Planta (2017) 245:749–764
DOI 10.1007/s00425-016-2642-z

ORIGINAL ARTICLE

Functional analysis of oxidative burst in sugarcane smut-resistant and -susceptible genotypes

Leila P. Peters[1] · Giselle Carvalho[1,3] · Milca B. Vilhena[1] · Silvana Creste[2] · Ricardo A. Azevedo[1] · Claudia B. Monteiro-Vitorello[1]

Received: 7 August 2016 / Accepted: 13 December 2016 / Published online: 21 December 2016

Abstract

Main Conclusion **Smut pathogen induced an early modulation of the production and scavenging of reactive oxygen species during defence responses in resistant sugarcane that coincided with the developmental stages of fungal growth.**

Sporisorium scitamineum is the causal agent of sugarcane smut disease. In this study, we characterized sugarcane reactive oxygen species (ROS) metabolism in response to the pathogen in smut-resistant and -susceptible genotypes. *Sporisorium scitamineum* teliospore germination and appressorium formation coincided with H_2O_2 accumulation in resistant plants. The superoxide dismutase (SOD) activity was not responsive in any of the genotypes; however, a higher number of isoenzymes were detected in resistant plants. In addition, related to resistance were lipid peroxidation, a decrease in catalase (CAT), and an increase in glutathione S-transferase (GST) activities and an earlier transcript accumulation of ROS marker genes (*CAT3*, *CATA*, *CATB*, *GST31*, *GSTt3,* and *peroxidase 5-like*). Furthermore, based on proteomic data, we suggested that the source of the increased hydrogen peroxide (H_2O_2) may be due to a protein of the class III peroxidase, which was inhibited in the susceptible genotype. H_2O_2 is sensed and probably transduced through overlapping systems related to ascorbate–glutathione and thioredoxin to influence signalling pathways, as revealed by the presence of thioredoxin h-type, ascorbate peroxidase, and guanine nucleotide-binding proteins in the infected resistant plants. Altogether, our data depicted the balance of the oxidative burst and antioxidant enzyme activity in the outcome of this interaction.

Keywords Antioxidant enzymes · Biotic stress · Hydrogen peroxide · Phytopathogen · Reactive oxygen species · *Sporisorium scitamineum*

Abbreviations

CAT	Catalase
GST	Glutathione S-transferase
MDA	Malondialdehyde
ROS	Reactive oxygen species
SA	Salicylic acid
SOD	Superoxide dismutase
hpi	Hours post-inoculation

Introduction

Plants have developed an efficient defence system against pathogens (Molina and Kahmann 2007), and an early response is one of the strategies acquired for plants' survival. One of the initial defence reactions in plants after

G. Carvalho and M. B. Vilhena contributed equally to this manuscript.

Electronic supplementary material The online version of this article (doi:10.1007/s00425-016-2642-z) contains supplementary material, which is available to authorized users.

✉ Claudia B. Monteiro-Vitorello
cbmontei@usp.br

[1] Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ), Universidade de São Paulo, Av. Pádua Dias 11, PO BOX 83, Piracicaba, SP 13400-970, Brazil

[2] Centro Avançado de Pesquisa Tecnológica do Agronegócio de Cana-IAC/APTA, Rod. Antonio Duarte Nogueira, Km 321, Ribeirão Preto, SP, Brazil

[3] Current address: Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Ilha Solteira, SP 15385-000, Brazil

pathogen recognition is the increased production of reactive oxygen species (ROS) (Torres 2010; Del Río 2015). This ROS burst, mostly consisting of superoxide anion and hydrogen peroxide (H_2O_2) at the site of invasion, is regarded as a core component of the early plant immune response (Doehlemann and Hemetsberger 2013). In plant cells, ROS are produced via plasma membrane-localized NADPH oxidase, cell wall peroxidases (class III peroxidases) (Torres 2010), and pathways, such as photosynthesis, photorespiration, and respiration (Gratão et al. 2005). In plants, ROS increase during the infection process by pathogens and, for instance, high concentrations of H_2O_2 may contribute to: (1) the strengthening of host cell walls via crosslinking of glycoproteins; (2) lipid peroxidation (membrane damage); (3) pathogen growth inhibition; (4) induction of gene expression; and (5) acting as a signalling molecule (Mittler et al. 1999). In addition, ROS in incompatible interactions culminate in localized cell death, called the hypersensitive response (HR), which may increase the host resistance to biotrophic pathogens (Barna et al. 2012).

