(This is a sample cover image for this issue. The actual cover is not yet available at this time.)

Plant Physiology and Biochemistry 54 (2012) 10–16

Contents lists available at SciVerse ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Temporal accumulation of salicylic acid activates the defense response against *Colletotrichum* in strawberry

Carlos F. Grellet-Bournonville [a], Martín G. Martinez-Zamora [a], Atilio P. Castagnaro [a,b], Juan Carlos Díaz-Ricci [a,*]

[a] *Departamento Bioquímica de la Nutrición, Instituto Superior de Investigaciones Biológicas (CONICET- UNT) and Instituto de Química Biológica "Dr. Bernabé Bloj", Universidad Nacional de Tucumán, Chacabuco 461, 4000 Tucumán, Argentina*

[b] *Sección Biotecnología de la Estación Experimental Agroindustrial Obispo Colombres (EEAOC)-Unidad Asociada al INSIBIO, Av. William Cross 3150, Las Talitas (4101), Tucumán, Argentina*

A R T I C L E I N F O

Article history:
Received 5 November 2011
Accepted 31 January 2012
Available online 8 February 2012

Keywords:
Strawberry
Defense response
Salicylic acid
Anthracnose
Colletotrichum

A B S T R A C T

Many authors have reported interactions between strawberry cultivars and pathogenic microorganisms, yet little is known about the mechanisms triggered in the plant. In this paper we examine the participation of the salicylic acid (SA) signaling pathway involved in the response of *Fragaria x ananassa* cv. Pájaro plants to pathogens. Strawberry plants were challenged with the virulent strain M11 of *Colletotrichum acutatum*, or with the avirulent strain M23 of *Colletotrichum fragariae* which confers resistance to the former. Our study showed that the isolate M23 induced a temporal SA accumulation that was accompanied with the induction of *PR-1* gene expression in strawberry plants. Such events occured after the oxidative burst, evaluated as the accumulation of hydrogen peroxide and superoxide anion, and many hours before the protection could be detected. Similar results were obtained with exogenously applied SA. Results obtained supports the hypothesis that strawberry plants activate a SA mediated defense mechanisms that is effective against a causal agent of anthracnose. In contrast, plants inoculated with M11 did not show oxidative burst, SA accumulation or *PR1* gene induction. This is the first report about a defense response signaling pathway studied in strawberry plants.

1. Introduction

Plants are constantly in contact with potential pathogens; however, disease development rarely occurs. This is because plants have various preformed and inducible defense mechanisms to protect themselves against the attack of pathogens. Such defense mechanisms include many alterations in the plant cell, namely: change in ion fluxes across to plasmatic membrane, oxidative burst, cytoplasmic accumulation of calcium, phosphorylation and dephosphorylation of proteins, synthesis of vegetal hormones (*i.e.* salicylic acid, ethylene, jasmonic acid), cell wall reinforcement, and synthesis of antimicrobial metabolites. Additionally, the accumulation of pathogenesis-related proteins (PR proteins) and other peptides also occur, establishing an effective plant immunity [1–3].

The success of the plant induced defense mechanisms depends on the suitable and early recognition of the pathogen and the activation of downstream signals that would lead to disease resistance. In an incompatible interaction, in which no disease occurs, a specific pathogen race called 'avirulent' is rapidly recognized by a specific plant cultivar (resistant host). On the contrary, compatible interaction occurs when a virulent pathogen is not recognized by the plant and the defense response is insufficient or critically delayed to halt the infection. In these cases the host plant is susceptible and will be infected and diseased.

It is well known that salicylic acid (SA) plays an important role in plant defense. Participates on the establishment of the basal resistance against pathogens as well as in the activation of the systemic acquired resistance (SAR) which confers a systemic defense against a broad-spectrum of pathogens [4,5]. The involvement of SA as a key signal in plant defense mechanisms was known from early reports. Applications of SA analogs on tobacco plants induced resistance to *Tobacco Mosaic Virus* or TMV [6]. Other studies published later showed an increase of SA levels took place in a TMV resistant tobacco variety upon infection with this virus [7]. The accumulation of SA was also reported in the resistant cultivar

Abbreviations: SA, salicylic acid; M23, isolate of *Colletotrichum fragariae*; M11, isolate of *Colletotrichum acutatum*; hpi, hours post inoculation; *PR-1*, pathogenesis-related gene 1.

* Corresponding author. Tel.: +54 381 424 8921; fax: +54 381 424 8921x37.
E-mail address: juan@fbqf.unt.edu.ar (J.C. Díaz-Ricci).

0981-9428/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.plaphy.2012.01.019

SCM334 of *Capsicum annuum* upon infection with *Phytophthora capcisi* [8], in *Arabidopsis thaliana* challenged with the avirulent isolate EMWA of *Peronospora parasitica* [9], and in the resistant cv. Kinchaku of pear plants infected with *Venturia nashicola* [10]. In contrast, a smaller and delayed SA accumulation was reported in compatible interactions between the isolate NOCO of *P. parasitica* with *Arabidopsis*, and *V. nashicola* with suceptible pear cultivar Kousui [9,10].

Additional evidence of the participation of SA on defense mechanisms came from studies with transgenic tobacco and *Arabidopsis* plants that expressed the *nahG* gene of *Pseudomonas putida*, which converts SA to catechol. These plants were unable to accumulate SA, to induce *PR* genes expression and the resistance to avirulent pathogens was compromised [11,12]. In cucumber and tobacco, it has also been reported the increased of SA levels in the phloem sap during the SAR activation [13–15]. Other studies have shown that SA synthesized in the pathogen inoculated leaf was transported to the un-inoculated regions of the plant [16,17]. However, despite SA being capable of moving from challenged to the uninfected tissues, experiments with graft involving rootstocks from *nahG* transgenic plants suggested that SA is not the moving signal required for SAR establishment [18]. In addition, Maldonado et al. [19] described a mutant of *Arabidopsis* (*dir1*) defective in the ability to deploy SAR upon infection with the avirulent pathogen *Pseudomonas syringae*. The mutation occurred in a lipid transfer protein (LTP) found in petiole exudates, suggesting a key role of this protein in SAR systemic signaling. However, although SA may not be the moving signal, it is required for the establishment of SAR in the whole plant.

