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ORIGINAL ARTICLE

Soft mechanical stimulation induces a defense response against *Botrytis cinerea* in strawberry

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Abstract

Key message **Genes associated with plant mechanical stimulation were found in strawberry genome. A soft mechanical stimulation (SMS) induces molecular and biochemical changes in strawberry plants, conferring protection against *Botrytis cinerea*.**

Abstract Plants have the capacity to induce a defense response after exposure to abiotic stresses acquiring resistance towards pathogens. It was reported that when leaves of *Arabidopsis thaliana* were wounded or treated with a soft mechanical stimulation (SMS), they could resist much better the attack of the fungal pathogen *Botrytis cinerea*, and this effect was accompanied by an oxidative burst and the expression of touch-inducible genes (*TCH*). However, no further work was carried out to better characterize the induced defense response. In this paper, we report that *TCH* genes were identified for first time in the genomes of the strawberry species *Fragaria ananassa* (e.g. *FaTCH2, FaTCH3, FaTCH4* and *FaCML39*) and *Fragaria vesca* (e.g. *FvTCH2, FvTCH3, FvTCH4* and *FvCML39*). Phylogenetic studies revealed that *F. ananassa TCH* genes exhibited high similarity with the orthologous of *F. vesca* and lower with *A. thaliana* ones. We also present evidence that after SMS treatment on strawberry leaves, plants activate a rapid oxidative burst, callose deposition, and the up-regulation of *TCH* genes as well as plant defense genes such as *FaPR1, FaCHI2-2, FaCAT, FaACS1* and *FaOGBG-5*. The latter represents the first report showing that *TCH*- and defense-induced genes participate in SMS-induced resistance in plants, bringing a rational explanation why plants exposed to a SMS treatment acquired an enhance resistance toward *B. cinerea*.

Abbreviations

dpi	Days post infection
SMS	Soft mechanical stimulation
dpSMS	Days post SMS treatment
hpSMS	Hours post SMS treatment
mpSMS	Minutes post SMS treatment
ROS	Reactive oxygen species
TCH	Touch-inducible genes

Introduction

Plants have evolved specialized defense mechanisms to cope with biotic and abiotic stresses. Among the most common abiotic stresses should be mentioned: the temperature, irradiation, drought, flooding, salinity and mechanical. Plants have the capacity to sense not only strong but also subtle mechanical stimuli and, depending on the causal agent, a conjunction of biochemical, morphological and physiological responses are triggered. A classic example of a severe stimulus is the wind, which induces hardness and diameter increase of tree trunk (Scurfield

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1973; Wilson and Archer 1977). On the other hand, the mechanical stimulation of a trapped prey in Venus Flytrap (*Dionaea muscipula*) illustrates a delicate stimulus. In this case, the plant is able to count and integrate mechano-electrical signals (Bohm et al. 2016).

In field, plants are naturally exposed to a wide range of mechanical stimuli, such as bending by wind (Anten et al. 2005), brushing or contact among themselves (Markovic et al. 2016), sprinkle water (Braam 2005), rubbing (Saidi et al. 2010; Benikhlef et al. 2013), and others, which ought to be technologically considered in order to take advantage of them. Such a case is reported by Iida (2014) in which Japanese farmers achieved higher yield in wheat and barley crops by treading on them. This practice is known as "Mugifumi" ("Mugi" referred to wheat and barley crops, and "fumi" to the action of tread).

Cross-tolerance is the phenomenon by which plants activate an immune response after exposure to a particular stress and acquire the capacity to tolerate a different one (Foyer et al. 2016). It has been reported that a mechanical damage caused by insect feeding on plant tissue induced a global defense response rendering plants resistant towards pathogens of opposite lifestyle such as necrotrophs like *Botrytis cinerea* (Chassot et al. 2008; García et al. 2015), biotrophs like *Uromyces fabae* (Walters et al. 2006) and hemibiotrophs like *Pseudomonas syringae* (Francia et al. 2007). It was also reported that a delicate stimulus that did not cause cellular damage induced resistance against *B. cinerea* in *Arabidopsis thaliana*. This stimulus is known as soft mechanical stimulation (SMS) and the defense response triggered depends on an initial and rapid calcium influx, transient reactive oxygen species (ROS) generation, and activation of touch-inducible genes (*TCH*) encoding calmodulin and calmodulin-like proteins (Benikhlef et al. 2013). Although studies carried out in the model plant *A. thaliana* help to gain knowledge about SMS-induced resistance in plants, there was no information about whether this induced defense mechanism was shared by other plant species.

Strawberry is a horticultural crop of great importance throughout the world. However, strawberry plants are very susceptible to many pathogens, especially fungi like *B. cinerea* and *Colletotrichum* spp., causal agents of gray mold and anthracnose, respectively, being the most important fungal pathogens of the crop. They are responsible for large economic losses not only in strawberry but also in various other crops (Kim et al. 2006; Salazar et al. 2007; Dean et al. 2012). The fact that application of large amounts of fungicides is the most widespread practice to control fungal infections in this crop led to the urgent necessity to count with a phytosanitary management practice with lesser or even no impact on the environment, human and animal health. In this context, cross-protection induced by a soft mechanical stress becomes an interesting alternative to toxic agrochemicals to control diseases in strawberry.

In the present work, we report for first time that *TCH* genes are present in the genomes of *F. ananassa* and *F. vesca;* and through phylogenetic studies we could establish that they share high similarity, whereas present lower similitude with *A. thaliana* orthologous. We further demonstrate that SMS treatment induces a defense response that was effective against *B. cinerea*, which was characterized by a ROS burst (O_2^- and H_2O_2), cell wall reinforcement by callose depositions, and the expression of *TCH* genes (e.g. *FaTCH2, FaTCH3, FaTCH4*, and *FaCML39*) associated with SMS, and defense-related genes (e.g. *FaPR1, FaCHI2-2, FaCAT, FaACS1* and *FaOGBG-5*).