However, due to ROS toxicity, antioxidant compounds and enzymes work in conjunction to maintain the steady-state level in plant cells (Apel and Hirt 2004; Gratão et al. 2005). Among these enzymes are superoxide dismutase (SOD, EC 1.15.1.1), catalase, (CAT, EC 1.11.1.6), glutathione S-transferase (GST, EC 2.5.1.18), and others (Ghelfi et al. 2011; Peters et al. 2014). Moreover, it is suggested that plant hormones, such as salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA), can influence ROS and antioxidant enzyme activation in this process (Barna et al. 2012).

The biotrophic fungus *Sporisorium scitamineum* is the causal agent of sugarcane smut, one of the most important diseases of this crop. The teliospore germination of *S. scitamineum* occurs in the bud and internode surface of sugarcane, following the appressorium formation on the inner scales of the young buds. The fungus entrance in the bud meristem occurs between 6 and 36 h after the teliospore deposition (Sundar et al. 2012). It has been reported that the colonization pattern of *S. scitamineum* differs between resistant and susceptible sugarcane genotypes (Carvalho et al. 2016). In susceptible infected plants, the hyphae are progressively built up within sugarcane tissues, culminating in the formation of a whip-like structure in the primary meristem, compromising culms quality and productivity (Dalvi et al. 2012; Sundar et al. 2012).

The use of resistant varieties is one of the most effective, safe, economical, and environmentally sound approaches to control smut in sugarcane. Some studies have demonstrated that resistance to *S. scitamineum* is associated with chemical barriers, such as the presence of phenylpropanoids, flavonoids (Fontaniella et al. 2002; de Armas et al. 2007), free and conjugated glycoproteins, and an increase of polyamines in sugarcane buds (Millanes et al. 2008). Glycoproteins prevent the correct arrangement of microtubules and cause nuclear fragmentation defects, contributing to germinative failure of teliospores (Sánchez-Elordi et al. 2016). Likewise, resistance may be associated with the presence of trichomes, and the quantity of scales present in buds (da Gloria et al. 1995). Furthermore, resistant plants infected with *S. scitamineum* present an increase of phenylalanine ammonia lyase enzyme activity (de Armas et al. 2007) and cell wall lignification (Santiago et al. 2012). It has been reported in sugarcane that the gene nonexpressor of pathogenesis-related 1 (*NPR1*) was upregulated (RT-qPCR analysis) in response to SA and *S. scitamineum* (Chen et al. 2012). This gene plays a pivotal role in systemic acquired resistance in plants (Cao et al. 1997). In addition, regarding the oxidative burst, the *poxN* gene for peroxidase (*ScSs36*) was found to be weakly induced in smut-susceptible plants at 24 h post-inoculation (hpi), whereas it was upregulated in the resistant plants at 72 hpi (LaO et al. 2008). The catalase gene (*ScCAT1*) was associated with *S. scitamineum*, because CAT activity in smut-resistant plants was higher than that in susceptible plants (Su et al. 2014). Recently, Su et al. (2016) revealed that antioxidant enzymes (SOD, CAT, ascorbate peroxidase (APX), and other peroxidases) are useful biochemical indicators of smut resistance. Nevertheless, at present, there are few studies comparing both ROS production and antioxidant enzymes in susceptible- and resistant-sugarcane genotypes upon inoculation with *S. scitamineum*. The present results are expected to provide a better understanding of the sugarcane resistance mechanisms against *S. scitamineum* and should help the management of selection strategies aiming at the development of resistant cultivars.

Materials and methods

Biological materials and ethical statement

Two sugarcane genotypes were used to investigate the stress response after inoculation with *Sporisorium scitamineum* (Syd.) Piepenbr & Oberw. 2002 (=*Ustilago scitaminea* Sydow & P. Sydow) (Piepenbring et al. 2002): clone IAC66-6, the smut-susceptible genotype: (1), and cultivar SP80-3280, the smut-resistant genotype (2) (Fig. S1a). The plants were maintained at the experimental field of the Departamento de Genética, ESALQ, USP. The IAC66-6 clone and public domain variety SP80-3280 were kindly provided by the Centro Avançado de Pesquisa Tecnológica do Agronegócio de Cana, IAC/APTA.

Sporisorium scitamineum SSC39 teliospores were obtained from a diseased plant of the RB925345

intermediate-resistant variety and were maintained for subsequent experiments in the Genomics Laboratory (ESALQ, USP) (Taniguti et al. 2015).

No special permits were necessary for the teliospores or genotypes used in the experiments, because this project was developed in collaboration with IAC researchers. This work does not involve endangered or protected species.