SA and functional analogs such as 2,6-dichloroisonicotinic acid (INA) and *S*-methyl 1,2,3-benzothiadiazole-7-carbothioate ester (BTH), also induce defense responses in either experimental model and plants with agronomical interest. It has been reported that INA and BTH can protect wheat from powdery mildew caused by *Erysiphe graminis* f sp. *tritici* and from leaf rust caused by *Puccinia recondita* [20]. Likewise, it has been demonstrated that BTH protects maize from *Peronosclerospora sorghi*, the causal agent of downy mildew [21], while INA activates defense gene expression and resistance to powdery mildew in barley [22]. Homologs of NPR1, an important component of SA pathways, has been identified in rice, tobacco, tomato, apple, wheat and orange [4], suggesting conservation of these pathway between dicots and monocots.

On the other hand, plant PR proteins have been defined as host proteins that are induced, either local and systemically, in response to a pathogen attack, avirulent or virulent [23]. Plants are able to regulate the expression of specific PR genes depending on the challenging pathogen. It is well known that the expression of *PR-1* gene is closely associated with SA signaling pathways in several plant species [24,25]; for this reason PR genes are routinely used as a suitable reporter of SA mediated defense responses.

Although the literature about induction of disease resistance in plants is abundant, limited evidence about strawberry plants is available. It has been reported that the application of BTH in strawberry plants induced resistance to *Phytophthora cactorum* and *Phytophthora fragariae* [26], depending on concentration and time between treatment and inoculation. Chitosan treatment on strawberry plants also induced resistance to *P. cactorum* [26] and to *Botrytis cinerea* [27] in dose and time dependence. Hukkanen et al. [28] have demonstrated that BTH induced accumulation of phenolic compounds and improved resistance to powdery mildew in strawberry plants. A recent comprehensive review about strawberry plant defense mechanism includes many molecular aspects but none is about signaling pathways [29]. Also, few molecular studies are available about strawberry plant gene expressions in response to *Colletotrichum* spp. infection [30,31]. Results about biochemical and morphological changes in the cv. Pájaro of strawberry plants infected with *Colletotrichum* strains were presented by Salazar et al. [32]. Furthermore, although it has been reported that certain *Colletotrichum* strains can induce a defense response in strawberry [32,33], nevertheless, the mechanisms involved in such defense response remains poorly studied.

Results presented in this paper demonstrate a strong involvement of SA on the development of the defense response when strawberry plants are exposed to the avirulent isolate M23 of *Colletotrichum fragariae*. Evidence is also presented suggesting that a time dependent gene expression program is triggered upon a peak accumulation of SA.

2. Results

2.1. Phytopathogenicity tests

In Fig. 1 we present the results obtained of phytopathogenicity tests carried out with strawberry plants of the cultivars Pájaro and Chandler when inoculated with the isolate M23 or M11. Outcomes show that the cv Pájaro exhibited a highly resistant phenotype (DSR = 1) when inoculated with the isolate M23, whereas highly susceptible (DSR = 5) when inoculated with the isolate M11. Control experiments consisted in plants of the cv. Pájaro treated with water (Fig. 1, C1) and plants of the cv. Chandler treated with the isolate M23 (Fig. 1, C2). The latter was carried out to test the pathogenic property of the isolate M23 with a susceptible cultivar. Results showed that the isolate M23 behaves as highly virulent with the cv. Chandler (DSR = 5, Fig. 1, C2). These results highlight the specificity in the interactions among a particular isolate and two different cultivars of the same plant species.

2.2. Pathogen induced oxidative burst

With the aim to corroborate the induction of defense mechanism, the accumulation of reactive oxygen species in strawberry leaves challenged with M23 or M11 pathogen was evaluated. Leaflets were sampled from plants at 6 h post inoculation (hpi). As shown in Fig. 2, the avirulent pathogen M23 induced a strong accumulation of superoxide radicals and hydrogen peroxide in leaves tissues, visualized as blue deposits and green spots, respectively. Further experiments revealed that such accumulation was transient decreasing up to the level of not treated plants after

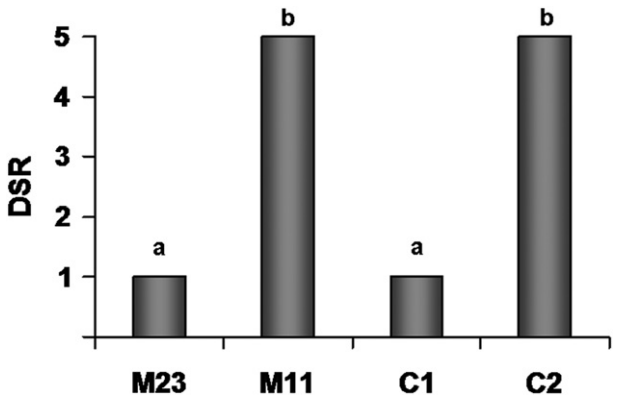

Fig. 1. Disease severity rating (DSR) of the cv Pájaro when inoculated with the isolates M23 of *C. fragariae* (M23) and M11 of *C. acutatum* (M11). C1 corresponds to cv. Pájaro treated with water, and C2 to cv. Chandler treated with the isolate M23. DSR values were evaluated 30 days after inoculation. Bars represent the average values of three independent experimental replicates ($n = 6$). DSR values with different letters correspond to statistically different (Fisher test, $p \leq 0.05$).