Materials and methods

Plant material

Plants of *Fragaria ananassa* Duch. cv. Pájaro were *in vitro* propagated at the Strawberry BGA (Banco de Germoplasma Activo at Universidad Nacional de Tucumán, Argentina). Briefly, runner meristems obtained from healthy plants were propagated in MS medium (Murashige and Skoog 1962). Then, plants were transferred to soil substrate and grown and maintained during 14–16 weeks in phytotron under controlled conditions: 28 °C, 70% relative humidity (RH), and 16-h light cycle (350 µmol m^{-2} s^{-1}).

SMS treatment

SMS treatment (soft mechanical stimulation) was performed as described previously (Benikhlef et al. 2013). Briefly, each leaflet of every leaf in a plant was gently rubbed on both sides of the main vein for ten successive times between thumb and forefinger, exerting a slight pressure. For phytopathological assays, plants were inoculated with *B. cinerea* (5×10^4 spores ml^{-1}) 30 min post SMS treatment (mpSMS).

Botrytis cinerea cultures

B. cinerea strain BMM was kindly provided by Brigitte Mauch-Mani (University of Neuchâtel, Switzerland). The fungus was grown for 2 weeks on Difco (USA) potato dextrose agar (PDA) at 22 °C and 16 h light cycle (200 µmol m^{-2} s^{-1}).

Spore suspensions and inoculation

Preparation of spore suspension and infection was performed as previously described by Hael-Conrad et al. (2015). Briefly, a 6 µl droplet of a *B. cinerea* spore suspension

(5×10^4 spores ml^{-1}) was applied on the adaxial side of leaflets next to the central vein in SMS-treated and not treated (control) plants, and were immediately transferred to infection chambers during 48 h at 20 °C, 100% RH and darkness. After this time, plants where transferred to disease evaluation chambers (20 °C, 70% RH and 16-h light cycle with 350 µmol m^{-2} s^{-1}). Gray mold disease symptoms were evaluated at different days post infection (dpi) by measurement of the lesion size (diameter in mm).

Cellular integrity and hyphae staining

Fungal hyphae and dead plant cells were stained by boiling inoculated leaves for 5 min in 0.2% aqueous Evans Blue solution. Stained leaves were cleared in chloral hydrate (2.5 g ml^{-1}) at room temperature by gentle shaking until wash solution was no longer colored. Then leaves were imbibed in glycerol 20% for 1 h and observed under bright field using a Leica DMR microscope. Three leaves (included the three leaflets) of three plants were evaluated ($n = 27$), and the assay was repeated three independent times.

Reactive oxygen species (ROS) detection and quantification

Hydrogen peroxide (H_2O_2) and superoxide ion (O_2^-) were detected in situ by histochemical staining with 3,3′-diaminobenzidine (DAB) (Thordal-Christensen et al. 1997) and nitroblue tetrazolium (NBT) (Doke 1983), respectively. SMS-treated and untreated (control) leaflets were collected 30 mpSMS and stained with fresh solutions of NBT or DAB during 4 or 8 h, respectively. H_2O_2 and O_2^- were also evaluated at 1, 2 and 4 h post SMS (hpSMS). Stained leaflets were cleared in ethanol 96° at room temperature by gentle shaking until no colored solution was observed. Pictures were taken with a digital camera (Panasonic lumix fz70). Brownish or blue deposits, for H_2O_2 or O_2^-, respectively, were quantified with ImageJ version 1.44 (NIH). Three leaves (including the three leaflets) of three plants were used ($n = 27$), and the assay was repeated three independent times.

Intracellular H_2O_2 was also assessed in foliar discs with the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Sigma-Aldrich). Briefly, 0.5 cm diameter leaf discs of SMS-treated and untreated (control) leaflets were punched obtained at 30 mpSMS and placed into a 96-well plate. Discs were previously incubated with 50 µM of H_2DCF-DA (Sigma-Aldrich) in a standard modified W5 buffer (150 mM NaCl, 5 mM KCl, 125 mM $CaCl_2$, 500 mM sucrose and 2 mM MES, pH 5.6) (Fujikawa et al. 2014; Martos et al. 2015). Fluorescence was measured using a spectrofluorometer (Perkin Elmer LS55 UK) at $\lambda_{ex} = 485$ nm, and $\lambda_{em} = 525$ nm. Three leaves (including the three leaflets) of three plants were used ($n = 27$), and the assay was repeated three independent times.

Callose deposition

Callose deposition was evaluated in SMS-treated and untreated (control) strawberry leaves after *B. cinerea* infection. SMS-treated and untreated plants were inoculated 30 mpSMS with *B. cinerea* (5×10^4 spores ml^{-1}) and immediately transferred to infection chambers under controlled conditions as described previously. Callose deposition was analyzed in both SMS-treated and untreated tissue at the site of infection (SI) and the tissue surrounding the SI (TS) at 1 cm radial distance from it, at 2, 4 and 6 dpi by aniline blue staining as described by Hael-Conrad et al. (2017) with minor modifications. Briefly, leaflets were collected and decolorized using a half-diluted solution of ethanol 96% and lactic acid (3:1). The clearing solution was changed several times until no chlorophyll was visualized. Translucent leaflets were re-hydrated in 50% ethanol for 2 h and incubated in water before staining with 0.01% aniline blue (Sigma-Aldrich) in 150 mM K_2HPO_4 (pH 9.5) at room temperature. Samples were mounted on slides with 30% glycerol and examined with a fluorescence microscope (BXS1 U-LH 100HG, Olympus), using a blue excitation filter (U-MWB2, 330–385 nm). Pictures were taken with an Olympus Video/Photo camera attached to the microscope. Three leaves (including the three leaflets) of three plants were used ($n = 27$), and the assay was repeated three independent times.