Inoculation procedure and time-course collection data

Single-bud sets of 10-month-old healthy plants of both genotypes (Fig. S1a) were surface disinfected and incubated for 16 h at 28 °C according to Carvalho et al. (2016). These bud sets were drop inoculated with 20 μL of a suspension containing 5×10^6 teliospores mL^{-1} in 0.01% (v/v) Tween-20, whereas controls were inoculated with 20 µL of sterile saline solution (0.85%) in 0.01% (v/v) Tween-20 (mock inoculated). All of the inoculated bud sets were maintained in vermiculite at 28 °C under conditions of 12 h light/12 h dark and 85% relative humidity. Twenty buds of each genotype were collected at each of the time point of 6, 12, 24, 48, and 72 h post-inoculation (hpi) and were maintained at −80 °C until further experiments (Fig. S1b).

Microscopy analysis of fungal structures in plant tissues

Infected bud scales collected at 6, 12, 24, 48, and 72 hpi were detached from buds and were used to detect fungal structures stained with lactophenol-cotton blue (10 g phenol, 10 mL glycerol, 10 mL lactic acid, 0.02 g cotton blue, and 10 mL deionized water) (Tuite 1969). Light microscopy analyses were conducted in an Optika B-350 microscope (Optikam B5 digital camera) for all time points. The experiment was performed with three biological replicates. The percentage of spore germination was obtained at 6 and 12 hpi (100 spores were counted for each replicate) (James 1973). The percentage of appressorium was quantified observing at least 100 germinated spores for each replicate (Apoga et al. 2004). Statistical analysis was performed as described in the "Experimental design and statistical analysis".

ROS localization of sugarcane–*Sporisorium scitamineum* interaction

Changes in reactive oxygen species (ROS) production in sugarcane bud tissues as a result of *S. scitamineum* infection were assessed. To detect superoxide ions, scales were excised from inoculated and mock-inoculated buds and vacuum infiltrated for 1 h in 0.1% (w/v) nitroblue tetrazolium (NBT) solution in 50 mM potassium phosphate buffer (pH 6.5) (Hückelhoven et al. 2000). Bud scales were then placed into a 0.15% trichloroacetic acid (TCA) in ethanol and chloroform (4:1; v/v) solution to make tissues clear in appearance (de Freitas and Stadnik 2012). After 72 h, they were maintained in the dark in 50% glycerol solution until light- microscopy analysis (Optika B-350; Optikam B5 digital camera). Histochemical detection of H_2O_2 was performed according to Hückelhoven et al. (2000), with modifications. A similar protocol was followed to detect superoxide, but NBT was used instead with a 1% (w/v) DAB (3,3′ diaminobenzidine) solution in 50 mM potassium phosphate buffer (pH 3.8, adjusted with HCl).

Biochemical analysis

For all of the biochemical assays described below, 20 sugarcane buds (mock inoculated and inoculated) of each genotype were collected at 6, 12, 24, 48, and 72 hpi. The sugarcane buds were maintained in liquid nitrogen during sampling and were subsequently stored at −80 °C until further analysis.

Hydrogen peroxide concentration

The content of H_2O_2 was determined as described by Alexieva et al. (2001). Sugarcane buds (100 mg) were homogenized in 1 mL of 0.1% (m/v) TCA. The homogenates were centrifuged at 12,000*g* for 10 min at 4 °C, and 200 µL of supernatant was added to 200 µL of 100 mM potassium phosphate buffer (pH 7.0) and 800 µL of 1 M potassium iodide (KI). The absorbance was read at 390 nm (Perkin Elmer Lambda 40). The H_2O_2 content for all samples was determined using a known H_2O_2 concentration curve as a standard. The result was expressed in µmol g^{-1} fresh weight.

Lipid peroxidation assay

Membrane damage was determined by estimating the content of thiobarbituric acid reactive substance (TBARS) following the method of Heath and Packer (1968). A total of 100 mg of powdered sugarcane buds was homogenized in 1 mL of 0.1% (w/v) TCA solution and centrifuged at 12,000*g* for 10 min at 4 °C. Next, 250 µL of the supernatant from the TCA extraction was added to 1 mL of a solution containing 20% (w/v) TCA and 0.5% (w/v) TBA. The samples were incubated for 30 min at 95 °C and centrifuged for 5 min at 12,000*g*. Malondialdehyde (MDA) was monitored by absorbance measurements at 535 and 600 nm in a Perkin Elmer Lambda 40 spectrophotometer, and the concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}. The result was expressed in nmol g^{-1} fresh weight.