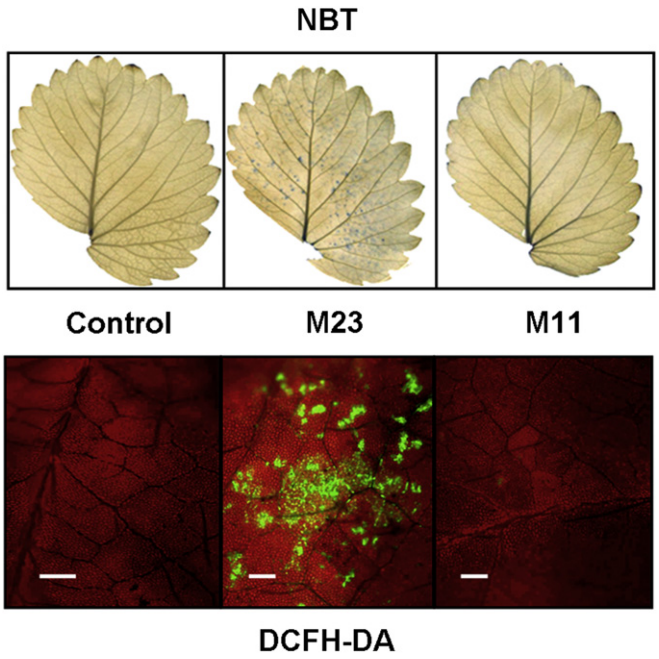

Fig. 2. Reactive oxygen species induced after different treatments. Six hours after M23, M11 or SDW (control) inoculation, plant leaves were harvested and stained with NBT or DCFH-DA to visualize superoxide radicals (blue deposit) and hydrogen peroxide (green spots) production, respectively. DCFH-DA stained leaves were observed by fluorescence microscopy. Scale bar represent 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

12 h (not shown). The oxidative burst observed suggests that the host plant can recognize M23 and activate defense signals. On the other hand, leaves of plants inoculated with the virulent isolate M11 or with water (control) showed no accumulation of reactive oxygen species, implying that a defense mechanism was not triggered.

2.3. SA accumulation in infected strawberry plants

In Fig. 3 the time course of SA level in petiole sap of plants inoculated with the isolate M23 of *C. fragariae* or with the isolate M11 of *Colletotrichum acutatum* relative to water treated plants is presented. Results show that SA content increased after inoculation with M23, reaching to maximum at 48 hpi, where the SA content was up to 2 fold with respect to water treated (control) plants. This time course study also showed that after 48 hpi the level of SA decreased reaching to same levels of control plants 96 hpi (Fig. 3). However, in M11 inoculated plants, a clear accumulation of SA was not observed. It should be noted that disease symptoms associated to M11 infection began to appear between 72 and 96 hpi whereas no symptom was observed in M23 infected plants. Accordingly, these results strongly suggested that a time dependent SA mediated defense mechanism was taking place in strawberry plants treated with avirulent isolate M23 in contrast those treated with the virulent isolate M11.

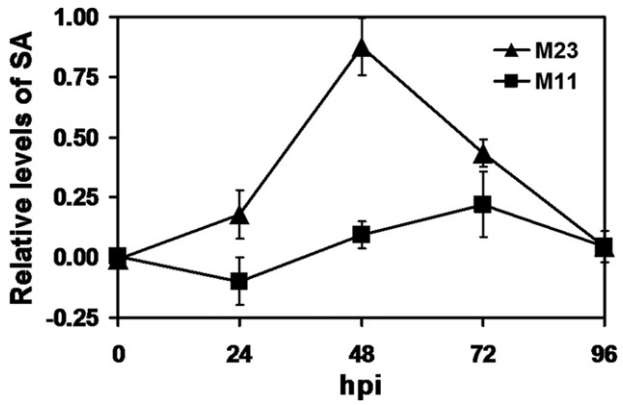

Fig. 3. Time course change of SA content in strawberry plants inoculated with M23 or M11 fungal pathogens. Results are expressed as the ratio of SA content of inoculated plants with respect to control (not inoculated) plants, at different hours post inoculation (hpi). Values represent the average of three independent experimental replicates ($n = 6$) with standard deviation (σ_{n-1}).

2.4. Expression analysis of PR-1 gene

In this study the specific sequence of *Fragaria x ananassa PR-1* gene was used. As shown in Fig. 4A, the analysis of *FaPR-1* expression was performed in strawberry plants at 24, 48, 72 and 96 hpi with M23 or M11, respectively. Results showed that *FaPR-1* was clearly induced during the first 48 h after M23 inoculation respect to water treated plants, being abruptly repressed at 72 and 96 hpi. The latter can be better visualised in Fig. 4B where the expression to each timepoint and pathogen assayed was referred to the respective water treated control.

On the other hand, the expression of *FaPR-1* in plants treated with M11 showed no change during the first 24 hpi being repressed after that time (Fig. 4B).

Since the highest expression of *FaPR-1* was observed at 48 hpi coinciding the maximal accumulation of SA (Fig. 3), the expression of *FaPR-1* in plants treated with exogenously applied SA was investigated. Results revealed an evident induction of the *FaPR-1* gene 72 h after SA treatment (Fig. 4A). Interestingly, the level of expression of *FaPR-1* showed no difference at 48 h after treatment with SA exogenous. These results suggested that at least 72 h are required to establish a response to exogenously applied SA in strawberry plants.

2.5. SA induced protection

Exogenously applied SA treatments on strawberry plants at different time prior to M11 inoculation, were performed to evaluate the effect of SA induced (protection) defense response over the time. Results presented in Fig. 5A show that when the time elapsed between the SA treatment and the inoculation with M11 increased, the DSR decreased. Symptom severity at 72 and 96 h were statistically less than DSR values determined at 0 h and methanol treated plants used as control. SA treated and inoculated after 0–48 h, and controls plants were severely affected or dead (DSR gt; 4.5). In contrast, plants inoculated 72 or 96 h after SA treatment recovered and produced new and healthy leaves beyond the 30 days post M11 inoculation (data not shown).

These results highlights the importance of time elapsed between the defense induction and the challenge with the pathogen for the full activation of the defense mechanisms.