RNA extraction and qPCR analysis

Total RNA extraction was performed from the youngest totally expanded leaflet from each of 3 control or SMS-treated plants ($n = 3$). Leaflets were harvested 0.5 and 48 hpSMS pooled, weighted, immediately frozen in liquid nitrogen, pulverized and kept at − 80 °C until further use. RNA was extracted from 75 mg of the pool with the RNAqueous-4PCR kit (Ambion), purity and integrity was assessed spectrophotometrically at 230, 260 and 280 nm (Biospec mini, Shimatzu) and by electrophoresis (1% agarose gel), respectively. Before performing reverse transcriptase reaction, RNA (4 µg) was treated with DNaseI (Ambion) to remove possible genomic DNA contamination. Retrotranscription was carried out on cDNA using the SuperScript II Reverse Transcriptase (Invitrogen) according to manufacturer instructions. qPCR reactions were performed using iQ SYBR Green Supermix (Bio-Rad) in a 7500 Real-Time PCR System (Applied Biosystems). Gene expression values from each sample were normalized to the endogenous gene *FaEF-1α* (*Elongation Factor-1a*) previously described as a stable reference gene for *F. ananassa* (Guidarelli et al. 2011; Amil-Ruiz et al. 2013). All primers used in this study

(Table 1) were designed using the software Primer Express (Applied Biosystems). PCR primers efficiency and Ct values were calculated using LinRegPCR software that takes into account fluorescence in the exponential phase of amplification of each real-time PCR reaction (Ramakers et al. 2003). These profiles were estimated in relation to *FaEF-1α* reference gene using fgStatistics software (Di Rienzo 2011), based on previously published algorithms (Pfaffl 2001). Gene expression is reported as the ratio between SMS-treated and untreated expression levels.

For the selected defense-related genes *FaPR1, FaCHI2-2*, and *FaOGBG5*, primers were designed from free access *F. ananassa* nucleotide sequences available in the GenBank at the NCBI web site (https://www.ncbi.nlm.nih.gov/genbank/). However, since the sequences for *F. ananassa* homologous to *AtEF-1, AtTCH2, AtTCH3, AtTCH4*, and *AtCML39* genes were not available, an indirect primer design strategy was applied. Briefly, *F. vesca* sequences of the orthologues genes of *A. thaliana* (Benikhlef et al. 2013) were obtained, and by BLAST-N analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) sought along the *F. ananassa* genomes (Hirakawa et al. 2014; http://strawberry-garden.kazusa.or.jp) to identify contigs containing the candidate genes. Then, *A. thaliana* corresponding protein sequence and *F. ananassa* DNA contigs were used to protein-based prediction of mRNA and protein sequences with the software FGENESH+ (Solovyev 2007) for each *F. ananassa* gene. To confirm the identity of the predicted protein, a BLAST-P search was carried out. The primers used to analyze the expression of touch- and defense-induced genes in *F. anannasa* are mentioned in Table 1.

Finally, phylogenetic relationship among the predicted *F. ananassa* protein sequences and corresponding orthologs from *A. thaliana*: AtTCH2 (NP_198593), AtTCH3 (NP_181643), AtCH4 (NP_200564), AtCML39 (NP_177790) and from *F. vesca* (XP004288041.1, XP004308823.1, XP004293369.1, and XP011464987.1, respectively) was inferred by the construction of a neighbor-joining tree, using MEGA software (version 2.1) (Kumar et al. 2001).

Accession numbers

The *FaEF-1α, FaTCH2, FaTCH3, FaTCH4* and *FaCML39* nucleotide sequence data reported here are available in the Third Party Annotation Section of the GenBank database under the accesion numbers: BK009992, BK010270, BK010271, BK010272, and BK010273 respectively. Other sequence data used in this article can be found in the GenBank database under the following accession numbers: *FaPR1* (AB462752), *FaCHI2-2* (AF320111), *FaOGBG-5* (JN204374) from *F. ananassa*; and: *FvCAT* (XM004300633.2) and *FvACS1* (XM004288870.2) from the wild strawberry *F. vesca* previously used in *F. ananassa* by Guerrero-Molina et al. (2014).

Table 1 Primers used for qPCR in this study

Gene	Primer sequences	Protein name
FaPR1	Fw 5′-TGGCCCTTATGGTGAAAACC Rv 5′-CACAGCAGATGTGCCTGATTAAGT	Pathogenesis-related protein 1
FaCHI2-2	Fw 5′-CCTCAGGGAAACAAACCATCA Rv 5′-CGCAGATGGATTCCAACGT	Chitinase type II
FvCAT	Fw 5′-TTTTCCCACCATCCAGAAAGTC Rv 5′-TGGAATTCCCAGGTCATTCAAA	Catalase enzyme
FaOGBG-5	Fw 5′-CCTTCAAAGAACCACC Rv 5′-CACATCTCTGGCACAG	Endo-b-1,3-glucanase
FvACS1	Fw 5′-GAAGGGTAATGCTGGGTTGTTT Rv 5′-CCCTTGTTGGTGTGTCCCAAGT	1-amino-cyclopropane-1-carboxylate synthase
FaTCH2	Fw 5′-GCGCAGAGGAGTTGTCTCTTGT Rv 5′-ACAAGCTCCAAGGCTGCAAT	Calmodulin-like protein 24
FaTCH3	Fw 5′-ACAGCATATGCAATCGACCAAT Rv 5′-TCCGCCGCCTCATCAT	Calmodulin-like protein 12
FaTCH4	Fw 5′-CGACGATTGGGCGACAAT Rv 5′-TGAAAGGAGCTGCCTTCCA	Xyloglucan-endo-transglucosylase/hydrolase
FaCML39	Fw 5′-GAGGGCTCAGGCTGCATAAC Rv 5′-CCCAACCTGCCCAGCAT	Calmodulin-like protein 39
FaEF-1α	Fw 5′-CCCCCACTTGGTCGTTTTG Rv 5′-TGATGACTCCCACAGCAACAG	Elongation factor Tu-1

Statistical analysis

Statistical analysis for ROS quantification and SMS-induced protection was carried out using the InfoStat statistic software (Di Rienzo et al. 2013). Differences between means were analyzed by *T* test (P value < 0.05) and significant differences were indicated with asterisks. qPCR data was analyzed using fgStatistics software interface (Di Rienzo 2011) (P value < 0.05) and significant differences in mean expression values between control- and SMS-treated plants were indicated with asterisks.