Antioxidant enzyme extraction and activity assays

One gram of fine sugarcane bud powders were homogenized (2:1, buffer volume: fresh weight) in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM DL-dithiothreitol (DTT), 2 mM β-mercaptoethanol, and 5% (w/w) polyvinylpolypyrrolidone (PVPP). The homogenates were centrifuged at 12,000*g* for 30 min at 4 °C, and the supernatants were stored in separate aliquots at −80 °C prior to enzymatic analysis. The concentration of protein was determined using bovine serum albumin as the standard (Bradford 1976).

SOD activity staining

SOD activity staining was carried out as described by Beauchamp and Fridovich (1971) and optimized by Azevedo et al. (1998). The non-denaturing 12% PAGE gels were loaded with 30 µg of biological extract protein, and electrophoresis was carried out with a constant current until migration was completed. After non-denaturing PAGE separation, the gel was incubated in the dark in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium, and 0.3% N,N,N′,N′-tetramethylethylenediamine. One unit of bovine liver SOD (Sigma) was used as a positive control of activity. After 30 min, the gels were rinsed with distilled deionized water and then were illuminated in water until the development of achromatic bands of SOD activity on a purple-stained gel. SOD isoenzyme characterization was performed as described by Giannopolitis and Ries (1977) and as modified by Azevedo et al. (1998). SOD isoenzymes were distinguished by their sensitivity to inhibition by 2 mM potassium cyanide and 5 mM hydrogen peroxide.

CAT total activity determination

CAT activity was assayed as described by Aebi (1984) and modified by Gratão et al. (2012) at 25 °C in a reaction mixture of 1 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 2.5 µL of H_2O_2 (3% solution). The reaction was initiated by the addition of 25 µL of protein extract, and the activity was determined by following the decomposition of H_2O_2 according to the changes in absorbance at 240 nm. CAT activity is expressed as µmol min^{-1} mg^{-1} protein.

GST total activity determination

The methodologies described by Booth et al. (1961) and modified by Ghelfi et al. (2011) were used to determined GST activity. The activity was assayed spectrophotometrically at 30 °C in a mixture containing 900 µL of 100 mM potassium phosphate buffer (pH 6.5), 25 µL of 40 mM 1-chloro-2,4-dinitrobenzene (CDNB), 50 µL of 1 mM GSH, and 25 µL of enzyme extract. The reaction mixture was followed by monitoring the increase in absorbance at 340 nm over 3 min. GST activity was expressed as µmol min^{-1} mg^{-1} protein.

Protein preparation

In view of the cytological and biochemical changes induced during the infection process, we sought to analyse proteins associated with an oxidative burst. The time point selected for protein extraction corresponded to the increase in H_2O_2 and lipid peroxidation concentration (72 hpi) detected in the resistant genotype. Proteins were extracted from sugarcane buds according to the protocol described by Hurkman and Tanaka (1986) with modifications. Bud samples (50 mg) were finely powdered in liquid nitrogen and homogenized in 800 µL of extraction buffer (1% PVPP, 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris–HCl pH 7.5, 500 mM EDTA, 1 mM (PMSF), and 2% b-mercaptoethanol). Phenol (800 µL) was added, and the mixture was homogenized for 30 min at 4 °C and finally centrifuged at 10,000*g* for 30 min. The upper phenol phase was removed and re-extracted two times with extraction buffer as above. Proteins were precipitated from the final phenol phase with two volumes of saturated ammonium acetate in methanol overnight at 4 °C and were pelleted by centrifugation at 10,000*g* for 30 min. The protein pellets were solubilized in lysis buffer (7 M urea and 2 M thiourea). The concentration of protein was determined using bovine serum albumin as the standard (Bradford 1976).

Mass spectrometry MS/MS and data analysis

For protein analysis, an aliquot of 4.5 µL of proteins from peptide digestion was separated in C18 (100 mm 6100 mm) RP-nano UPLC (nanoAcquity, Waters) coupled with a Q-Tof Premier mass spectrometer (Waters) with a nanoelectrospray source at a flow rate of 0.6 µL/min. The gradient was 2–90% acetonitrile in 0.1% formic acid over 45 min. The nanoelectrospray voltage was set to 3.5 kV, a cone voltage of 30 V, and the source temperature was set to 100 °C. The instrument was operated in the 'top three' mode, in which one MS spectrum is acquired followed by MS/MS of the top three most-intense peaks detected. After MS/MS fragmentation, the ion was placed on an exclusion list for 60 s and for the analysis of endogenous cleavage peptides, real-time exclusion was used. The spectra were acquired using the software MassLynx v.4.1, and the raw data files were converted to a peak list format (mgf) without summing the scans by the software Mascot

Distiller v.2.3.2.0, 2009 (Matrix Science Ldt.) and searched against (9747 sequences, 3345,870 residues) using Mascot engine v.2.3.01 (Matrix Science Ltd.), with carbamidomethylation as fixed modifications, oxidation of methionine as variable modification, one trypsin missed cleavage, and a tolerance of 0.1 Da for both precursor and fragment ions. The Scaffold software was used to calculate the normalized spectral counts and to validate peptide and protein identifications (Nesvizhskii et al. 2003), considering the scoring parameters (95% of peptide confidence level identification, 99% peptide probability, and at least two unique peptides) to obtain a false discovery rate (FDR) of less than 1% for proteins and peptides.

