The Resistance of Carnation (Dianthus caryophyllus L.) to Fusarium oxysporum f.sp. dianthi is a Multigene-Multivariate Phenomenon

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Abstract

Floriculture is one of the most crucial crop industries worldwide. The carnation is the second more exported/imported flowers in the world. The disease most affecting the carnation crop is Fusariosis, produced by the ascomycete *Fusarium oxysporum* f.sp. dianthi. Little is known about the genetics of the resistance to *Fusarium* in carnations. In this job, different genes implicated direct or indirect forms in the defense mechanisms were identified using mRNAseq and RT-qPCR techniques. Some of these genes were involved in basal metabolism, genes implicated in the primary response to the pathogen, and genes kind pathogens-related proteins (PRs). These genes in different carnation varieties present overexpression or in contrast subexpression and determine somehow the resistant or susceptible phenotype to *Fusarium oxysporum*. Some of them are directly related to cell wall remodeling. Different genes are involved in the resistant response in carnations in different varieties; so, each variety elaborates a response in a different form from the other varieties; even more, the same gene is expressed differently in the different resistant varieties.

Introduction

Carnation (*Dianthus caryophyllus* L.) is the most representative species of the Dianthus genus. The major use of carnation flowers is as a vase flowers. The annual trade worldwide is about USD 25 billion, and in some cases is one of the most important income for some countries (Singh et al. 2019). Perennial plants of a woody base, with a maximum height of a meter, with long internodes (Whealy 1992). Linear leaves, opposite, acuminate, and glaucous, with a color that could change from green to gray. Flowers with fragrance to clove, bisexual, with radial symmetry, four to ten stamens, surpass the ovary, and terminal inflorescence with a cylindrical calyx (Whealy 1992).

The fungus *Fusarium oxysporum* f.sp. dianthi (FOX) is a complex of species that causes vascular wilt in carnation, which is the crop's most limiting disease, commonly known as fusariosis (Castaño et al. 2014). Introducing crop-resistant varieties is the most efficient way to control the disease since the use of pesticides has the highest economic and environmental cost (Cuervo et al. 2009). Biological control is another strategy used to control FOX, but its genetic plasticity produces genetic resistance quickly. The best approach is integrated management that agrees with fungicides and controller microorganisms (Erazo et al. 2021). Additionally, few jobs directed to study and identify responsible genes for plant resistance to FOX have been carried out. Soto and collaborators (2012) phenotypically evaluated the resistance to FOX in hybrid varieties and estimated that the resistance is governed by more than two genes and with additive genetic action. Noman and collaborators (2017) developed the transformation of carnations with the jasmonate methyl transferase gene for Fusarium tolerance. Phytoanticipins such as 3-hydroxyacetophenone were identified in 2005. Ardila and collaborators (2014) studied the activity of peroxidases and enzymes of phenylpropanoid routes in resistant varieties. Rodrigues and collaborators (2016) reported some proteases and reactive oxygen species (ROS) using commercial carnation varieties. Studies in the apoplast of stems and roots infected with FOX have permitted the identification of some flavonoids and fenolenonas that could participate in the resistant response (Ardila et al. 2018).

This work was directed to identify the variations in the levels of expression of genes involved in the pathogen response, which were identified in the transcriptome of elicited carnation cells (in previous work) of plants resistant and susceptible to FOX under in vitro conditions.

Material and Methods

Biological

Carnation plants produced in 2018, in a breeding program in the UMNG in Cajicá, a municipality of the department of Cundinamarca in Colombia, were used in this study. Plant code SH2, susceptible to FOX, was produced from varieties of not commercial carnations. Plant code 395 resistant to FOX was produced crossing the commercial variety Kaly (susceptible) and Candy (resistant) and plant code F1B1 resistant was obtained in an F2 cross from the previous cross between Kaly and Candy, and both (395 and F1B1) were used as resistant plants to FOX. Simultaneously, we used plants code 6455 susceptible, produced too in the previous cross F2. The seeds that produced the hybrid plants used here were germinated in vitro culture with MS media Murashige and Skoog (1962), sucrose 35g/L, and Phytagel 3g/L. The plants were propagated in the same medium and maintained at 26°C in the laboratory with a light 6000-lux and 16 hours/day by four weeks. The plants (clones of each code) were set in a greenhouse and maintenances for three months with fertilization until each plant had stems of more than four knots to proceed with the inoculation. One hundred and fifty plants of each code (SH2, 395, F1B1, and 6415) were evaluated for resistance with a pathogenic isolate of FOX (results not shown here) to check the resistance and susceptible character of each clone of plants used here.

The vascular wilt characteristic symptoms were evaluated one week after the inoculation, each seven days and during 18 weeks, according to the next symptoms scale (Soto et al. 2012): 0 = healthy plants without symptoms, 1 = plants with symptoms of turgor loose, 2 = plants with yellowing symptoms, 3 = plants with severe wilting and 4 = died plants. When the time of symptoms register was finished, we took each plant that was inoculated and we re-insulated the pathogen with the goal to corroborate the plant infection.