2.6. Inhibition of M11 growth mediated by SA

In order to investigate whether the plant protection effect observed was due to a growth inhibition effect of SA over M11 or an effective defense response induced by SA, *in vitro* growth inhibition experiment was performed. Different concentrations of SA solutions were loaded in wells made in soft agar containing M11 conidia. Results showed that SA at 5 mM did not inhibit the M11 growth whereas at 50 mM the halo was barely perceptible and at

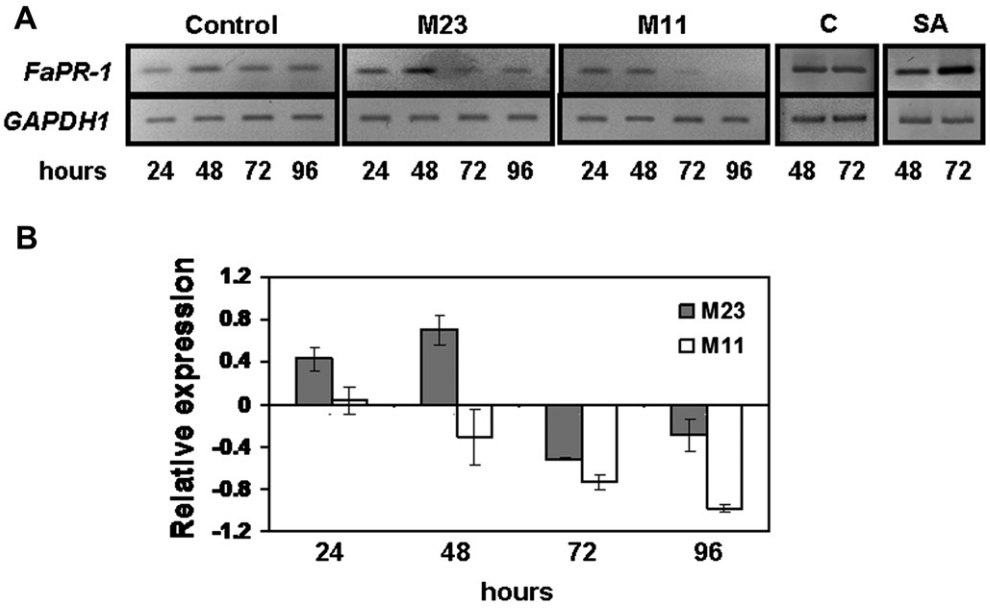

Fig. 4. Time course of *FaPR-1* gene expression in response to fungal inoculations or to SA treatment. (A) Semi-quantitative RT-PCR assay on leaves sampled at indicated hours after inoculation with M23, M11 or treatment with SA. *GAPDH1* gene was used as loading control. (B) Relative expression of *FaPR-1* gene after M23 or M11 inoculation, calculated as the ratio of *FaPR-1* expression change in inoculated with respect to control plants. Control and C denote plants treated with SDW or 30% methanol (SA solvent), respectively. Results correspond to average values coming from four replicates and two independent experiments ($n = 6$) with standard deviation (σ_{n-1}).

500 mM the halo indicates a strong inhibitory effect (Fig. 5B). These results let us to rule out that the effect observed in the plant protection experiments shown in Fig. 5A is due to a direct effect of SA on M11 growth.

3. Discussion

Many interactions between strawberry cultivars and pathogens of *Colletotrichum* genus have been reported [30–32]. Salazar et al. [32] presented a strong evidence of the induction of a defense response in strawberry plants mediated by the avirulent isolate of *C. fragariae* and effective against the virulent pathogen *C. acutatum* isolate M11. Interestingly, these authors also showed that the time elapsed between the first inoculation with the avirulent pathogen and the second inoculation with the virulent one was crucial to inhibit the growth of the latter. They observed that a complete protection was achieved when the time interval between inoculations was 96 h or more. However, the mechanism involved in strawberry resistance to these fungal pathogens was not investigated.

Many works have shown that SA and its functional analogs, *e.g.* INA and BTH, induce defense responses against pathogens in different plant species including wheat, maize, barley, among others [20–22]. All this information confirmed the key role assigned previously to SA as signal of acquired resistance in plants.

Our results showed that strawberry plants inoculated with avirulent pathogen M23 exhibited a notorious accumulation peak of SA in petiole sap 48 h after inoculation whereas no accumulation was observed when inoculated with M11. This behavior strongly suggested that SA also played an important role on signaling the defense response in strawberry. The latter was further confirmed with the results obtained of the expression of *PR1* gene. However, if we compare the time required for conidia germination [32] and oxidative burst we would observe that the peak of SA showed up

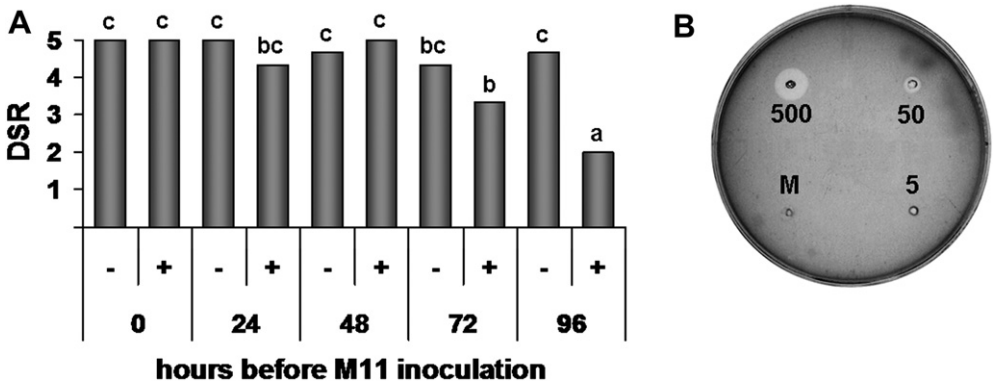

Fig. 5. Effect of SA on the susceptibility of strawberry plants and on the growth of the virulent isolate M11. (A) Disease severity rating (DSR) of strawberry plants treated (+) or not treated (−) with SA (5 mM) 30 days after the inoculation with M11. Plants were treated with SA or with 30% methanol (control) 0, 24, 48, 72 and 96 h before the inoculation with M11. (B) Effect of different concentration of SA (5, 50, 500 mM) in 30% methanol on the growth of M11. M, denotes a control sample containing only 30% methanol. Bars represent the average values of three independent experimental replicates ($n = 6$). Different letters on bars represent statistically different values (Fisher test, $p \leq 0.05$).

much later, indicating that the SA signaling is downstream of the pathway but required the former signals.

