences downloaded from the Plant Comparative Genomics portal are shown in Fig. 4.

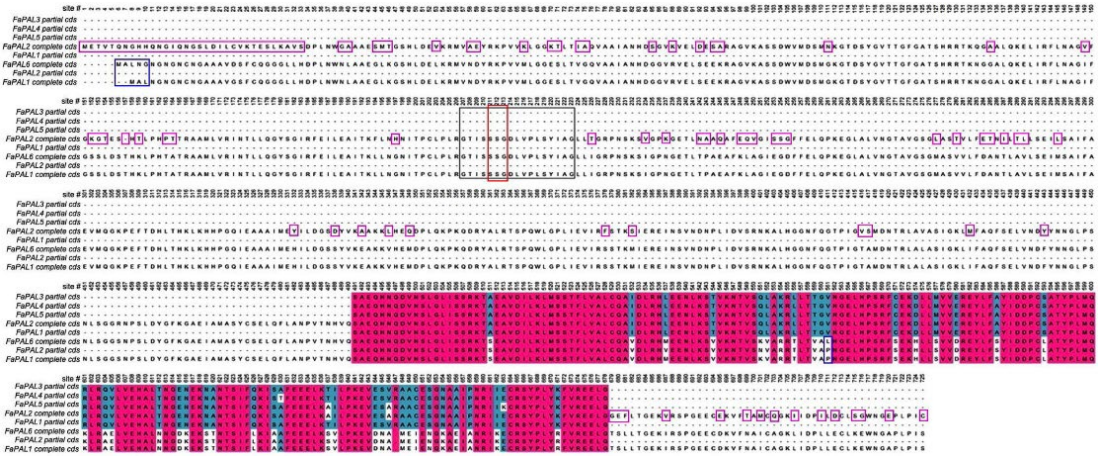

Fig. 4. Complete amino acid sequences of FaPAL1, FaPAL2 and FaPAL3. Magenta boxes are the differences between FaPAL 2, and FaPAL1 - FAPAL6. Blue boxes are differences between FaPAL1 and FaPAL6. Black box is the active site and red box is the catalytic triad. Pink and green boxes are the conserved sequences of the partial *FaPAL1, FaPAL2, FaPAL3*, *FaPAL4* and *FaPAL5*.

The only differences between FaPAL1 and FaPAL6 are the first amino acids (6-10) and the L x P change (561). The active site GTISSSGDLVPLSYIAG and the catalytic triad SSG are conserved in the three complete sequences. FaPAL1 and FaPAL6 have a 99 % identity, and FaPAL2 has a 98 % identity in comparison to them. The partial *FaPAL*-cDNAs respective sequences of *FaPAL3*, *FaPAL4* and *FaPAL5* are like *FaPAL2*.

***FaPAL* gene structure, domains identification, conserved motifs, and promoter elements**

After aligning the obtained complete *FaPAL1* and *FaPAL2* cds sequences with the whole genomes of *Fragaria x ananassa* Camarosa, and of *Fragaria x ananassa* Royal Royce, seven *FaPAL* genomic sequences were recognized which formed two defined gene families [43]: four *FaPAL* genes (Fxa7Ag201940.1, Fxa7Bg201899.1, Fxa7Cg101819.1, and Fxa7Dg101690.1) in chromosome 7 aligned with *FaPAL1* cds; and three *FaPAL* genes (Fxa6Ag101540.1, Fxa6Cg101365.1, and Fxa6Dg101303.1) in chromosome 6 aligned with *FaPAL2* cds. The conserved motifs, domains identification and gene structure of the seven *FaPAL* genomic genes, is shown in Fig. 5. Conserved motif 18 is twice repeated in *FaPAL2* gene family members, domains at amino end are different between the two families and introns of *FaPAL1* gene family members are shorter.