With the symptoms register, using the severity scale at the time of the experiment, we estimated the area under the disease progress curve (AUDPC), using this equation: AUDPC = $\sum((Xi + X\ i + 1)/2) \times ((T\ (i + 1) - Ti))$, i = 1, where Ti= # days between the inoculation and the date of sampling, Xi = the rating using the symptoms scale, (T(i + 1) - Ti) = time in days between two readings, (Soto et al., 2012). With the AUDPC date of each parental and each F1 plant, as well as with the detailed observation of the symptoms in the time, we estimated phenotypes using frequency analysis.

Leaf of the evaluated plants of each code was put on MS media, supplied with 2-4-D hormone (1 ppm), (Filgueira 2011), in in vitro conditions to produce undifferentiated cells or not-innate stem cells. The cells obtained were re-cultured in the same media no more than two times before being used for the elicitation assays.

FOX was recollected and isolated from unhealthy plants with vascular wilt symptoms. The plants were disinfected with sodium hypochlorite 1% and ethanol 70%. Fragments of 5 mm of symptomatic stems were cultured in PDA media. Once the fungus appeared, a monosporic culture was performed in PDA and conidia were re-culture in the same media (Filgueira 2011). For the next experiments, the fungus was cultured in Czapek Dox (liquid media) for seven days at 24°C. The isolated used here were identified like FOX, using morphological characterization and molecular taxonomy with the sequencing of PCR amplicons of the regions ITS 1, 2, and 4 between the 10S and 25S intergenic regions and elongation factor EF1 (result not presented here).

Elicitation in dual culture

Three grams of undifferentiated cells of the different varieties (SH2, 395, F1B2, and 6455) were cultured in a Petri dish with 30mL of MS solid media, supplied with 2-4-D for seven days. 10uL of $1x10^6$ conidia/mL solution was located in the opposite region of the Petri dish where the plant stem cells were growing. This dual culture was maintained for seven days, taking care of the fungus mycelia that did not touch the plant cells (Filgueira 2011).

mRNA obtained and relative RT-qPCR

Elicited cells of hybrids F1B2 and 395 resistant (R) and SH2 and 6455 susceptible (S) were contrasted with not elicited cells of all hybrids used here. In a similar form, all hybrids were used to obtain the mRNA. The isolation and purification of mRNA were developed using the Trizol method with the NucleoSpin mRNA kit of MachereyNagel. The concentration of the obtained mRNA was measured using the Qubit RNA BR Assay Kit of Thermo Fisher Scientific and the integrity was estimated using agarose 1% electrophoresis in TBE buffer (89mM Tris base, 89mM boric acid, and 2mM EDTA). The kit One Step Green RT-qPCR with MMLV & hot-start Taq DNA Polymerase of Sigma-Aldrich was used to perform the amplification and relative quantification in an equipment qPCR Line-Gene 9600 Plus of BIOER Technology. Each elicitation and RT-qPCR experiment with the different genes and with each variety of carnation used here were repeated in triplicate.

Gene pool

All the genes studied here were selected from a previous study, where the carnation transcriptome of resistant and susceptible elicited cells with FOX was studied (J.J. Filgueira unpublished data). A group of genes that were involved in basal metabolism was selected because of its variation during the elicitation; genes involved in the primary response to the pathogen and pathogens-related proteins (PRs) genes were selected. All the primers listed in Table 1 were first tested with the genomic DNA of all four varieties through PCR, before the cDNA amplification, and the amplicons were sequenced by the Sanger method in Macrogen Korea, to corroborate the unique relation between oligos and genes (results are not presented here).

Table 1
List of forward and reverse primers. In front of each gene name appears the ID of the gene in the Carnation DB https://carnation.kazusa.or.jp/.