RNA extraction and gene expression analysis

The time points selected for gene expression analysis corresponded to appressorium formation (24 hpi) of *S. scitamineum* in the susceptible genotype and the increase in H_2O_2 and lipid peroxidation concentration (72 hpi) in the resistant genotype. The genes analyzed were selected from previous work based on differential expression (analysed by RNAseq) in plants infected with *S. scitamineum* (Schaker et al. 2016). In addition, other genes coding for proteins identified as present or absent during the proteomic assay were selected for gene expression analysis. RNA extraction was performed using Trizol® (Sigma) and the Direct-zol™ RNA MiniPrep Kit (Zymo Research) according to the manufacturer's instructions. Total RNA was treated with DNAse (Sigma), and RNA quality was verified in agarose gel. The primers were manually designed and the quality verified using Gene Runner (http://www.generunner.net/) and Beacon Designer™ Free Edition (http://www.premierbiosoft.com) softwares (Table S1). To confirm the absence of genomic DNA contamination, PCR assays were performed with samples. All RT-qPCRs were conducted in the 7500 Fast Real-Time PCR System (Applied Biosystems) using GoTaq® One-Step RT-qPCR System Kit (Promega). A reaction mixture containing 50 ng of RNA, 6.5 µL of GoTaq® qPCRMaster Mix, 0.2 µM of each primer, 0.25 µL of GoScript™ RT Mix, and nuclease-free water to a final volume of 12.5 µL was used for three biological replicates and two technical replicates. The cycling conditions were as follows: 37 °C for 15 min, 95 °C for 10 min.; 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. Primer specificity was confirmed obtaining the dissociation curve for every reaction. Sugarcane housekeeping genes encoding for polyubiquitin (Papini-Terzi et al. 2005) and 14:3:3 (Rocha et al. 2007) were used to normalize expression signals. PCR efficiencies and Ct values were obtained using the LinReg PCR program (Ramakers et al. 2003). The relative changes in the gene expression ratios were calculated using the REST software (Pfaffl et al. 2004). Control samples (mock-inoculated plants) were used as calibrators.

Experimental design and statistical analysis

The experiment was performed in a completely randomized design, and each treatment was conducted on three biological replicates. The significance of the observed differences was verified using Student's t test ($P < 0.05$). All statistical analyses were carried out using the R software (URL http://www.r-project.org).

Results

Sporisorium scitamineum development delayed in sugarcane tissues of resistant plants

To understand the initial sugarcane reaction to *S. scitamineum*, we investigated the infection process of the fungus in smut-susceptible and -resistant plants. Sugarcane bud scales were used to analyse the production of ROS in a time-course experiment, at 6, 12, 24, 48, and 72 hpi, monitored by light microscopy. The results showed that the fungus presented well established infection stages and differentiated structures in both the analysed genotypes (susceptible and resistant). These modifications comprised filament formation upon recognition of the host surface and the development of infection structures, such as the appressorium. At 6 hpi, 81% of teliospores were germinated in the susceptible genotype (Fig. 1a), forming promycelium (Fig. 2a-1, b-1); however, in the resistant genotype SP80-3280, teliospore germination had initiated only in 36% of teliospores (Fig. 2a-6, b-6), reaching a maximum rate at 12 hpi (53%) (Fig. 1a).

Appressorium formation was quantified by observing lactophenol-cotton blue-stained fungal filaments on bud scale surfaces. In the susceptible genotypes, 71% of the hyphal tips developed an appressorium (Figs. 1b, 2a-3) at 24 hpi, whereas in the resistant genotype, only 43% of the tips developed an appressorium later at 48 hpi (Fig. 1b, 2b-9). At 72 hpi, the formation of an extensive network of filaments in both genotypes was observed (Fig. 2a-10, b-10).