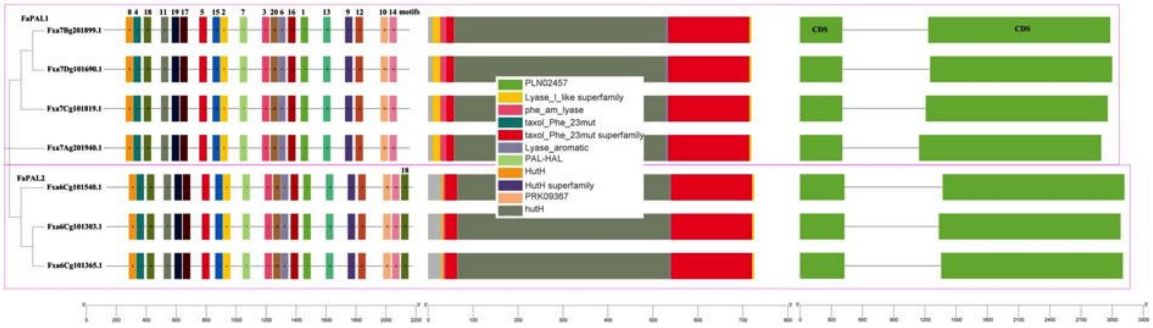

Fig. 5. Conserved motifs, domains identification and gene structure of *FaPAL* enzymes and genomic genes. Two *FaPAL* gene families are recognized. *FaPAL1* gene family has four members and *FaPAL2* gene family has three members.

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FaPAL promoter *cis*-active elements

The *cis*-active elements identified in the 2,000 bp promoter regions of the seven *FaPAL* genomic genes are shown in Fig. 6. The two *FaPAL* gene families are clearly distinguished by the number of *cis*-active elements related to abiotic/biotic stress, development, light, phytohormone and unknown conditions.

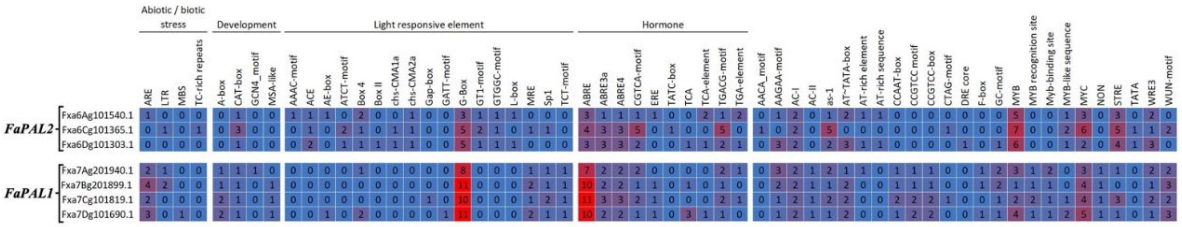

Fig. 6. Analysis of *cis*-regulatory elements in the promoter regions of all *FaPAL* genomic genes, showing the two *FaPAL* gene families, under the respective stress or studied conditions.

Considering se existence of at least 1 responsive element in the promoter regions of the two *FaPAL* gene family members under specific conditions, the number of responsive elements per family are shown in Table 4.

Table 4. Promoter *cis*-active elements identified in the two *FaPAL* gene families.

Responsive elements / Factor	Abiotic/biotic stress	Development	Light responsive	Hormone	Unknown	Total
FaPAL2 gene family	3	1	14	10	17	45
FaPAL1 gene family	3	4	8	9	21	45

To date, promoters of both *FaPAL* gene families have the same number of responsive elements; however, and according to these data *FaPAL2* gene family is more light-responsive than *FaPAL1* gene family, and this one seems to be more responsive to developmental factors. Even at unknown conditions, the *cis*-active elements in promoters of both gene families are consistent per family, e.g., AACA-motif, AT-rich seq, DRE core, and TATA elements are present only in *FaPAL2* but not in *FaPAL1* gene family. On the other hand, AC-II motif, CCAAT-box, CCGTCC- motif and box, F-box, MYB-recognition site, Myb-binding site and NON are present only in *FaPAL1* but not in *FaPAL2*. It is remarkable the huge amount of G-box light-responsive and ABRE hormone-responsive elements in promoters of *FaPAL1* gene family members. This distribution may change as far as the unknown factors could be associated to specific elements, and new elements and factors can be formally recognized.