Gene	Symbol	Forward	Reverse
Alcohol dehydrogenase I (18771)	ADH	aaggaatgcgctcactgcaaatcc	atgtcgatttcaagcgtccatgcg
Arginine decarboxylase (38063)	ADC	aattccgccgtcgtttgcttcttc	tatcggtcctggttgcacttcaca
Flavonol 3-O-glucosyltransferase (56048)	GTI	agacatcgagctcttcatgctggct	accgatacaccacttggcttccact
Ethylene insensitive like 3 (54048)	EIL3	ggatgaggactgtactgcgatggg	ctgcagctgatcaccggtttcc
Anthranilate N-benzoyltransferase (4247)	HCBT	gtgccctactatccaatggcgggta	aaggggttgcaggaaaggtctccaa
Ethylene receptor gene (2767)	ETR1	tgttgtcgcagttcgggtccc	acgtgtcgcttgaacctttgcat
Phenylalanine ammonia lyase (137519	PAL	cttgccttcctctgcgcgga	atggggactcaattttggcggc
Anthocyanin 5-O-glucosytransferase (11039)	UGT	gaaaatcgctccgtcgagggttgag	ccatagcggccttcttcagctcatg
Beta-1,3-glucanase (10925)	GLU	ggcttgttactgaaggctgga	gctacaacacctgaactgcc
Chitinase 4-like glycosyl hydrolase (25028) β-Glycosidase glycoside hydrolase (6768)	CTS BGLU	tcggtactggcacgaatgtc ccgcattggctgatggattg	gtaacaccgaatttggcgca gcgatggtccaggtttggta
Aldose 1-epimerase (28123)	AEP	cctgcaaggagctggatca	cgtaaacgcaagtacgacgc
Thaumatin like protein (3959)	TLP	tgcctacaaatgccgtgaca	tggcatcaatcatcgcaacg
α and β Amylase (105)	ABAM	ggctactcaagaggctcagg	ccaaacaaccgtcccaggaa
Homologous to peroxidases (2878)	PER	tcgcagacgtgggtttaaca	cggcatcagactggaagagg
Catalase (31671)	CAT	accgacacatgattgggtcc	ctcagcttcacgaacagggt
Malate dehydrogenase (18362)	MDH	tcacaacccgacttaccgtc	acctgtttgacaggcactcc
Jasmonic acid mediated signaling pathway (4214)	MAPK	agagcgacagtgctcgtaat	aagtcaacagctgcaggaga
Polygalacturonase inhibiting protein (31653)	PGIP	aaaaccggtctcacttggct	tgctggcaaaagagtcagcc
Salicylic acid mediated signaling pathway (13044)	RDC	tcccctcgtaggtcgtgatt	tgctggcaaaagagtcagcc
Receptor-Like Kinase (9311)	RLK	tggacccagatggaggagtt	atctcctactcataacgcggc
Ribulose bisphosphate carboxylase / oxygenase	RuBP	acccttcgttgggttgaagt	tcacggtatacgaacgcctc

Gene	Symbol	Forward	Reverse
(48496)			
Fructokinase (55984)	FRK	caatgtcactgtggcaccct	aagcttgctcaaaagcgcac
Ribosomal protein L7 (22284)	RPL	ttgtcgtcatggctgctgata	aatcacgggcctggagact
Xyloglucan endotransglucosylase/hydrolase (17615)	XTH	caggcttctgggtctggttt	gtggtggggtcgaaccatag
Glyceraldehyde-3-phosphate dehydrogenase (8698)	GAPDH	ttggcatcgttgagggtctc	ctggcaggtcttgtggatgt
Tonoplast intrinsic protein gamma (50626)	TIP	gccaacgtatccccatggaa	ctcgtagatgacaccggcaa

Statistical analysis

The RT-qPCR data of each gene in resistant and susceptible cells of each code used here were obtained in triplicate and the experiment of elicitation that was present before was developed in triplicate for each variety. The quantitative raw data and the quantitation of the amplification results of all genetic sequences were studied and obtained with the software of the LineGene 9600 Plus Fluorescent Quantitative Detection System of BioER. The Quantitative Raw Data (QRD) obtained from the qPCR simple lecture was plotted fluorescence vs. cycle number and the tendency line was calculated with the data of each curve. The linear equation was obtained from the tendency line. The comparison between the different RT-qPCR repetition lectures was performed using the slope of the line, which is the rate of change of the equation. Subsequently, calculated the cumulative distribution of the three repetitions and then we calculated the maxima distance between the different linear equations.

Results

Elicitation and total RNA analysis

The methodology of elicitation assay of the cells on in vitro culture of the different carnation varieties used in this study has been evaluated in other studies, where the plant cells were demonstrated to respond in the presence of FOX at in vitro dual culture. The resistance and susceptibility results to FOX were corroborated with the assays in the greenhouse with plants infected with a pathogenic isolate of FOX (results not shown here). In this condition (the dual culture), the plant cells respond by producing substances, that in the case of the resistant varieties limit the fungus growth, demonstrating that the carnation cells recognize the presence of the fungus through the culture media (Fig. 1. a and b). Consequently, the fungus releases substances into the culture media that were recognized by the cells without existing physical contact between them. The growth rate of FOX in the dual culture with two carnation varieties is presented in Fig. 1a, where it is possible to see that the fungus growth is affected by plant cells and the different fungistatic activity of each variety. For varieties 6455(S) and SH2(S), the growth ratio was 0.95:1 about the controlled fungus growth without cells and for the varieties, F1B2(R) and 395(R) was 0.7:1 in relation to the control without plant cells (Fig. 2. a.).

a. Dual culture with carnation-resistant cells (395) where it is possible to observe an inhibition halo on the fungus growth. b. Dual culture with susceptible cells (SH2), where the inhibiting halo is not observed, and the fungus growth is heading toward the stem cells. c and d. Electrophoresis of total RNA of not elicited (c) and elicited (d) cells of carnation varieties A: SH2 (S), B: 395(R), C: F1B2(R), and D: 6455(S).

a. Graphic that presents the fungus growth in the presence of the carnation cells of the different varieties in dual culture. The lowest fungal growth value is seen in the presence of the resistant varieties 395 and F1B2 and the highest fungal growth value is noticed in the presence of susceptible varieties SH2 and 6455. b. The graphic shows the production of total RNA of different carnation varieties elicited and not elicited. c. Here, it is presented the effect of the elicitation with FOX on the mRNA production in the different carnation varieties used, (E) elicited carnation cells and (NE) not elicited carnation cells.