Results

SMS induces an oxidative burst

With the aim to study whether a soft mechanical stimulus (SMS) induced a defense response in strawberry plants, the occurrence of an oxidative burst was evaluated in SMS-treated leaflets (SMS-TL) (Fig. 1) and in leaf discs (Online Resource 1). SMS treatment induced an early and strong accumulation of H_2O_2 (Fig. 1a), O_2^- (Fig. 1b) and intracellular H_2O_2 (Online Resource 1) 30 mpSMS compared to untreated plants. However, no ROS was detected at later time points (Online Resource 2). SMS treatment did not cause cellular damage as non stained Evans-blue cells were observed (Fig. 1c). In contrast, leaflets from control plants which were deliberately forceps-wounded showed blue-stained dead cells (Fig. 1c). This result suggests that ROS burst was due to a defense response triggered by SMS, rather than by cellular damage.

Identification of *TCH* genes in strawberry

With the aim to evaluate whether *TCH* genes were induced during SMS treatment in strawberry plants, we first identify if the ortholog genes described in *A. thaliana* (Benikhlef et al. 2013) were present in *F. ananassa* genome. The latter was carried out by bioinformatics search, comparing sequences of *A. thaliana, F. vesca* and *F. ananassa*. Results revealed that the genes *FaTCH2, FaTCH3, FaTCH4* and *FaCML39* were present in *F. ananassa* genome and BLAST-P analysis suggested that they would also codify for calmodulin-like proteins. Phylogenetic analysis showed similarities among genes that belong to *Fragaria* genus (e.g. *F. vesca*

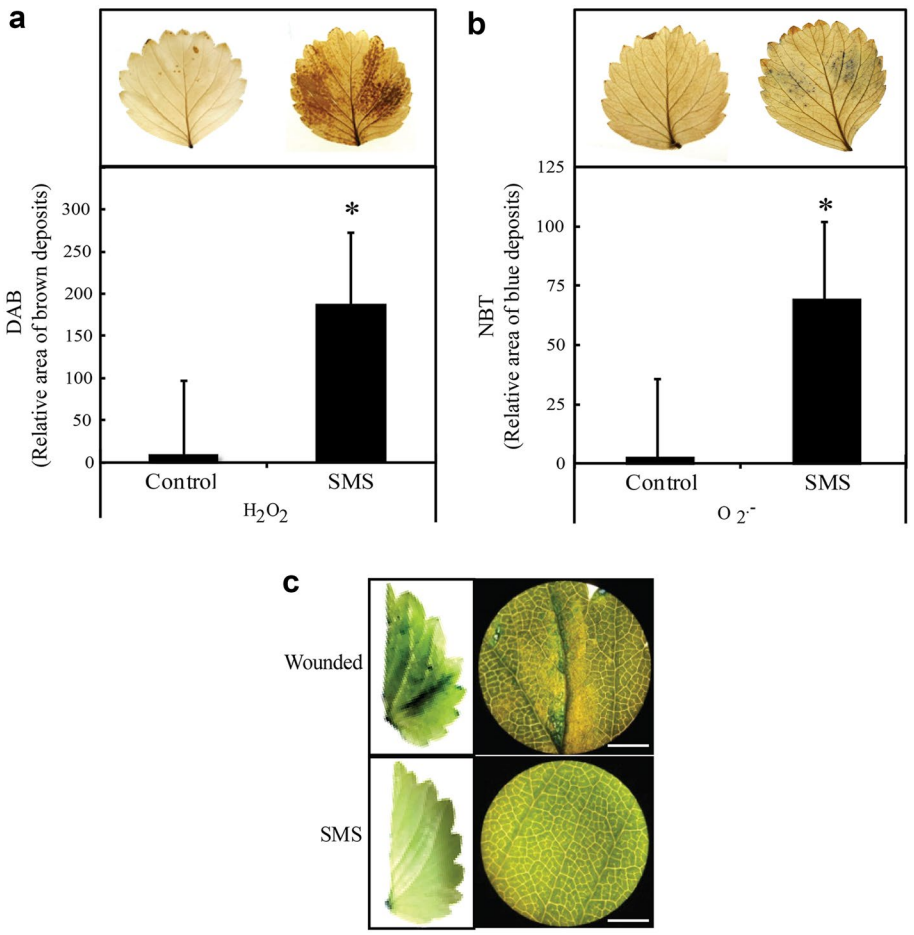

Fig. 1 ROS quantification and cellular integrity in response to SMS treatment in *F ananassa*. Control (not treated) and SMS-treated plants were evaluated for: **a** hydrogen peroxide (H_2O_2), **b** superoxide anion (O_2^-), and **c** cellular integrity 30 mpSMS (bar = 400 µm). H_2O_2 and O_2^- were visualized by DAB and NBT staining, respectively, and quantification was performed by measurement of brown (DAB) and blue (NBT) deposits with ImageJ software. Cellular integrity was visualized by Evans Blue staining under light microscope. Mean values ± SE were obtained from three independent assays ($n = 27$). One representative picture was used for illustration of each treatment. Different letters represent statistically significant differences with respect to control plants (*T* test, $P < 0.05$)

and *F. ananassa*) with the corresponding ortholog from *A. thaliana*, suggesting the existence of common ancestors (Fig. 2).

SMS induces the expression of touch- and defense-responsive genes

The expression level of *TCH* genes (*FaTCH2, FaTCH3, FaTCH4* and *FaCML39*) and defense-related genes *FaPR1* (pathogenesis-related protein1), *FaOGBG5* (a PR2 endo-beta-1,3-glucanase), *FaCHI2-2* (a PR3 chitinase type II), *FvCAT* (catalase), and *FvACS1* (1-amino-cyclopropane-1-carboxylate synthase) was evaluated in SMS-treated and untreated (control) strawberry leaves.