Sugarcane-resistant plants over-produce ROS earlier after *S. scitamineum* inoculation

The production of superoxide anion and H_2O_2 in infected bud scales was determined by the in situ oxidation of nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB), respectively. The results revealed that the main ROS compound produced is H_2O_2, and the response

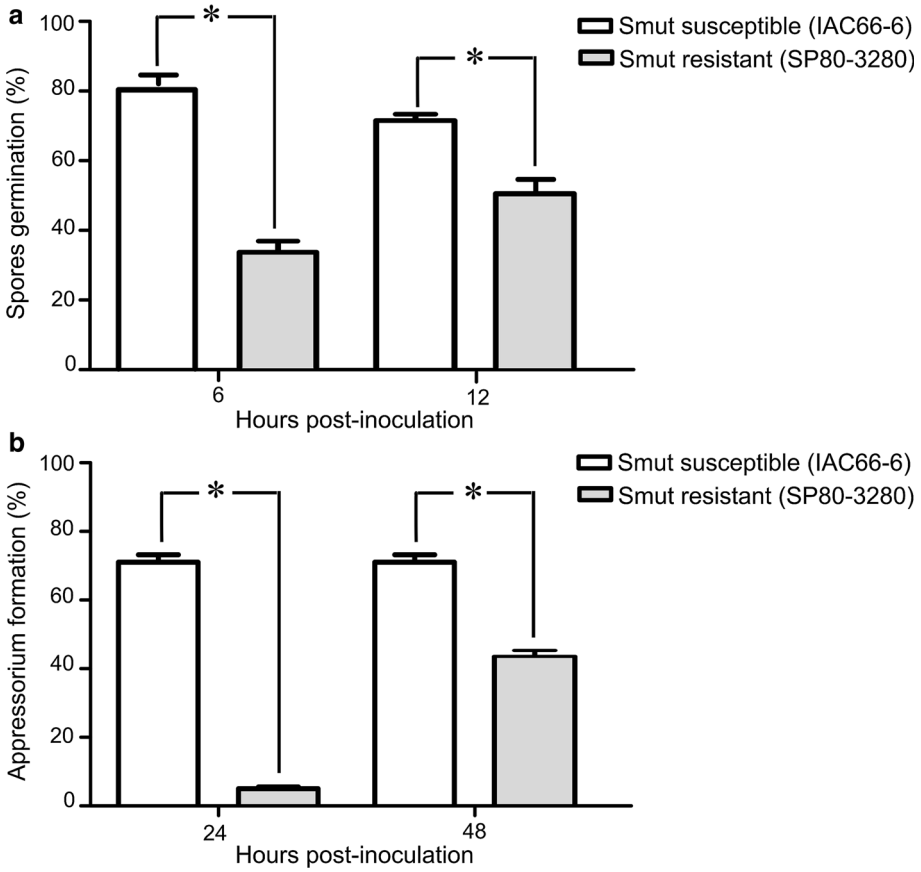

Fig. 1 Teliospore germination and appressorium formation of *S. scitamineum* on sugarcane bud scale surface in smut-susceptible and -resistant genotypes. **a** Teliospore germination (%). **b** Appressorium formation (%). *Bars* represent the standard deviations of three independent biological replicates. *Asterisk* represents statistically significant differences ($P < 0.05$) between the smut-susceptible and -resistant genotypes

became more evident at 72 hpi for both genotypes (Fig. 2b-10, b-10). This is the time when the network of filaments is well established for both genotypes. However, for resistant plants, H_2O_2 accumulation was initiated earlier at 6 hpi along with teliospore germination (Fig. 2b-6). Furthermore, H_2O_2 accumulation was also observed in the plant epidermal cells in direct contact with the appressorium at 48 hpi (Fig. 2b-9). As the colonization progressed, the fungal hyphae started to produce internal vesicular bodies, probably containing H_2O_2 (Fig. 2b-8, b-2, b-3). These vesicles were evident in hyphal growth at 12 and 24 hpi in the susceptible genotypes and at 24 hpi in the resistant one. However, at the other time points analysed for the genotypes, there was no accumulation of H_2O_2 in fungal vesicles.

The production of superoxide anion was also evaluated in sugarcane tissues infected with *S. scitamineum*. The results showed that the plant cells of the resistant genotype produced superoxide at 6 and 12 hpi (Fig. 2a-6, a-7). At these time points, the presence of superoxide anion was restricted to the surrounding areas of the promycelium hyphal tips. Superoxide production was not observed at any other time point analysed of the resistant genotype as well as at any observed time of the susceptible genotype using the proposed technique.

Biochemical assays allowed the quantification of H_2O_2 produced in both inoculated and non-inoculated buds of the two genotypes. The accumulation of H_2O_2 at 6, 48, and 72 hpi in smut-resistant plants was higher (23, 22, and 70%, respectively) than that in mock controls (Fig. 3a); however, for the other time points analysed, changes in the H_2O_2 content were not observed. Likewise, smut-susceptible buds did not exhibit the accumulation of H_2O_2 at all time points analysed (Fig. 3a).