Variation of the gene expression

All the oligos used in this study were initially evaluated with genomic DNA (results are not presented) and the corresponding amplicon was sequenced. The obtained sequence in each case was contrasted with the carnation KAZUSA genome database to confirm the presence of the amplified gene in the genome of the carnation variety used here. The relative expression level of all genes in cells of carnation varieties evaluated here was detected using the RT-qPCR technique. The data allowed us to determine the increase and decrease in the genic expression of genes involved in the basal metabolism, secondary metabolism, and the specific parasite's response during the elicitation with FOX.

Between the housekeeping genes, cinnamyl alcohol dehydrogenase I (ADH) were analyzed, which catalyzes the final step in the biosynthesis of monolignol, the main component of lignin. Lignin is deposited in the secondary cell wall and plays a role in plant defense against pathogens (Sibout et al. 2005). The resistant varieties presented an increase of 55.5% for ADH expression during elicitation compared with the susceptible varieties´ expression; conversely, in the susceptible elicited varieties, the increase did not exceed 24.6% in the two varieties under study (Fig. 3.a.b.) compared with not elicited cells. In the case of arginine decarboxylase (ADC), the activity is associated mainly with secondary metabolic processes, cell extension, and stress responses (Fortes et al. 2011). The resistant varieties increased the expression of ADC by 49.6% compared with the susceptible varieties, in these varieties; the increase during the elicitation did not exceed 4.9% (Fig. 3.a.b.) compared with not elicited cells. Another reference gene was Flavonol 3-O-glucosyltransferase (GTI); this enzyme is involved in the biosynthesis of secondary metabolites. The primary function of this enzyme is binding a glucoside onto a flavonol molecule, forming a flavonol 3-O-glucoside; it is through this mechanism that the enzyme converts anthocyanidins to anthocyanins as a part of the phenylpropanoid pathway (Yin et al. 2012). GTI increased its expression by 66.6% during the elicitation in susceptible varieties compared with an increase of 55.1% in resistant varieties.

Ethylene insensitive like 3 (EIL3), a probable transcription factor, acts as a positive regulator in the ethylene response pathway (Dolgikh et al. 2019). The resistant varieties reduced the expression by 25.6% and the susceptible varieties by 39.5; this case is the first reported in carnations of negative regulation post elicitation (Fig. 3.b.). The ribulose bisphosphate carboxylase (RuBP) gene produces an enzyme involved in the first major step in carbon fixation. Pathogen infection of plants results in the modification of photosynthesis during the activation of defense mechanisms (Rojas et al. 2014). An important decrease of -23.4% was detected in the

resistant varieties during the elicitation and a decrease of -19.1% in the susceptible varieties (Fig. 3.a.b.). The Fructokinase gene (FRK) is an important enzyme that catalyzes the key metabolic step of fructose phosphorylation, which is essential for plant growth and development, and sucrose is engaged in plant defense by activating plant immune responses against pathogens (Yao et al. 2017). Despite the above, in susceptible varieties, it was possible to observe an important variation in the expression of -4.3% and in resistant varieties a slight increase of 3.7%.

Tonoplast intrinsic protein gamma (TIP) (Rodriguez et al. 2016), is a gene that increases its expression during the elicitation in resistant varieties until 6.7%, but more importantly is the reduction of the expression in the susceptible varieties that reach – 7.7% in compared with the same not elicited varieties (Fig. 3.b.). In the genes involved in the primary response to the pathogen, we initially selected the anthranilate N-benzoyltransferase (HCBT), which catalysis the committed reaction of phytoalexin biosynthesis (Yang et al. 1998). The variation in the elicited susceptible variety was – 5.2% and in the elicited resistant varieties, the increase was 7.9% (Fig. 3.b.). The ethylene receptor gene (ETR1) is involved in the response to *Xanthomonas campestris* pv. vesicatoria in tomatoes, in the regulation of the ethylene receptor (Ciardi et al. 2000). In carnation, the resistant varieties increased the expression by 18.7% and the susceptible to 18% on average during the elicitation (Fig. 3.b.). Phenylalanine ammonia-lyase (PAL) is the most representative in the response to parasites and plays different roles in the metabolism of biotic stress (Cass et al. 2015). Like GT, GTI other genes involved in the phenylpropanoid metabolism, PAL showed important variation in elicited varieties, an increase in the expression of 22.5% for resistant, and 15.1% for susceptible (Fig. 3.b.).