Increase of SA levels were also found in the phloem sap of cucumber plants infected with *Colletotrichum lagenarium* [13], *P. syringae* [14] and in TMV infected tobacco [15], and these events preceded or paralleled an increase of *PR* genes expression and SAR development. It has been earlier reported that the SA produced after the infection was transported from the challenged leaf to distal leaves before appearance of systemic resistance [16,17]. Results presented by Darby et al. [34] using transgenic tobacco plants expressing the SA hydroxylase enzyme (*nahG*) targeted to the phloem space caused the depletion of SA at the phloem and the suppression of SAR. Accordingly, the increase of SA observed in petiole sap of the cv Pájaro of strawberry inoculated with the isolate M23 would also suggest the SA involvement as a systemic signal in mechanisms of disease resistance.

Conversely, although we have no insight about why M11 inoculated plants did not accumulate SA, results were coherent with the idea that SA accumulation is required to induce a defense response in strawberry plants. Incidentally, we could speculate that the failure to accumulate SA may be due to a virulence factor produced by the virulent strain M11 with the aim to overcome the plant defense response. It should be noted that the symptoms of M11 infection appear between 72 and 96 hpi.

In addition, results obtained about reactive oxygen species accumulation would bring a strong support to the idea that the defense mechanism was triggered upon M23 inoculation. Moreover, the fact that an oxidative burst was not observed in M11 challenged strawberries would bring further support to the hypothesis about the participation of an M11 derived virulence factors to suppress plant defenses.

Many studies have demonstrated that the expression of *PR-1*, *PR-2*, *PR-5* and other defense genes are associated to the SA signaling pathway [11,12]. However, it was later reported that *PR-2* and *PR-5* induction may not be necessarily associated to a peak accumulation of SA [35]. Results shown here provide a clear evidence that in strawberry the accumulation of SA triggered the expression of *PR-1* and both events are required for the full establishment of a defense response and plant protection. Furthermore, these outcomes strongly suggest that M23 is the responsible of the activation a SA dependent defense mechanism. Also, since strawberry plants inoculated with the virulent isolate M11 exhibited neither accumulation of SA nor induction of *PR-1*; highlights the SA involvement in strawberry defense mechanisms.

Another interesting result obtained is that the expression of the *FaPR-1* gene is downregulated after 48 h regardless the isolate used for inoculation, suggesting that a time dependent regulatory process is taking place. Whether this phenomenon is associated or not to the decrease of SA observed after 48 hpi is something that we cannot conclude from these results. However, since both events take place precisely at 48 hpi we may speculate that this behavior may be due to the activation of other signals. Jasmotes and ethylene are well known to exert antagonistic effects on SA signaling pathway [36], nevertheless more specific studies are required to understand this issue.

Faize et al. [10] also found a *PR-1* gene induction associated to increased SA levels in the resistant Kinchaku cultivar of pear plants upon infection with *V. nashicola*, however a peak accumulation was not clearly observed. They also reported that SA accumulation was notably delayed in the susceptible Kousui pear cultivar. These authors proposed that the SA would be an endogenous inducer factor of pear plant resistance to *V. nashicola*. They also suggested that as the increase of SA levels and the expression of *PR-1* gene in Kinchaku cultivar are positively correlated, the resistance attained would be SA dependent.

The fact that strawberry plants treated with exogenous SA exhibited a clear induction of *FaPR-1* gene, confirmed that its expression strongly responds to SA signaling. Exogenously applied SA also showed that at least 72 h are required to establish a clear protection against M11 infection. These results provide additional evidence that strawberry plants have a SA dependent defense mechanism effective against M11 infection. It should be noted nevertheless, that *FaPR-1* gene was not induced until 72 h after exogenous SA treatment, exposing clearly the importance of the time required to launch an effective SA induced protection. Similar results were obtained by Eikemo et al. [26] when inducing strawberry resistance to *P. cactorum* and *P. fragariae* pathogens by BTH application; although a gene expression study was not reported. More experiments would be necessary to elucidate whether this time is due to a slow translocation of exogenous SA into plant tissues or to a peculiar kinetic property of the signaling pathway that requires few days to activate and to achieve a full protection.

Finally, results obtained with the growth inhibition assay let us to discard that SA exerts a direct toxic effect on M11 isolate, supporting the idea that SA acts as inductor of defense against M11 infection in strawberry plants rather than an inhibitor.

In summary, results presented in this paper provide strong evidence that strawberry plants have a SA signaling associated defense mechanism that can be activated by the avirulent M23 isolate of *C. fragariae*. Also, the SA associated defense response is effective against a causal agent of anthracnose produced by the isolate M11 of *C. acutatum*, and the protection effect requires many days to be fully effective in strawberry plants. In contrast, the virulent isolate M11 would delay or suppress the activation of SA dependent defense response, bringing about plant susceptibility.

4. Methods

4.1. Plant materials, fungal cultures and inoculation conditions

Strawberry plants (*Fragaria x ananassa*) of the cv. Pájaro and cv. Chandler used in experiments and were kindly provided by the strawberry BGA (Banco de Germoplasma Activo at University of Tucumán, Tucumán, Argentina). Healthy plantlets were obtained from *in vitro* cultures and rooted in pots with sterilized substrate (humus: perlome, 2:1). Before using in experiments, plants were maintained for 14 or 16 weeks in growing cabinets at 28 °C, 70% RH with a light cycle of 16 h per day (450 μmol m^{-2} s^{-1}) and watered every other day with 50 ml of distilled water. All senescent leaves and petioles were removed periodically until 10 days before the inoculation experiments, leaving only three to four young healthy leaves. Plants of the cultivar Chandler were only used to test the pathogenicity of the *C. fragariae* isolate M23. This cultivar was previously characterized as susceptible to M23.