FaTCH2, FaTCH3 and *FaTCH4* gene expression was slightly induced (0.5-fold) with respect to the control at 0.5 hpSMS, whereas *FaCML29* expression was almost fourfold up-regulated (Fig. 3a). At 48 hpSMS all genes were onefold up-regulated compared to the respective control (Fig. 3a). The defense marker genes *FaPR1, FaOGBG-5, FaCHI2.2, FaCAT* and *FvACS1* were significantly induced only at 48 hpSMS. Whereas *FaPR1, FaCAT* and *FvACS1* exhibited a high up-regulation (sixfold), *FaOGBG5* and *FaCHI2.2* were less up-regulated (twofold) (Fig. 3b).

SMS induces a strong protection against *B. cinerea* in strawberry

Since SMS treatment was sufficient to induce some key parameters associated to defense response in strawberry plants (i.e. oxidative burst and defense genes), protection towards a necrotrophic fungal pathogen such as *B. cinerea* was evaluated. Lesion sizes in SMS-TL were smaller than in untreated leaves, indicating that SMS induced 74% of protection 4 days post infection (dpi) (Fig. 4a). Despite SMS-induced protection slightly decreased (62%) in SMS-TL 6 dpi, it was still significant with respect to the control (Fig. 4a). Therefore, we confirmed that SMS treatment induced a strong protection against *B. cinerea* in strawberry plants. Moreover, SMS-TL presented light-brownish, drier and superficial lesions circumscribed to the adaxial side of the tissue, which at the same time were confined to a defined area around the initial point of infection (Fig. 4b). By contrast, lesions in control leaflets looked like dark-brown and water-soaked, were deeper, reached the abaxial side of the tissue, and covered the entire necrotic area suggesting a more intense infection (Fig. 4b). Further studies demonstrated that whereas a slight and controlled oxidative burst was observed in SMS-TL, a stronger one occurred in the control (Fig. 5a). Finally, control leaflets presented long fungal hyphae extensively covering the infected area in comparison to shorter hyphae that barely covered SMS-TL (Fig. 5b).

SMS induces callose depositions

Since SMS activated a local defense response, it was expected that other downstream events were induced as well. Therefore, callose deposition was assessed in the site of infection (SI) and tissue surrounding the SI (TS) of SMS-treated and control plants (Fig. 6a). In SI tissue, callose was highly deposited around *B. cinerea* infection area 2 dpSMS, in comparison to the control in which fewer callose deposits were observed (Fig. 6b). From 4 up to 6 dpSMS there was no difference between control and SMS-treated plants (Fig. 6b). However, callose deposits were notoriously abundant in TS of SMS-treated plants at all times analyzed when compared to the controls, in which fungal hyphae predominated over callose (Fig. 6c).

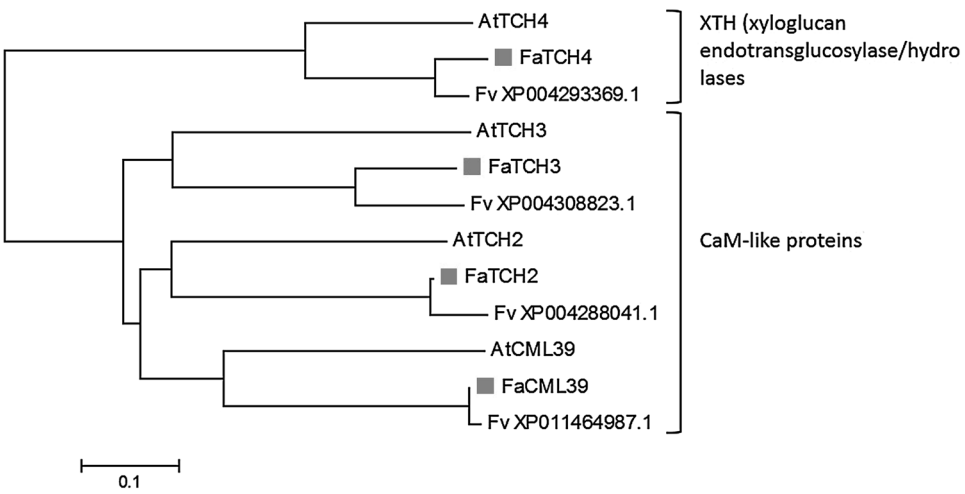

Fig. 2 Phylogenetic tree of the *TCH* genes. The tree was constructed based on the aminoacid sequences from *A. thaliana, F. ananassa* (cultivated strawberry) and *F. vesca* (wild strawberry) by the Neighbor-Joining method using MEGA 21 software