In strawberry, the most studied *PAL* genes have been *FaPAL1, FaPAL2* and *FaPAL6* genes. In a comparative genomic analysis of five Rosaceae species, just two genes were reported in *Fragaria vesca* (*FvPAL1* and *FvPAL2*) located in chromosomes 7 and 6 respectively [65]. A full-length *FaPAL6*-cDNA was isolated and respective gene expression was only detected in 100 % red fruit from Toyonoka and Camarosa cultivars [64]. However, the expression in Camarosa was stronger than in Toyonoka. Transcripts of *FaPAL1* and *FaPAL2* were studied but no correlation between the two PAL enzyme isoforms was detected, suggesting different roles or different regulation processes for the two isoforms [67]. An increase in the expression of *FaPAL1* was reported at the 50 % R stage in "Camarosa," "Crystal," "Monterey" and "Portola," varieties [66]. Recently, the expression of an undefined *FaPAL* gene was studied in transgenic strawberry calli overexpressing the B-box transcription factor *FaBBX22* gene, which plays a vital role in light-induced anthocyanin accumulation. A significant anthocyanin accumulation resulted by upregulating the expression of related biosynthetic genes as *FaPAL, FaANS, FaF30H,* and *FaUFGT1* [68]. Similarly, other undefined *FaPAL* gene was studied in transgenic strawberries where the transcription factor *FaMYB123* gene, which is ripening-related, receptacle-specific, and antagonistically regulated by ABA and auxin; was knockdown by expression of the FaMYB123-RNAi. The expression of the early genes *FaPAL*, *FaDFR*, *FaCHS*,

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FaCHI, *FaCAD*, and *FaCCR* was not different in transgenic receptacles [69]. Unfortunately, not any identification of the *FaPAL* genes detected in the previous works was provided. In this work, we show that the amino acid sequences, the conserved motifs, domains identification and gene structure as well as the *cis*-active elements of respective promoters, are also different between the two *FaPAL1* and *FaPAL2* gene families [70].

Light response is the most clean and non-invasive interaction that plants have with the environment because no physical contact is involved related to biochemical reactions. Hormones and abiotic responses are relatively easy to study considering that we may apply a pure hormone or a pure abiotic factor like metals and study the plant response. Developmental responses are more complicated because that involves specific developmental stages which are influenced also by hormones, light, and other factors. However, biotic responses to other organisms are most complicated to study because usually they involve several factors some biotic, and some abiotic, which may change depending on the stage of the biotic factor that is also influenced by development, light, and other intrinsic factors.

As mentioned before, the use of *Fof* as biotic factor will include biotic and abiotic factors, which may change as the fungal development is going along the infection time. So, to avoid these changes, we used chitosan and BABA as elicitors related to biotic factors, to study the specific expression of *FaPAL1* and *FaPAL2* genes.

Effect of elicitors on strawberry plantlets

Strawberry plantlets treated with chitosan and BABA solutions (0, 0.1, 1.0, and 10 mg/mL), were incubated at 25 ºC for different times as described before. Fig. S2 shows the result of addition of 1 mg/mL and 10 mg/mL of BABA and chitosan respectively, after 3 days incubation. At this time, 1 mg/mL BABA-treated plantlets looked like control, but at 10 mg/mL plantlets showed oxidation in brownish roots and stems and leaves were yellowish. At same time, chitosan-treated plantlets at 1 mg/mL shown brownish roots and stems and yellowish leaves, but at 10 mg/mL plantlets looked dark and quite damaged. After 3 days incubation, all plantlets were washed with DDW and placed into fresh MS medium. All plantlets except those treated with 10 mg/mL chitosan, recovered after a week or two. After these experiments, chosen conditions to induce defense responses and to carry out the gene expressions assays were 10 mg/mL BABA and 1 mg/mL chitosan by three days. After five days under these conditions all plantlet's tissues were dark (data not shown).

Genes expression comparisons

With the bioinformatic data, it was possible to design specific primers to differentiate between *FaPAL1* and *FaPAL2* gene expression (Table 2), and compare it with the other *FaWRKY1*, *FaPR1*, *FaCyf1*, *FaChi3*, *FaMBL1* defensive genes in strawberry plantlets treated with chitosan and BABA. The references genes for this experiment were *FaEf1α* and *FaGADPH2*. After treatments, RNAs were extracted, and qRT-PCR reactions were carried out. Expression data of the defense and *FaPAL* genes, were normalized with data from the reference genes and results are shown in Fig. 7.