Two locally purified and characterized isolates of *Colletotrichum* fungi were used in this work: the isolate M23 of *C. fragariae* and isolate M11 of *C. acutatum*. Strains were propagated on Potato Dextrose Agar (Sigma–Aldrich, St. Louis, U.S.A.) supplemented with streptomycin (300 μg ml^{-1}) and maintained at 28 °C under continuous white fluorescent light (200 μmol m^{-2} s^{-1}) until the medium was completely covered. Then the culture surface was gently scraped with a sterile scalpel to remove conidia and suspended in sterile distilled water (SDW). The conidial suspension obtained was filtered through sterile gauze to remove mycelial debris under axenic conditions. Suspension was then diluted with SDW (containing 0.01% Tween 20) to a final concentration of 1.5×10^6 conidia ml^{-1}, and applied to plants as a spray to run-off [32]. Immediately after inoculation, plants were placed at 100% relative humidity (RH), 28 °C and for 48 h in infection chambers, under a light cycle of 16 h day^{-1} (white fluorescent,

450 μmol m^{-2} s^{-1}). Then plants were moved to a growth chamber at 70% RH, 28 °C under same light cycle. Plants sprayed with SDW containing 0.01% Tween 20 and incubated under identical conditions were used as control.

4.2. Histochemical detection of reactive oxygen species

Accumulation of superoxide radicals and hydrogen peroxide was evaluated in leaves tissues from pathogen inoculated strawberry plants. Leaflets were sampled 6 h after M23, M11 or SDW inoculation and stained with Nitro Blue Tetrazolium (NBT, Sigma–Aldrich, St. Louis, U.S.A.) or 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Invitrogen, Carlsbad, CA, U.S.A.) probe. NBT staining was used to detect *in situ* superoxide anion and was carried out according to Grellet–Bournonville et al. [37]. Briefly, detached leaflets were immersed in 50 mM potassium phosphate buffer (pH 7.8) containing 0.1% NBT and 10 mM sodium azide, vacuum infiltrated (0.8 bar) during 2 min and incubated for 2 h in the dark (without vacuum). Finally leaves were immersed in 96% (v/v) ethanol to completely eliminate the chlorophyll. Superoxide production was visualized as blue formazan deposit within leaflet tissues.

Hydrogen peroxide was detected using DCFH-DA probe according to Boszó et al. [38]. Detached leaflets were immersed and vacuum infiltrated (see before) in freshly solution of 50 μM DCFH-DA in 10 mM sodium phosphate buffer (pH 7.4), and incubated 15 min in dark without vacuum. Leaves were then mounted and observed under Olympus System Microscope model BX51 equipped with U-LH100HG reflected fluorescence system, setting blue excitation filter (U-MWB2). Images were captured by Olympus Video/Photo Adapter (Olympus, Hamburg, Germany).

4.3. SA treatment, protection assay and in vitro effect on M11 growth

Plants were sprayed to run-off with 5 mM SA (Fluka, Lausanne, Switzerland) in 30% methanol and maintained in a grow chamber as mentioned above. Plants treatment with 30% methanol and incubated under the same conditions were used as control. Preliminary experiments in our laboratory indicate that the 5 mM SA treatment can protect strawberry plants against M11 infection, without producing phytotoxic effects.

Protection assays were performed in plants infected with M11, previously treated with SA 0, 24, 48, 72 and 96 h before inoculation. SA treatments were carried out under the same conditions as described above. SA-induced protection was evaluated by determining the disease severe rating (DSR) 30 days after M11 inoculation, according to the scale proposed by Delp and Milholland [39]. Control plants were treated with 30% methanol under the same conditions and evaluated at the same times as mentioned above.

Inhibitory effects of SA on M11 growth was assessed on Petri dishes. 5 ml melted soft agar (0.6% in water) containing 1.5×10^6 conidia ml^{-1} was overlaid onto plates of PDA medium, supplemented with streptomycin (300 μg ml^{-1}). 0.3 cm diameter wells performed on soft agar surface under axenic conditions were loaded with 10 μL of SA standard solutions (5, 50, 500 mM) diluted in 30% methanol. Plates were then incubated at 28 °C for 24 h under continuous white fluorescent light (200 μmol m^{-2} s^{-1}). Inhibition was visualized as growth fungus inhibition hales on soft agar. Control well was loaded with 10 μL of 30% methanol.

4.4. Semi-quantitative RT-PCR

Total RNA were obtained from leaves of strawberry plants at 24, 48, 72 and 96 h after inoculation with M23 or M11 pathogens, as well as from plants at 48 and 72 h after SA treatment [40]. Retrotranscription reactions were carried out using 5 μg of DNase-treated total RNA, following prescriptions suggested by SuperScript II RT protocol (Invitrogen, Carlsbad, CA, U.S.A.). RNA quantification was performed by spectrophotometry at 230, 260 and 280 nm. The semi-quantitative RT-PCR method was used to evaluate the expression of *FaPR-1* gene (GenBank ID: AB462752). The gene *GAPDH1* (GenBank ID: AF421492) was used as control to adjust the amount of cDNA in each treatment and to attain the same exponential phase PCR signal strength.

Specific primers used were: GAPDH1 (forward), 5′-CTACAGCAACACAGAAAACAG-3′; GAPDH1 (reverse), 5′-AACTAAGTGCTAATCCAGCC-3′; *FaPR-1* (forward), 5′-TGCTAATTCACATTATGGCG-3′; and a *FaPR-1* (reverse), 5′-GTTAGAGTTGTAATTATAGTAGG-3′. The PCR program used was: 94 °C for 7 min (initial step); cycles (see below) of 94 °C for 45 s, different annealing temperature (see below) for 1 min, 72 °C for 1.5 min, and a final extension of 72 °C for 10 min. The number of cycles used for each gene was adjusted to obtain the specific band amplified at the exponential phase of PCR reaction. Annealing temperatures for *FaPR-1* and *GAPDH1* genes were 57 °C and 52 °C, respectively. Amplified bands were visualized with agarose gel (2%) ethidium bromide-stained (10 μg ml^{-1}) and photographed under UV light with a digital camera. band observed were digitalized and quantified using Total Lab Quant software (Nonlinear Dynamics Ltd., Newcastle, UK). Relative expression of studied genes was calculated as the ratio of band strength in inoculated/treated leaves respect to control leaves. To ensure the absence of genomic DNA in each cDNA sample, *GAPDH1* primer sequences were designed to enclose an intronic region. Plants treated with SDW or 30% methanol and incubated under the conditions above mentioned and assayed at each time were used as control.