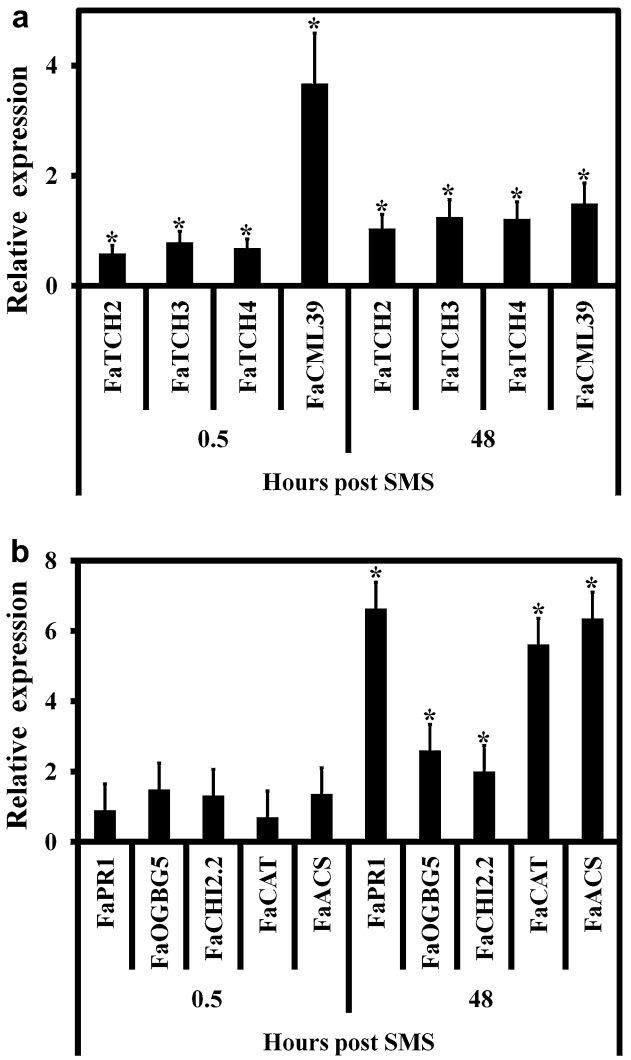

Fig. 3 qPCR analysis of touch-induced and defense-related genes in response to SMS treatment in *F. ananassa*. Expression of *TCH* and defense-related genes was evaluated in SMS-treated and untreated (control) plants. **a** *FaTCH2, FaTCH3, FaTCH4* and *FaCML39* genes were evaluated 0.5 and 48 h post SMS treatment (hpSMS), and **b** *FaPR1, FaOGBG-5, FaCHI2-2, FvCAT* and *FvACS1* defense-related genes at 0.5 and 48 hpSMS. Expression values were normalized with respect to that of reference gene (*FaEF-1α)* and to the controls. Bars represent main values ± SE from an assay with three technical replicates for each qPCR reaction ($n = 3$). Asterisks indicate a statistically significant difference between SMS-treated and untreated (control) plants (LinReg PCR software, $P < 0.05$)

Discussion

Strawberry plants are responsive to SMS by activation of an oxidative burst

It was previously reported that abiotic stimuli such as wounding or soft mechanical stimulation (SMS) conferred resistance against pathogens such as *B. cinerea* in *A. thaliana* (Chassot et al. 2008; Benikhlef et al. 2013). Nonetheless, since then, further reports about SMS and its relation to defense response in other plant species were not found. Hence, the question about whether the SMS is operative in other species and induce a defense response was not addressed. Thus, in the present work, we demonstrate that strawberry plants are responsive to SMS, and have the capacity to induce an effective defense response against *B. cinerea*.

It is well known that one of the earliest defense mechanisms triggered upon biotic stimuli is an oxidative burst as a result of ROS generation (Apostol et al. 1989; Alvarez et al. 1998). Here, we show that an abiotic stimulus such as SMS also induces an early accumulation of superoxide (Fig. 1a) and hydrogen peroxide (Fig. 1b) 30 mpSMS in strawberry, and by using the fluorescence probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) we observed that H_2O_2 was intracellularly accumulated (Online Resource 1). In line with our results, ROS were also detected within few minutes after touch-stimulation in soybean (Yahraus et al. 1995), parsley (Gus-Mayer et al. 1998) and Arabidopsis (Beneloujaephajri et al. 2013; Benikhlef et al. 2013). ROS have several biological effects (Laloi et al. 2004; Torres and Dangl 2005; Torres et al. 2006; Mittler 2016), in the early defense response activated upon biotic (Torres et al. 2006; Heller and Tudzynski 2011; Lehmann et al. 2015) and abiotic (Marino et al. 2012) stresses.

TCH genes are present in *F. ananassa* genome and are up-regulated after SMS treatment

TCH genes were reported for the first time in *A. thaliana* plants by Braam and Davis (1990). Many of them codify for calmodulin-like proteins (CML), such as *AtTCH2* (CML24) and *AtTCH3* (CML12) (Braam and Davis 1990; McCormack and Braam 2003; McCormack et al. 2005), *AtCML39* (CMSL39) (Bender et al. 2013), and for cell wall-modifying proteins like *AtTCH4* which encodes for a xyloglucan-endotransglucosylase/hydrolase (XTH) (Xu et al. 1995; Rose et al. 2002). Later, Lee et al. (2005) performed a wide genome identification of touch-regulated genes in Arabidopsis, revealing that over 2.5% of expressed genes were up-regulated in response to touch. However, the question about whether *TCH* genes were present in the genome of other plant species was not addressed. Therefore, our first effort was directed to identify the orthologous sequences from the diploid species *F. vesca*, presumably a phylogenetic ancestor of the octoploid species *F. ananassa* and use these sequences to identify candidates in its genome available at http://strawberry-garden.kazusa.or.jp (Hirakawa et al. 2014). The bioinformatic study allowed us to identify for first time, to our knowledge, the existence of the orthologs touch-induced genes in the cultivated strawberry *F. ananassa*, namely *FaTCH2, FaTCH3, FaTCH4* and *FaCML39*.

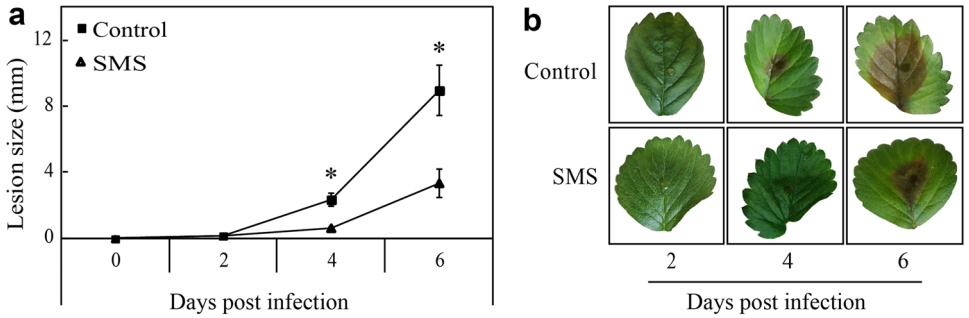

Fig. 4 SMS-induced protection against *B. cinerea* in *F. ananassa*. SMS-treated and untreated (control) plants were infected with *B. cinerea* (5×10^4 conidia ml^{-1}) 30 mpSMS. Evaluations were performed 2, 4 and 6 dpi. **a** Quantification of lesion size at different days post infection. Mean values ± SE were obtained from three independent assays ($n=27$). Asterisks indicate a statistically significant difference between SMS-treated and control plants (T test, $P<0.05$). **b** Appearance of *B. cinerea* lesions on leaflets