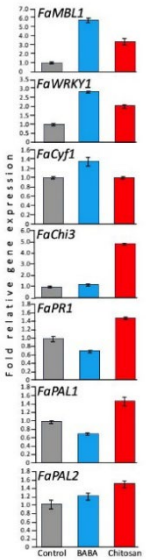

Fig. 7. Gene expression of assayed genes by qRT-PCR. All data were normalized with the *FaEF1α* and *FaGAPDH2* reference genes. All assays were performed by triplicate.

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Two different responses were noticed, *FaMBL1*, *FaWRKY1* and *FaCyf1* responded strongly to BABA having *FaMBL1* the strongest response. *FaCyf1* response was almost exclusive to BABA because it showed the same response to chitosan and control, although the BABA response was not so strong as in *FaMBL1* and *FaWRKY1*. *FaCyf1* response has been more related to the endogenous regulation of protein turnover during seed development and germination, and in programmed cell death [47]. *FaMBL1* and *FaWRKY1* showed similar responses to BABA and chitosan, being slightly stronger with BABA. In contrast, the other genes *FaChi3*, *FaPR1*, *FaPAL1* and *FaPAL2* responded strongly to chitosan having *FaChi3* the strongest response. *FaChi3* response was almost exclusive to chitosan because BABA response was like control, which was expected considering that chitinase release chitin fragments like chitosan, and these fragments seems to be specific for the induction of this gene, playing a critical role against fungal pathogens [71]. Also, *FaPR1* and *FaPAL1* showed a similar response to both elicitors, but the responses were stronger with chitosan. In these cases, controls were higher than BABA responses, suggesting a potential downregulation of respective gene expression by BABA. *FaPAL2* responded to both elicitors, being slightly stronger with chitosan.

Elongation factor-1α (*FaEF1α*) and the glyceraldehyde-3-phosphate dehydrogenase (*FaGAPDH2*) an essential l enzyme for carbohydrate metabolism in the cytoplasm genes, were used as reference genes for strawberry [72]. These two genes together with *FaRIB413,* a 18S rRNA which is a ribosome component*,* and *FaACTIN* were selected, identified, and validated as optimal reference genes for accurate normalization of gene expression in strawberry plant defense response studies. Respective analyses were focused on 13 strawberry genes, including those used as reference in this work. All 13 genes were tested by five different methodologies against different conditions like biotic stresses, ripening and senescent, SA/JA treatments as well as in different tissues, and strawberry cultivars [46]. The obtained results validated their use as good reference genes to compare the gene expression of other strawberry genes coding for a component of the protein synthesis machinery [73].

FaMBL1, is a member of the strawberry G-type lectin family, which is the most up-regulated gene in 24-h-infected white strawberries, suggesting a role in the low susceptibility of unripe stages [48]. It has an important role in the response to *Colletotrichum fioriniae* and *B. cinerea* fungal diseases in plants [74]. *FaPR1* encodes a protein structurally related to pathogenesis-related proteins [75]. This and other 16 genes, were identified as anti-anthracnose-related genes in fruits infected with *Colletotrichum gloeosporioides*, and together with *FaChi3*, both increased in response to *B. cinerea* infection [71,76]. *FaWRKY1* gene coding for IIc WRKY transcription factor is up regulated in strawberry following *Colletotrichum acutatum* infection, treatments with elicitors, and wounding and is the major negative regulatory factor in the resistance of *F. x ananassa* to *Colletotrichum acutatum* [77,78]. *FaCyf1* is member of the PR6 family (*FaCPI*) which encodes a phytocystatin, a multifunctional protein with the ability to inhibit endogenous cysteine proteinases as well as from plants, insects and phytopathogenic microorganisms with antifungal properties, it is expressed in leaves, roots, and seeds of the achenes, but not in the receptacle of strawberries [47,18]. Recombinant *FaCPI* expressed in *Escherichia coli* inhibited the growth of the phytopathogenic fungi *Botrytis cinerea* and *Fusarium oxysporum* [47], and nematodes [79]. *FaChi3* codes for β-1,3-glucanase (βGlu), a class III chitinase, associated with cell-wall degradation [25].