4.5. Measurement of endogenous SA

Content of SA in inoculated plants with M23 or M11 isolates was measured at 0, 12, 24, 48, 72 and 96 h post inoculation. Plants treated with SDW plus 0.01% Tween 20 subject to same conditions, were used as non infected control plants. Petiole sap was obtained from the excised petiole by extruding the fluid applying a slight pressure at the basal end, and collecting the sap using a micropipette. After collecting the drop (50 μL), this end was cut to avoid tissue damage and vascular sealing by callose deposition, and the procedure repeated along of the petiole. Collected sap were immediately dispensed into 1 ml of ice-cold ethanol absolute, acidified with HCl to pH 3.0. Extracts were pooled and the precipitate removed by centrifugation at 10,000 × g for 15 min. The supernatant was transferred to a new tube, completely dried under vacuum (Speed Vac Plus SC110A, SAVANT, Hobrook, New York, U.S.A.) and the dried weight of each sample was determined. Sample was then dissolved in 1 ml of 30% methanol. SA was separated by high performance liquid chromatography (Analytical HPLC System, Gilson, Middleton, Idaho, U.S.A.) using a C18 reversed phase column, Prodigy 5 ODS-2 (Phenomenex, Torrance, U.S.A.), with a gradient of water with 0.1% trifluoroacetic acid (solvent A) and methanol with 0.1% trifluoroacetic acid (solvent B). The gradient was performed from 30% to 100% of B in 20 min and then maintained for 10 min. SA contained in fractions was detected at 302 nm and collected for fluorometetric determination at $\lambda ex = 296$ nm and $\lambda em = 408$ nm using a spectrofluorometer (Photon Counting PC1, ISS, Owingen, Germany). SA content in each sample was calculated from fluorescence units of standard solutions subject to HPLC and fluorometric analysis. The latter allowed us to estimate the losses during the manipulation of samples. The content of SA were determined as μg mg^{-1} sap dried weight, and expressed as the change of the amount of SA (ΔSA) with respect to

the amount of SA detected in water treated (control) plants, divided by the SA content in this control plants.

4.6. Statistical analyses of data

The experimental design was randomized with 12 plants per experimental unit, with 6 plants corresponding to the inoculated and 6 to the control plants (see above). Experimental data obtained from phytopathological tests were analyzed with the Statistix software (Analytical Software, Tallahassee, U.S.A.). Analysis of variance (ANOVA) was used to evaluate the statistic significance of treatments by LSD test. Experiments were repeated three times to diminish the dispersion of DSR values.

Acknowledgments

This work was partially supported by the Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT, 26/D423), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 6441) and Agencia Nacional de Promoción Científica y Tecnológica (PICT 08-2105). CFGB, MGMZ are fellow and JCDR and APC are members of CONICET.