Sporisorium scitamineum infection induces lipid peroxidation in resistant sugarcane

We examined the extension of oxidative damage in sugarcane buds challenged with *S. scitamineum* by determining the content of lipid peroxidation (malondialdehyde, MDA). The results showed that the MDA content in inoculated plants of the susceptible genotype was not altered significantly throughout the experiment (Fig. 3b). Although a similar result was observed for the resistant genotype, an increase of 41% in the MDA content was detected at 72 hpi (Fig. 3b). There was a major intrinsic difference in MDA content between the smut-susceptible and -resistant genotypes regardless inoculation, with the susceptible genotype exhibiting a much higher lipid

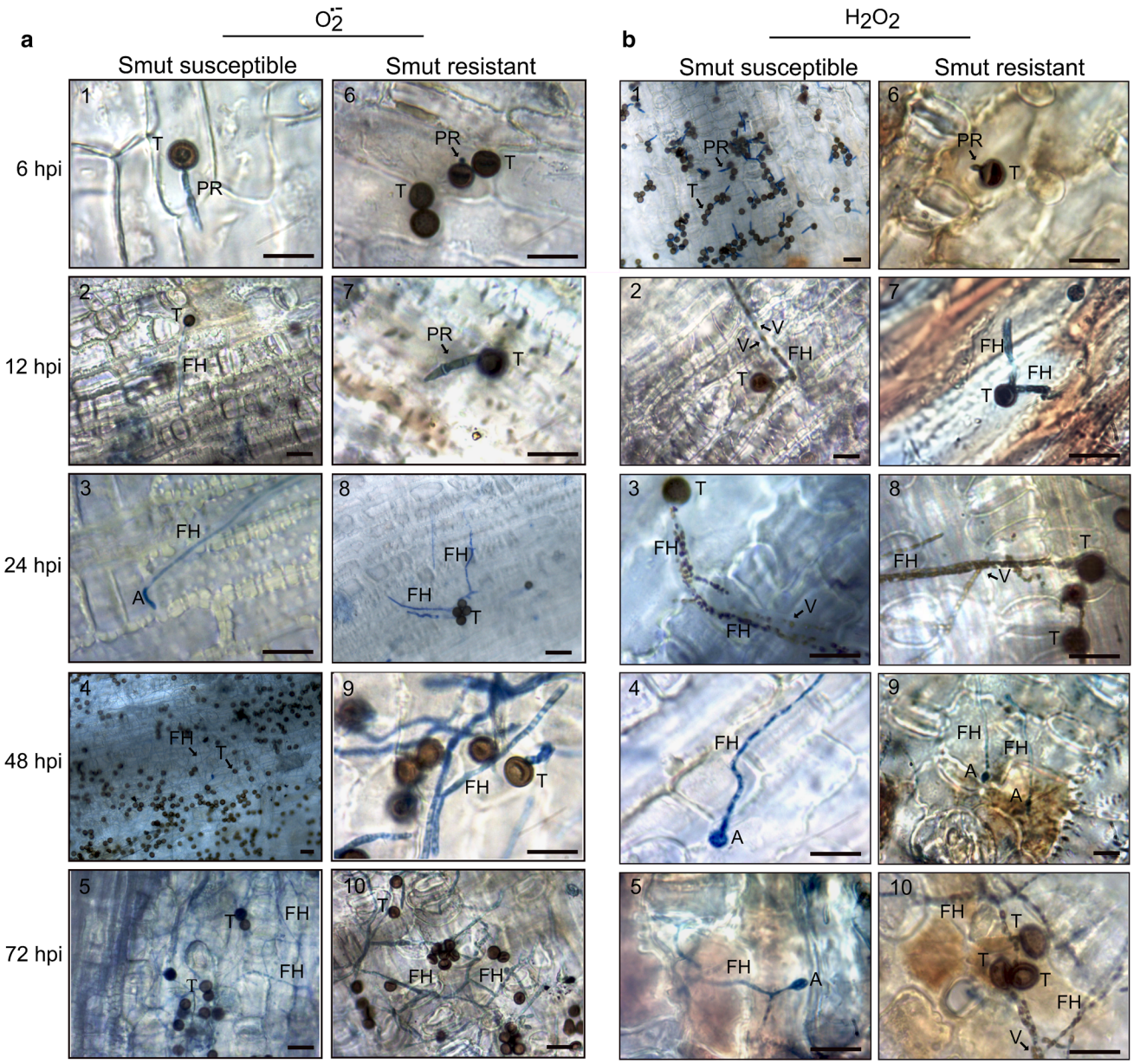

Fig. 2 Microscopic analysis of the *S. scitamineum* infection sites on inoculated sugarcane bud scale and ROS produced on sugarcane bud scales during *S. scitamineum* infection in a time course from 6 to 72 hpi. **a** Inoculated bud scales were stained with nitroblue tetrazolium (NBT) to detect the production of superoxide anion (formation of dark blue formazans) in sugarcane smut-susceptible (numbers of 1–5) and -resistant plants (numbers of 6–10). **b** Inoculated bud scales were stained with 3,3′-diaminobenzidine (DAB) to detect the production of H_2O_2 (DAB polymerization, brown) in sugarcane smut-susceptible (numbers of 1–5) and -resistant plants (numbers of 6–10). *A* appressorium, *FH* fungal hyphae, *PR* promycelium, *T* teliospore, *V* vesicular bodies. *Bar* 100 µm