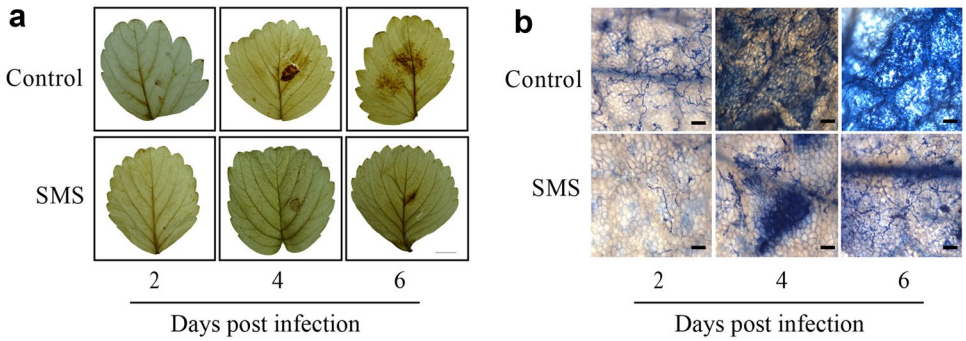

Fig. 5 Effect of SMS treatment during the interaction between *F. ananassa* and *B. cinerea*. **a** ROS burst, (H_2O_2) was visualized by DAB staining on leaflets. **b** *B. cinerea* hyphae growth was evidenced by Evans blue staining (bar = 50 µm). One representative picture of each treatment is presented

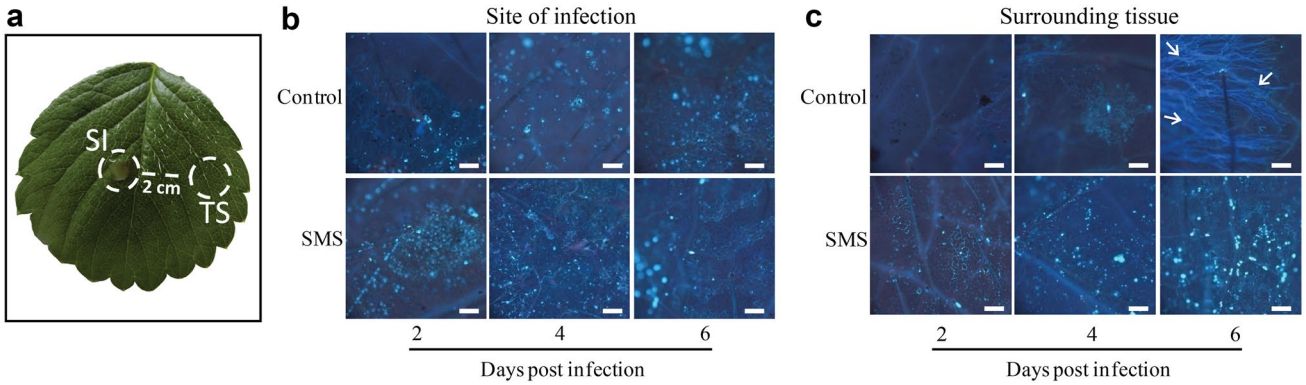

Fig. 6 SMS-induced cell wall reinforcement during the interaction between *F. ananassa* and *B. cinerea*. **a** Sketch of the evaluated areas in a strawberry leaflet, **b** callose deposition at the site of infection (SI), and **c** callose deposition at the tissue surrounding the SI (TS) from SMS-treated and untreated (control) plants. Callose deposits are visualized as bright light-blue spots along the tissue (bar = 50 µm). Hyphae are indicated with arrows in **c**. Three independent assays were performed ($n=6$). A representative picture of each treatment is presented

Blast-P analysis showed that they may also correspond to the same enzymes mentioned above in Arabidopsis. Interestingly, although the genes were clearly identified in strawberry, and exhibited a high similitude among the *Fragaria* species, their similarity was much lower when compared to the Arabidopsis genes (Fig. 2). The latter led us to pose the question about whether *TCH* genes were induced or not after SMS treatment in strawberry plants.

With the aim to answer that question, gene expression analyses were performed by qPCR. All *FaTCH* genes studied were up-regulated as early as 0.5 hpSMS treatment and the induction persisted 48 hpSMS (Fig. 3a). These results

suggest that CML24, CML12, XTH and CML39 proteins may participate in SMS-induced response in strawberry, probably being the latter one the most implicated in the signaling. Our results are in agreement with a previous report in which the *TCH* genes were up-regulated in response to different mechanical stimuli in Arabidopsis (Braam and Davis 1990). Therefore, it is likely that these calcium-binding proteins and the cell wall-modifying protein XTH are part of a conserved defense-induced mechanism in plants.

The initial biochemical and molecular evidences indicating that a defense response was triggered upon SMS treatment in strawberry were further reinforced by the fact that expression of defense-responsive genes was up-regulated in SMS-treated plants. *PR* genes are expressed under (a) biotic stresses conferring plant resistance towards pathogens (van Loon 1997; van Loon and van; Strien 1999; van Loon et al. 2006; Graham et al. 2003; Nürnberger et al. 2004). In the current study we demonstrated that *FaPR1*, which is a key marker gene of plant innate immunity, was up-regulated upon SMS treatment in about sixfold with respect to the untreated control 48 hpSMS (Fig. 3b), suggesting that PR1 participates in defense responses activated upon a mechanical stimulus in strawberry. Moreover, PR1 participation would be a common mechanism of induced defenses in strawberry under different kind of stresses since it was demonstrated previously that *FaPR1* expression was up-regulated in response to biotic stresses like a fungal avirulent isolate (M23 of *Colletotrichum acutatum;* Grellet-Bournonville et al. 2012) and a biological elicitor (AsES; Chalfoun et al. 2013; Hael-Conrad et al. 2017). Although there was reported that *PR1* expression was induced after a mechanical stimulus in parsley cell culture (Gus-Mayer et al. 1998), to our knowledge, this is the first time that *PR1* participation is reported after mechanical stimulation *in planta*.