Another gene that participates in the primary response and was evaluated here, the anthocyanin 5-O-glucosyltransferase (UGT), whose ectopic expression of potato tuber causes increased resistance to bacteria (Lorenc et al. 2005), presented a 24.1% increase in the expression in the resistant varieties and 16.7% in the susceptible ones. The difference, in this case, could be representative of the response to FOX (Fig. 3.b.). Ribosomal protein L7 (RPL) plays a role in no host disease resistance to bacterial pathogens (Nagaraj et al. 2016) and in the carnations present in the susceptible varieties, a decrease of -13.8% in the expression about the increase of 3.8% in the resistant varieties (Fig. 3.b.). Xyloglucan endotransglucosylase/hydrolase (XTH) is a gene that is present in a susceptible and resistant jute species and shows an opposite expression pattern following *Macrophomina phaseolina* infection (Shamir et al. 2012). In carnations, the susceptible varieties presented a decrease of -0.42% in the expression; on the contrary, the resistant varieties presented a slight increase of 3.1%. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a multi-functional enzyme involved in the regulation of ROS, autophagy, and plant immune responses (Henry et al. 2015). In the susceptible varieties, it presented a decrease in expression of -6.8%, in contrast with the resistant varieties that present an increase of 4.8% in the expression.

The values of the gene expression (increase or decrease) in the terminus of the difference between the expression without elicitation and during the elicitation with FOX of cells of the different carnation varieties used in this study permit us to predict the reason for the resistance and susceptible behavior. Genes with the highest significant increase in the expression of resistant varieties are ADC, CTS, ADH, and MDH. The second group of genes that increase its expression but less significantly are PER, RPL, TIP, HCBT, GLU, and GAPDH. It is unclear why some important genes that elaborate the immune response, decreased its expression in the resistant varieties, are the case of ABAM, GTI, AEP, TLP, and RDC.

a. Heatmap, red represents up-regulated genes and blue represents down-regulated genes, grey represents unchanged expression. b. The graphic presents comparatively the relative expression of all genes used in this study (relative expression = the expression of an elicited gene – the expression of not elicited gene); these values could be negatives or positives. The values at the bottom are expression frequencies.

In the PR gene group, we evaluated beta-1, 3-glucanase (GLU), which defends plants against fungal pathogens either alone or in association with chitinases and other antifungal proteins. They are grouped in the PR-2 family of PR proteins (Balasubramanian et al. 2012). Here, the difference between the expression of the GLU gene in the resistant varieties is 12.3% and in the susceptible ones 2.2%, which is slightly significant (Fig. 3.b.). Another PR gene, the chitinase 4-like glycosyl hydrolase (CTS), can enhance the plant's defense system as it acts on chitin, a major component of the cell walls of pathogenic fungi and renders the fungi inactive without any negative impact on the plants (Kumar et al. 2018). CTS presented an important increase both in resistant 70% and an increasingly less important in susceptible varieties of 27.1%, (Fig. 3.b.).

β-Glycosidase glycoside hydrolase (BGLU) a plant glycoside hydrolase involved in cell wall polysaccharide degradation (Dong et al. 2019), presented in resistant varieties an increase of 21.6% in the susceptible, that presented a variation in expression of 13.5%. In the case of the gene aldose 1-epimerase (AEP) that participates in abiotic and biotic stress control (Sheshukova et al. 2017), the gene presented an increase of 5.9% in the resistant varieties and 15.2% in the susceptible varieties (Fig. 3.b.). The genes of thaumatin-like protein (TLP) are a highly complex protein family associated with host defense and developmental processes in plants. They are classified as the PR-5 (Pathogenesis-Related-5) protein family (de Jesus et al. 2020). TLP presented an increase in the expression of 20.6% in the resistant elicited variety and 28.4% in the susceptible variety. The α and β amylase (ABAM) act in the induction and protective roles of maltose during temperature shock and biotic stress (Kaplan and Guy 2004). In the case of carnation, the resistant varieties present an increase of 3.9% in gene expression and 24.1% in susceptible. The gene homologous to peroxidases (PER) is part of a family that presents differential induction during the infection of some crop cereals by bacteria (Mir et al. 2015). Here, in the resistant varieties, an increase of 35% of the basal expression was observed, and in the susceptible varieties of 10.8% during the elicitation (Fig. 3.b.).

The case of the catalase gene (CAT) has been observed during the regulation, activity, and expression in the infection by *Phytophthora nicotianae* in tobacco (Blackman and hardham 2008). In the resistant carnation varieties, the gene increased its expression by 37% and in the susceptible ones by 29%. The malate dehydrogenase MDH gene, the enzyme that catalyzes the reversible oxidation of malate to oxaloacetate using the reduction of NAD to NADH (Schereier et al. 2018), is presented an important decrease in its basal expression of -18% in susceptible varieties, in comparison with a slight increase in the resistant varieties of 6% (Fig. 3.b.). The jasmonic acid-mediated signaling pathway (MAPK), intervenes in the jasmonic acid (JA) and its precursor, referred to as jasmonates (JAs), are important molecules in the regulation of many physiological processes in plant growth and development, and particularly in the mediation of plant responses to biotic and abiotic stresses (Ruan et al. 2019). This gene shows an increase in expression in resistant varieties of 15.6% and in susceptible varieties of 7.9%. The polygalacturonase - inhibiting proteins (PGIP) are cell wall proteins that inhibit the pectin-depolymerizing activity of polygalacturonases secreted by microbial pathogens and insects (Kalunke et al. 2015). This gene presented an increase in the resistant varieties of 24% compared with the increase in the susceptible by 18.1%.