References

[1] J.M. McDowell, J.L. Dangl, Signal transduction in the plant immune response, Trends Biochem. Sci. 25 (2000) 79–82.
[2] M. Odjakova, C. Hadjiivanova, The complexity of pathogen defense in plants, Bulg. J. Plant Physiol. 27 (2001) 101–109.
[3] C.M.J. Pieterse, A. Leon-Reyes, A. Van der Ent, S.C.M. Van Wees, Networking by small-molecule hormones in plant immunity, Nat. Chem. Biol. 5 (2009) 308–316.
[4] R. Chaturvedi, J. Shah, Salicylic acid in plant disease resistance, in: S. Hayat, A. Ahmad (Eds.), Salicylic Acid-A Plant Hormone, Springer, Dordrecht, The Netherlands, 2006, pp. 335–370.
[5] C. An, Z. Mou, Salicylic acid and its function in plant immunity, J. Integr. Plant Biol. 53 (2011) 412–428.
[6] R.F. White, Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco, Virology 99 (1979) 410–412.
[7] J. Malamy, J.P. Carr, D.F. Klessig, I. Raskin, Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection, Science 250 (1990) 1002–1004.
[8] M. Ueeda, M. Kubota, K. Nishi, Contribution of jasmonic acid to resistance against *Phytophthora* blight in *Capsicum annuum* cv. SCM334, Physiol. Mol. Plant P 67 (2006) 149–154.
[9] B. Mauch-Mani, A.J. Slusarenko, Production of salicylic acid precursors is a major function of phenylalanine ammonia–lyase in the resistance of Arabidopsis to *Peronospora parasitica*, Plant Cell 8 (1996) 203–212.
[10] M. Faize, L. Faize, M. Ishizaka, H. Ishii, Expression of potential defense responses of Asian and European pears to infection with *Venturia nashicola*, Physiol. Mol. Plant P 64 (2004) 319–330.
[11] T. Gaffney, L. Friedrich, B. Vernooij, D. Negrotto, G. Nye, S. Uknes, E. Ward, H. Kessmann, J. Ryals, Requirement of salicylic acid for the induction of systemic acquired resistance, Science 261 (1993) 754–756.
[12] T.P. Delaney, S. Uknes, B. Vernooij, L. Friedrich, K. Weymann, D. Negrotto, T. Gaffney, M. Gut-Rella, H. Kessmann, E. Ward, J. Ryals, A central role of salicylic acid in plant disease resistance, Science 266 (1994) 1247–1250.
[13] J.P. Métraux, H. Signer, J. Ryals, E. Ward, M. Wyss-Benz, J. Gaudin, K. Raschdorf, E. Schmid, W. Blum, B. Inverardi, Increase in salicylic acid at the onset of systemic acquired resistance in cucumber, Science 250 (1990) 1004–1006.
[14] J.B. Rasmussen, R. Hammerschmidt, M.N. Zook, Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv *syringae*, Plant Physiol. 97 (1991) 1342–1347.
[15] N. Yalpani, P. Silverman, T.M.A. Wilson, D.A. Kleier, I. Raskin, Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco, Plant Cell 3 (1991) 809–818.
[16] V. Shulaev, J. Léon, I. Raskin, Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? Plant Cell 7 (1995) 1691–1701.
[17] W. Mölders, A. Buchala, J.P. Métraux, Transport of salicylic acid in tobacco necrosis virus-infected cucumber plants, Plant Physiol. 112 (1996) 787–792.
[18] B. Vernooij, L. Friedrich, A. Morse, R. Reist, R. Kolditz-Jawhar, E. Ward, S. Uknes, H. Kessmann, J. Ryals, Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction, Plant Cell 6 (1994) 959–965.
[19] A.M. Maldonado, P. Doerner, R. Dixon, C.J. Lamb, R.K. Cameron, A putative lipid transfer protein involved in systemic resistance signaling in Arabidopsis, Nature 419 (2002) 399–403.
[20] J. Gorlach, S. Volrath, G. Knauf-Beiter, G. Hengy, U. Beckhove, K.H. Kogel, M. Oostendorop, T. Staub, E. Ward, H. Kessmann, J. Ryals, Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat, Plant Cell 8 (1996) 629–643.
[21] S.W. Morris, B. Vernooij, S. Titatarn, M. Starrett, S. Thomas, C.C. Wiltse, R.A. Frederiksen, A. Bhandhufalck, S. Hulbert, S. Uknes, Induced resistance responses in maize, Mol. Plant-Microbe Interact. 11 (1998) 643–658.
[22] K.H. Kogel, U. Beckhove, J. Dreschers, S. Münch, Y. Rommé, Acquired resistance in barley, Plant Physiol. 106 (1994) 1269–1277.
[23] L.C. Van Loon, E.A. Van Serien, The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins, Physiol. Mol. Plant P 55 (1999) 85–97.
[24] J.F. Antoniw, W.S. Pierpoint, Purification of a tobacco leaf protein associated with resistance to virus infection, Biochem. Soc. Trans. 6 (1978) 248–250.
[25] K.A. Lawton, K. Weymann, L. Friedrich, B. Vernooij, S. Uknes, J. Ryals, Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene, Mol. Plant-Microbe Interact 8 (1995) 863–870.
[26] H. Eikemo, A. Stensvand, A.M. Tronsmo, Induced resistance as a possible means to control diseases of strawberry caused by *Phytophthora* spp, Plant Dis. 87 (2003) 345–350.
[27] M.V.B. Reddy, K. Belkacemi, R. Corcuff, F. Castaigne, J. Arul, Effect of pre-harvest chitosan sprays on post-harvest infection by *Botrytis cinerea* and quality of strawberry fruit, Postharv. Biol. Technol. 20 (2000) 39–51.
[28] A.T. Hukkanen, H.I. Kokko, A.J. Buchala, G.J. McDougall, D. Stewart, S.O. Kärenlampi, R.O. Karjalainen, Benzothiadiazole induces the accumulation of phenolics and improves resistance to powdery mildew in strawberries, J. Agric. Food Chem. 55 (2007) 1862–1970.
[29] F. Amil-Ruiz, R. Blanco-Portales, J. Muñoz-Blanco, J.L. Caballero, The strawberry plant defence mechanism: a molecular review, Plant Cell Physiol. 52 (2011) 2190–2199.
[30] A. Casado-Díaz, S. Encinas-Villarejo, B.d.L. Santos, E. Schiliró, E.M. Yubero-Serrano, F. Amil-Ruíz, M.I. Pocovi, F. Pliego-Alfaro, G. Dorado, M. Rey, F. Romero, J. Muñoz-Blanco, J.L. Caballero, Analysis of strawberry genes differentially expressed in response to *Colletotrichum* infection, Physiol. Plant 128 (2006) 633–650.
[31] A.A. Khan, D.S. Shih, Molecular cloning, characterization, and expression analysis of two class II chitinase genes from strawberry plant, Plant Sci. 166 (2004) 753–762.
[32] S.M. Salazar, A.P. Castagnaro, M.E. Arias, N.R. Chalfoun, U. Tonello, J.C. Díaz Ricci, Induction of a defense response in strawberry mediated by an avirulent strain of Colletotrichum, Eur. J. Plant Pathol. 117 (2007) 109–122.
[33] N.R. Chalfoun, A.P. Castagnaro, J.C. Díaz-Ricci, Induced resistance activated by a culture filtrate derived from an avirulent pathogen as a mechanism of biological control of anthracnose in strawberry, Biol. Control 58 (2011) 319–329.
[34] R.M. Darby, A. Maddison, L.A.J. Mur, Y.M. Bi, J. Draper, Cell-specific expression of salicylate hydroxylase in an attempt to separate localized HR and systemic signaling establishing SAR in tobacco, Mol. Plant Pathol. 1 (2000) 115–123.
[35] C. Nawrath, J.P. Métraux, Salicylic acid induction deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation, Plant Cell 11 (1999) 1393–1404.
[36] A. Verhage, S.C. van Wees, C.M. Pieterse, Plant immunity: it's the hormones talking, but what do they say? Plant Physiol. 154 (2010) 536–540.
[37] C.F. Grellet-Bournonville, J.C. Díaz-Ricci, Quantitative determination of superoxide in plant leaves using a modified NBT staining method, Phytochem. Anal. 22 (2011) 268–271.
[38] Z. Bozsó, P.G. Ott, A. Szatmári, A. Czelleng, G. Varga, E. Besenyei, É Sárdi, É Bányai, Z. Klement, Early detection of bacterium-induced basal resistance in tobacco leaves with diaminobenzidine and dichlorofluorescein diacetate, J. Phytopathol. 153 (2005) 596–607.
[39] B.R. Delp, R.D. Milholland, Evaluating strawberry plants for resistance to *Colletotrichum fragariae*, Plant Dis. 64 (1980) 1071–1073.
[40] A.B. Iandolino, F. Goes da Silva, H. Lim, H. Choi, L.E. Willams, D.R. Cook, High-quality RNA, cDNA, and derived EST libraries from grapevine (*Vitis vinifera* L.), Plant Mol. Biol. Rep. 22 (2004) 269–278.