β-1,3-endoglucanases (endoβGlu) and chitinase type II (Chi2) enzymes are cell wall-modifying proteins induced upon stress, and are codified by *PR2* and *PR3* genes family, respectively (van Loon et al. 2006; Mercado et al. 2010; Wang et al. 2012). In strawberry, the genes *FaOGBG5* and *FaCHI2.2* codifying for an endoβGlu (GenBank: AEQ01058.1, Martinez-Zamora et al. 2012) and for a class II chitinase (GenBank: AF320111.1), were previously reported. In the present study, we determined that both genes were slightly but significantly up-regulated upon SMS treatment with respect to controls 48 hpSMS (Fig. 3b). Thus, it is likely that these enzymes also participate in SMS-induced immunity in strawberry. These results are in agreement with previous studies where it was demonstrated that a chitinase and β-1,3-glucanase participate in the resistance towards *Cladosporium cucumerinum* in cucumber plant stimulated by a gentle mechanical pressing (Zhao et al. 2005). Some other evidences that glucanases are required for plant resistance towards pathogens reinforce our results (Conrath 2006).

Regarding to *FaCAT* gene, which encodes for a catalase in strawberry (Guerrero-Molina et al. 2014), it exhibited an up-regulation of about fivefold after SMS treatment with respect to the control 48 hpSMS (Fig. 3b). Since catalases are highly active enzymes with H_2O_2-metabolizing activity (Mittler et al. 1999, 2004; Mittler 2002; Willekens et al. 1997), it is likely that the up-regulation of *FaCAT* observed yields the low H_2O_2 accumulation observed after 48 hpt (Fig. 5a).

The *FvACS1* gene is a key biosynthetic enzyme of the gaseous phytohormone ethylene (ET). ET has an important role in the defense response against pathogens (Thomma et al. 1998), and mediates a wide range of physiological processes in plants (Abeles et al. 1992; Wang et al. 2002). In the present study we observed that *FvACS1* was strongly up-regulated (over sixfold) in SMS-treated plants with respect to the control 48 hpSMS (Fig. 3b). Similarly, mechanical stimulation induced an early up-regulation of *ACS* gene in *Vigna radiata* plants (Botella et al. 1995). Based on our results, it is plausible that ET is synthesized via ACS and participates in the SMS response.

SMS treatment induces protection against *B. cinerea* in *F. ananassa*

Induced protection by mechanical stresses, such as wounding caused by high or soft pressure, in Arabidopsis against *B. cinerea* was already reported (Chassot et al. 2008; Benikhlef et al. 2013). Nevertheless, to our knowledge, cross-protection induced by mechanical stress has not been reported in strawberry. In the present study, we demonstrated that the application of a soft mechanical stimulus is sufficient to trigger a defense response effective to control grey mold in strawberry (Fig. 4).

In an attempt to characterize the interaction between SMS-induced strawberry plants and *B. cinerea*, we further analyzed ROS production. We observed that the oxidative burst induced after *B. cinerea* infection in SMS-treated plants was clearly restricted within the lesion, in contrast to the more extended burst that underwent untreated control plants, which spread within and around the fungal lesion (Fig. 5a). These results let us speculate that *B. cinerea* induced the oxidative burst for its own benefit in control plants, but possibly by the action of catalases, ROS production was much more controlled in SMS-treated plants. In line with these findings, hyphal growth in SMS-treated plants was clearly restricted in comparison to the controls (Fig. 5b). Our results agree with many reports that propose that *B. cinerea* induces an uncontrolled ROS production as an infection strategy (Govrin and Levine 2000; van Kan 2006; Choquer et al. 2007; Williamson et al. 2007; Torres 2010; O'Brien et al. 2012). However, they disagree with Benikhlef et al. (2013), who found that

ROS production was responsible for the induced resistance against *B. cinerea* in Arabidopsis.

Cell wall reinforcement may also be responsible for the SMS-induced protection against *B. cinerea* since high callose deposits were observed both in the site of infection (SI) and tissue surrounding the SI (TS) (Fig. 6b, c). An indirect correlation between callose deposition and hyphae propagation along the TS in SMS-treated leaves was observed (Fig. 6c) in comparison to the direct correlation observed in the control in which hyphae predominated in a tissue without callose (pointed with arrows in Fig. 6c). Our results suggest that cell wall strengthening prevent fungal hyphae penetration, in agreement with previous reports showing that callose deposition prevents fungal penetration in Arabidopsis (Eggert et al. 2014; Ellinger et al. 2013; Jaffe et al. 1985), and contributes to the innate immune response in strawberry plants (Amil-Ruiz et al. 2011).

To conclude, in this work we present for the first time evidence that touch-induced genes are present in *F. ananassa* genome and also, that a mechanical stimulation induces a defense response in strawberry, which is highly effective to control grey mold. This defense response is characterized by an oxidative burst, callose deposition and up-regulation of touch- and defense-related genes expression. These results would also contribute to gain knowledge on mechanical stimulation in strawberry, and provide a suitable alternative to replace agrochemicals on the control of fungal diseases in this crop.

Author contribution statement RHTG and FJRS have contributed equally to this work. RHTG, FJRS, VHC and MFGM are CONICET fellows; MGMZ and JCDR are CONICET members. VHC, MGMZ, MFGM and JCDR conceived and designed the experiments. RHTG and FJRS, performed the experiments, analyzed and interpreted the data. RHTG, FJRS and VHC wrote the manuscript, and JCDR and MGMZ revised critically the article. All authors approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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