The salicylic acid-mediated signaling pathway (RDC) gene, where the small phenolic molecule salicylic acid (SA) plays a key role in plant defense (Ng et al. 2011), shows, in this case, an increase in susceptible varieties of 36.1% in relation to an increase in the resistant varieties of 25.3%. Finally, the receptor-like kinase (RLK) forms the largest family of receptors in plants and is critical to recognizing pathogen-associated molecular patterns and modulating the plant immune responses to invasive fungi, including cereal defenses against fungal diseases (Thapa et al. 2018). This gene presents a litter bit significant increase in the resistant varieties 33.8% about the susceptible ones 26.6% (Fig. 3.b.).

Discussion

The *in vitro* model of the relation of host/parasite used here showed robustness to evaluate the cell response to the pathogen's presence. This model has been evaluated in other studies in our laboratory (Filgueira 2011) and facilitates analysis because it reduces the number of variables in the experiment. The analysis of the total RNA obtained from the elicited and not elicited varieties (Fig. 2. b.) Showed that the cells of resistant elicited varieties with FOX increased the production rate of the total RNA 4.4 times on average, compared with the not elicited cells (Fig. 1.c. and d.). Variety 6415(S) elicited showed an increase of 85.9% production of total RNA compared with SH2(S), which increased the total RNA production rate by 76.3%. Variety 395(R) increased the production of total RNA by 80.3% compared with variety F1B2(R), which enhanced it by 78.4%. It is clear that the increase in the rate of total RNA is more or less the same in the elicited resistant and susceptible varieties (Fig. 2.b.). These tendencies were also observed when we only quantify the expression of the mRNA of the different genes in the resistant and susceptible varieties with or without elicitation (Fig. 2.c.).

The highest variation in the gene expression level of elicited cells was present in CTS, ADH, ADC, PER, and MDH. Chitin and its modified form chitosan induce host defense responses in vascular plants (Debnath et al. 2022), and CTS is directly related to the production of chitin in carnations. Second, lignin not only acts as a barrier to pathogen invasion, but the phenylpropanoid pathway responsible for lignin biosynthesis may also be recruited for the defense such as the synthesis of phenolic compounds including phytoalexins, stilbenes, coumarins, and flavonoids (Malinovsky et al. 2014) and in carnation, ADH is related to the production of lignin. In another way, MDHI is an enzyme that transforms oxaloacetate into malate and in the tricarboxylic acid cycle is transformed into glutamate; the glutamate in the arginine pathway participates in the polyamide biosynthesis with the interaction of the ADC gene; in carnation, simultaneously, a polyamide (p-coumaroyl amide) is transformed in lignin (Bassard et al. 2010). Finally, peroxidases are implicated in cell elongation via the generation of reactive oxygen species (ROS) through the hydroxylic cycle (Passardi et al. 2004), and PER in carnation is a gene of the peroxidase family that presented high activity during the elicitation.

The second group of genes, which is integrated by PAL, UGT, GLU, BGLU, CAT, MAPK, RLK, and TIP, presented a moderated level of overexpression, but this was very consistent. PAL, the most important gene in the phenylpropanoid pathway in carnations, is overexpressed in resistant varieties. The anthocyanin 5-O-glucosyltransferase (UGT) in plants is related to anthocyanin and flavonoid biosynthesis, which are part of the signaling pathway in the pathogen response (Yonekura et al. 2012). Otherwise, β-1,3-glucanases (GLU) can destroy the cell walls of fungi, and the phenylpropanoid pathway and PR proteins are stimulated by the GLU activities (Aziz et al. 2007). Second, glycosylation is the major regulator of phenylpropanoid pathway activity in plants (Le Roy et al. 2016), and the expression of genes such as BGLU in carnations could play an important

role in the response activation. Similarly, catalases are powerful antioxidants and work in the stimulation of the phenylpropanoid pathway by biotic and abiotic elicitation (Ampofo and Ngadi 2021), in the same way, the gene CAT of carnation could be activating the flavonoid biosynthesis that favors the FOX response.