The fact that *FaPAL1* and *FaPAL2* responded similar to *FaChi3* and *FaPR1,* may indicate that these genes responded preferentially to the cell wall destruction to either a fungal attack as potential pathogens having chitin or to physical damage, in both cases to repair fast and properly the cell wall integrity. As shown in Fig. 7, *FaPR1* and *FaPAL1* showed a similar response to BABA and chitosan; however, control responses in both cases were higher than BABA responses, suggesting a potential inhibition of these genes by BABA. In *Arabidopsis thaliana*, it was reported that no *PR-1* transcript accumulation after BABA treatment was observed, and mRNAs for the plant antifungal proteins defensin and thionin, respectively, responsive to both JA and ethylene or JA alone, were not induced after BABA treatment [80]. On the other hand, the resistance of blueberry to leaf spot increased after BABA induction, also the activity of PPO, POD, PAL, and β-1,3-glucanase enzymes increased in blueberry leaves [81]. However, same authors mention that after transcriptome sequencing, differential expression results showed that BABA upregulated 900 differential genes and downregulated 531 differential genes [81]. This means that BABA not always induce defensive genes, but it can even inhibit expression of specific genes. In our case, the difference in the gene expression between *FaPAL1* and *FaPAL2* to BABA treatment, could means that *FaPAL2* was responsible for the lignin production under both treatments, but *FaPAL1* could be used preferentially for phenolics production,

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although it was inhibited by BABA treatment. When chitosan was present, both *FaPAL* genes responded strongly because the menace of a fungal attack and possible damage to the cell wall requires an urgent response, and that's more important over the production of any other compound.

Although the action mechanism of these two elicitors is not yet understood, their effects are fast and severe in both cases. As described in Fig. S2, after 3 days of exposure to 1 mg / mL chitosan, more severe damage was found in plantlets than with 10 mg /mL BABA, resulting in oxidation of the tissues. Only BABA treated plantlets were able to recover after washing and culturing in fresh medium, suggesting that BABA was easily washed out in comparison to chitosan, which may remain attached after washing and is just partially soluble in acid solutions. BABA is soluble in water and is induced in plants after biotic and abiotic stresses [32], but the biosynthetic pathway is still unresolved. It is known that BABA "priming" plant defense, results in a wide pathogen's protection by the potentiation of the SA-inducible defense genes expression, and/or by induction of abscisic acid (ABA) biosynthesis and signaling pathways [82,83]. BABA also enhances the biosynthesis and accumulation of primary and secondary metabolites. Plants derived from BABA-treated plants have a higher resistance to pathogens enhancing PR gene expression, suggesting an inherited priming by the next generation [84]. After exogenous application, BABA moves through the plant as being transported through the systemic tissue. However, it wasn't detected in the derived plants, suggesting a lack of transference through the seeds [84].

There are several reports of the effects of chitosan and BABA on the phenylpropanoid pathway, but studies were focused mainly of the PAL enzyme activity or phenolics production, and in field studies. Application of chitosan to soybean leaf tissues caused increased activity of PAL enzyme. The enzyme activity was dependent on the chain length of the oligomers and time after treatment. The pentamer of chitosan produced the maximum PAL activities at 36 h after treatment as compared to controls [85]. In a study on the effects of foliar applications of chitosan in field condition, it was found an increase in plant growth and fruit yield (up to 42% higher) and had significantly higher contents (up to 2.6-fold) of carotenoids, anthocyanins, flavonoids and phenolics compared to untreated control in strawberry fruit [86]. It has been reported that BABA treatment at 10 mM attenuated postharvest gray mold decay in strawberry fruits by signaling H_2O_2 accumulation, leading to higher b-1,3-glucanase, chitinase and *PAL* gene expression (unspecified gene) along with increased levels of chitinase and PAL enzyme activities [87]. Strawberry fruits treated with BABA at 25 mM may be ascribed to providing sufficient intracellular ATP, higher ROS scavenging enzyme activity resulting in diminishing H_2O_2 accumulation, higher PAL enzyme activity resulting in higher phenols and anthocyanins accumulation as well as DPPH scavenging capacity, and lower PLD and LOX enzyme activity [88]. All resulting in maintaining membrane integrity, representing by lower malondialdehyde accumulation. However, and as far as we know, our work is the first one to study the effect of these elicitors on the *FaPAL* specific gene expression.