In the case of MAPK, which functions in the signaling pathway of jasmonic acid, this gene is related to both the lignin production via p-coumaroyl and the phenylpropanoid pathway via phenylalanine conversion in cinnamate (Yadav et al. 2020). In the case of the RLKs, recent studies have focused on the importance of this gene that acts in both broad-spectrum, elicitor-initiated defense responses and as dominant resistance (R) genes in race-specific pathogen defense (Goff and Ramonell 2007). Finally, is unclear the role that plays a gene-like LIT in the defense mechanisms against FOX, they are presumed to play an important role in plant defense responses against biotic and abiotic stressors (Afzal et al. 2016).

Conclusion

It was clear here that the carnation resistance to FOX is multigene and multivariate and it is impossible to attribute the resistance phenomena to a single gene. Multigene, due to the participation of different genes, including those that belong to the basal metabolism, and multivariate because different resistant varieties respond using a different combination of genes. In many cases studied here, was present an increase in the expression in both resistant and susceptible varieties showing that the susceptible varieties also respond to FOX presence, but the combination of genes used by the susceptible does not be conducted to avoid the infection.

In this work, we identified two pathways that play an important role in plant defense against FOX in carnations. The first one is the production pathway of lignin and chitin that are involved in the remodeling of the cellular wall, where directly or indirectly participate genes such as ADC, ADH, MDH, CTS, and PER; these groups of genes are the major components and are responsible for the increased transcriptional change. Genes such as PAL and MAPK that are involved in the phenylpropanoid pathway could contribute to the remodeling of the cell wall, increasing the production of lignin.

The other pathway that is central to the response to FOX is the phenylpropanoid pathway; genes like PAL and UGT play here a principal role in flavonoid biosynthesis. Others such as GLU participate in eliciting the phenylpropanoid way, and BGLU is an important regulator of this pathway. Second, CAT stimulates the phenylpropanoid way. These two pathways are interconnected via MAPK and act synergistically, adding their effects to the result of the resistant phenotype observed in the 395 and F1B2 varieties. Previously demonstrated that carnations present a complex response strategy with the activation or deactivation of multiple genes.

Declarations

Authors' contributions

William Andres Gomez Corredor analysis RT-qPCR, Daniela Londoño Serna generation NGS-seqRNA libraries and Juan Jose Filgueira Duarte working with RT-qPCR and NGS-seqRNA resoults.

Data availability statement

The datasets generated during and/or analyzed during the current study are available in the Carnation DB https://carnation.kazusa.or.jp/ The ID of the each gene used here appeared in table 1.

Conflict of interest statement

In our manuscript do not exists conflict of interest.

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Figures

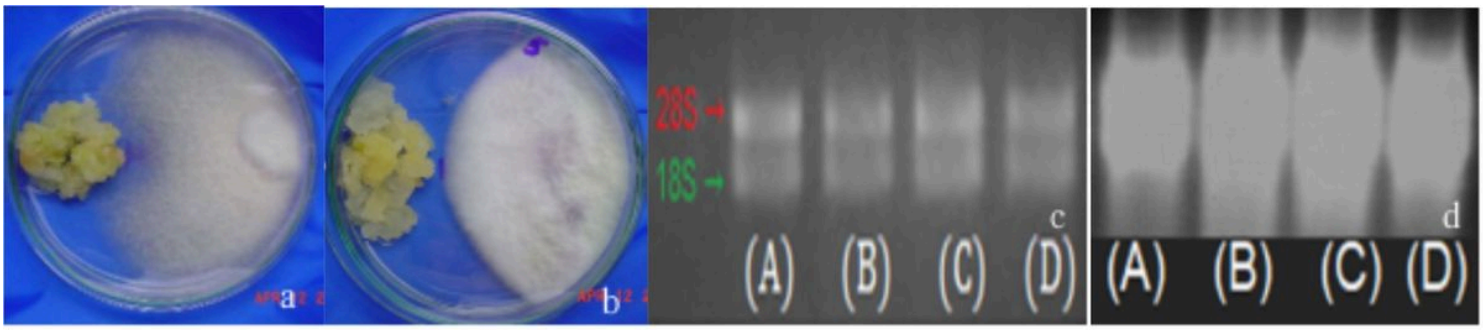

Figure 1

Elicitation effects.

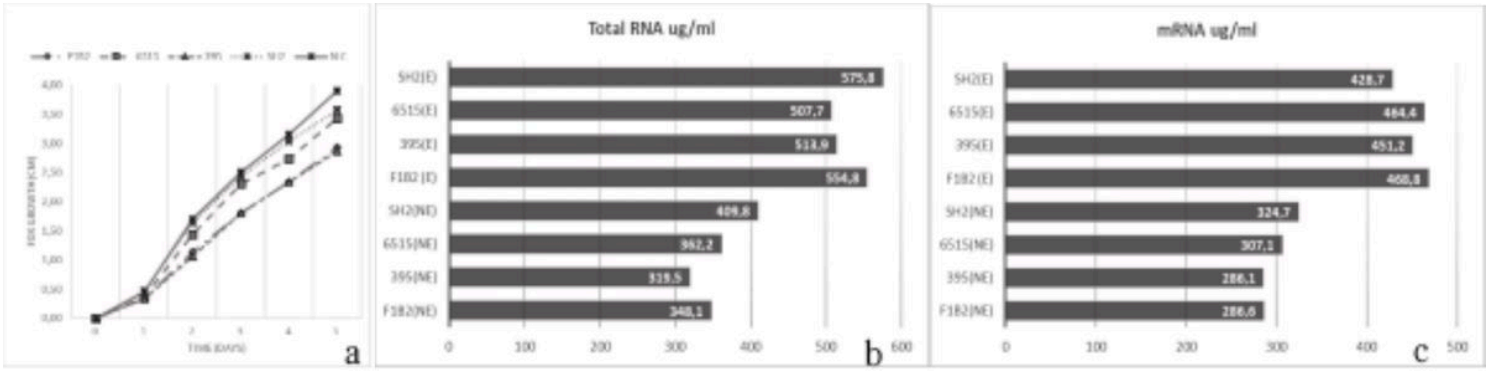

Figure 2

Plant cells and fungus responses in the dual culture.

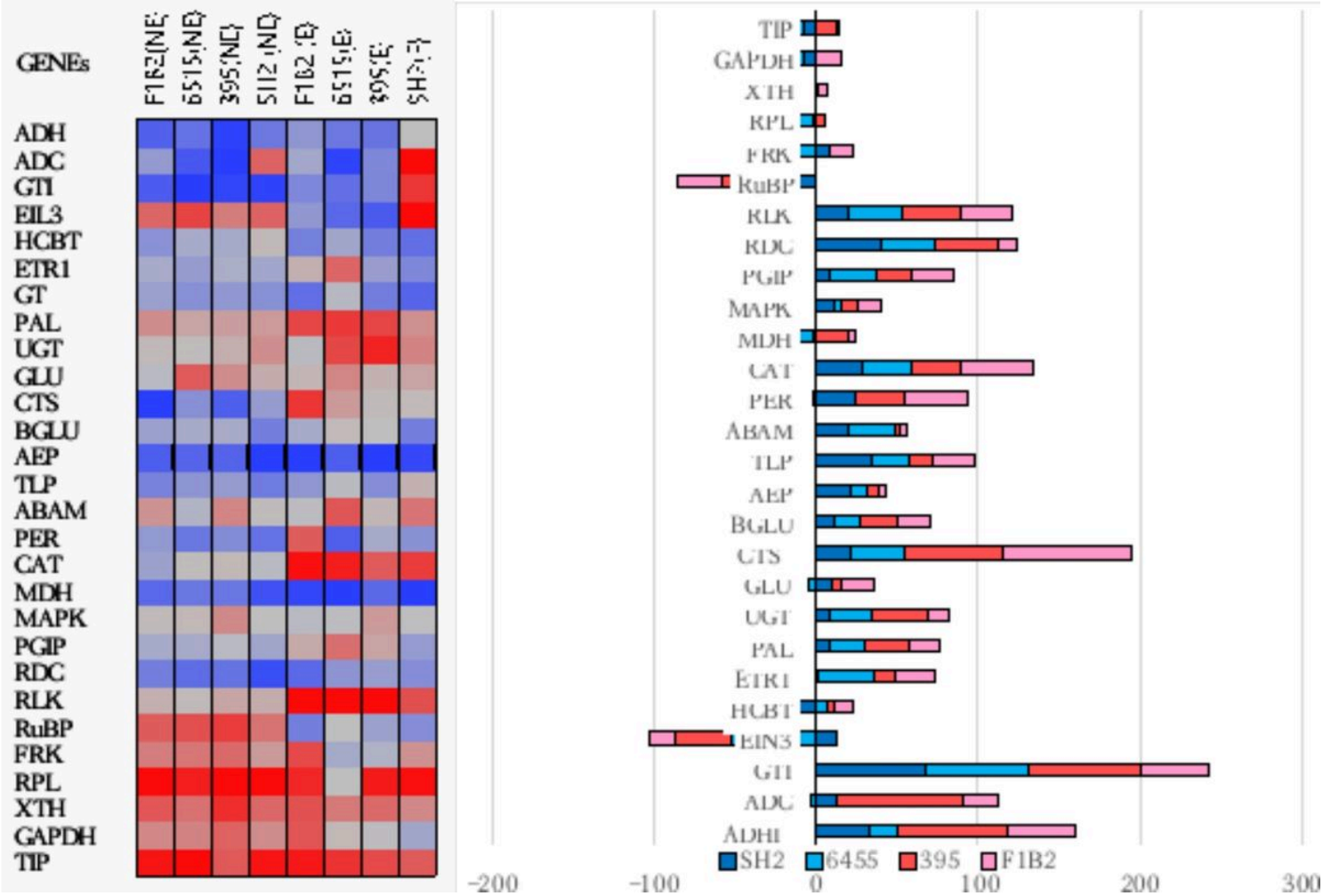

Figure 3

Gene expression variations during the cell elicitation.