The *cis*-active elements related with biotic / abiotic stress shown in Fig. 6 have been found in other strawberry genes, as well as in genes associated with other factors like development, ripening, and others [89,90]. Also, transcription factors binding these elements and controlling several process and functions in the plant are known and are being studied [91]. Our *in vitro* system seems appropriated to look for the transcription factors responding specifically to chitosan and BABA, and to find the *cis*-active elements associated.

Different members of the *PAL* gene family have been identified in many higher plants, including 7 in *Arabidopsis thaliana* [92,93], 12 in *Citrullus lanatus* [94], 5 in *Salix viminalis* [95], 13 in *Cucumis sativus* [96], 12 in *Juglans regia* L [97], 6 in *Medicago truncatula* [98], 9 in *Oryza sativa* [99], 7 in *Fragaria x ananassa* [100], 8 in *Sorgum bicolor* [101], and 14 and 11 in *Solanum lycopersicum* and *Solanum pennellii*, respectively [102]. Considering the amount of *PAL* genes present in plants, and the key roles these genes may have in their physiology and biochemistry; it is important to identify which *PAL* gene(s) is(are) oriented toward a specific metabolic condition(s), to understand how the overall response of plants is concerted. The relevance of this knowledge was demonstrated in two varieties of sorghum where *SbPAL6* and *SbPAL8* showed low expression in sorghum shoots, but expression of remaining genes was significantly induced except *SbPAL5,* and a strong induction of *SbPAL1* at an early stage of infestation suggested its primary defense role in sugarcane aphid infestation [101]. Also, in cultivated (*Solanum lycopersicum*) and wild tomatoes (*Solanum pennellii*), 4 *PAL* genes were up-regulated, and 3 genes were down-regulated after root-knot nematode infection [102]. This result provided a potential application for the subsequent selection of tomato *PAL* genes for root-knot nematode resistance. A study across 29 *Oenothera* species analyzed the

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molecular evolution of two major biological processes: 1.- regulation of defense responses to fungi, bacteria, viruses, nematodes, and insects; and 2.- regulation of the phenolic biosynthetic process and hormone-based metabolism [103]. Same authors found 1,568 phenolic related genes arranged into 83 multigene families that varied widely across the genus. A rapid phenolic evolution (fast rate of genomic turnover) involving 33 gene families exhibited large expansions, gaining about 2-fold more genes than they lost. *PAL* and 4-coumaroyl: CoA ligase (*4CL*) genes showed the most significant expansions and contractions [103]. So, we can expect a variability and flexibility in *PAL* gene families, according to the changing environmental and biotic conditions. Global warming and increasing pollution may have a key role in this variability.

Conclusions

The *in vitro* system used in this work, allowed us to certify that there is not a statistical difference in *Fof* tolerance between 'Camino Real' and 'Nikté' strawberry varieties, and the results are reproducible. Not only by the phenotypic analysis but also by the results of phenolic and flavonoid compounds, which can be more precisely analyzed. Also, molecular analysis of gene expression and others, may be done in a more precisely and controlled way as demonstrated here. However, although these results may not be extrapolated directly to the field, due to the huge amount of other uncontrolled factors, we can have faster results and valuable knowledge about the behavior of the plant and the pathogen to design more accurate defensive strategies. The bioinformatic of *FaPAL* genes, resulted in the analysis of the two gene families, *FaPAL1* and *FaPAL2*. In our case, that allowed us to associate the difference in the response of *FaPAL1* from *FaPAL2* to chitosan and BABA with some *FaPAL* gene family and *cis*-active elements in the respective promoters, and this can help to assay other molecular approaches. This knowledge may be ver useful in evolutive studies and to identify more specifically which gene family and perhaps members, are involved in specific responses of strawberry to different stimuli. This can also help to design specific strategies for genomic edition of strawberry, focused on the search for better and more adapted varieties depending on the clime, soils, biotic and abiotic factors typical from different regions, and to improve specific nutraceutical and organoleptic properties.

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