peroxidation rate (about 70%) during the entire course of the experiment (Fig. 3b).

Sporisorium scitamineum alters the activities of antioxidant enzymes in sugarcane

The overall enzyme activity patterns related to ROS scavenging (SOD, CAT, and GST) were distinct in the resistant and susceptible genotypes. SOD activity was determined by non-denaturing PAGE staining for isoenzyme identification (Fig. 4a, b). The results revealed the existence of five isoenzymes characterized as Mn/SODs (SOD I, II, and III) and Cu–Zn/SODs (SOD IV and V) in the smut-susceptible genotype (Fig. 4a), and ten isoenzymes identified as Mn/SODs (SOD I, II, III, and IV) and Cu–Zn/SODs (V, VI, VII, VIII, IX, and X) in the smut-resistant genotype (Fig. 4b). However, SOD activity did not exhibit any major visible changes or specific alterations

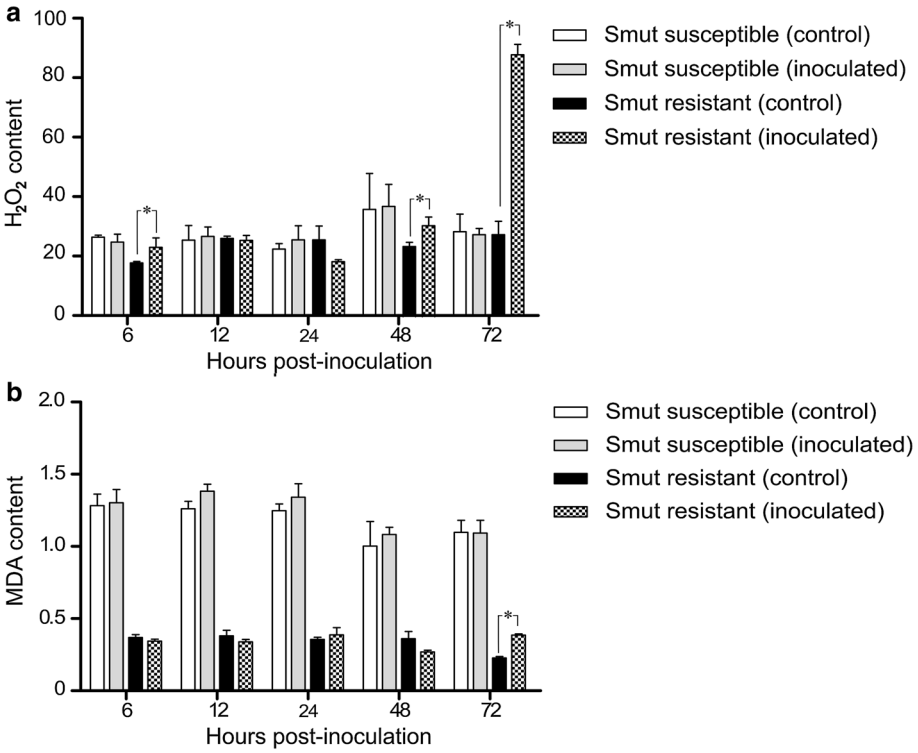

Fig. 3 Detection of H_2O_2 and lipid peroxidation caused by *S. scitamineum* in sugarcane buds. **a** Effects of *S. scitamineum* infection on the H_2O_2 content (μmol g^{-1} fresh weight) (quantitative results). **b** Effects of this fungus on malondialdehyde (MDA) content (nmol g^{-1} fresh weight) in susceptible- and resistant-sugarcane genotypes over the time course from 6 to 72 hpi. Values represent the means from three independent biological replicates ±SD. *Asterisk* represents statistically significant differences ($P < 0.05$) between control buds (mock inoculated) and inoculated buds

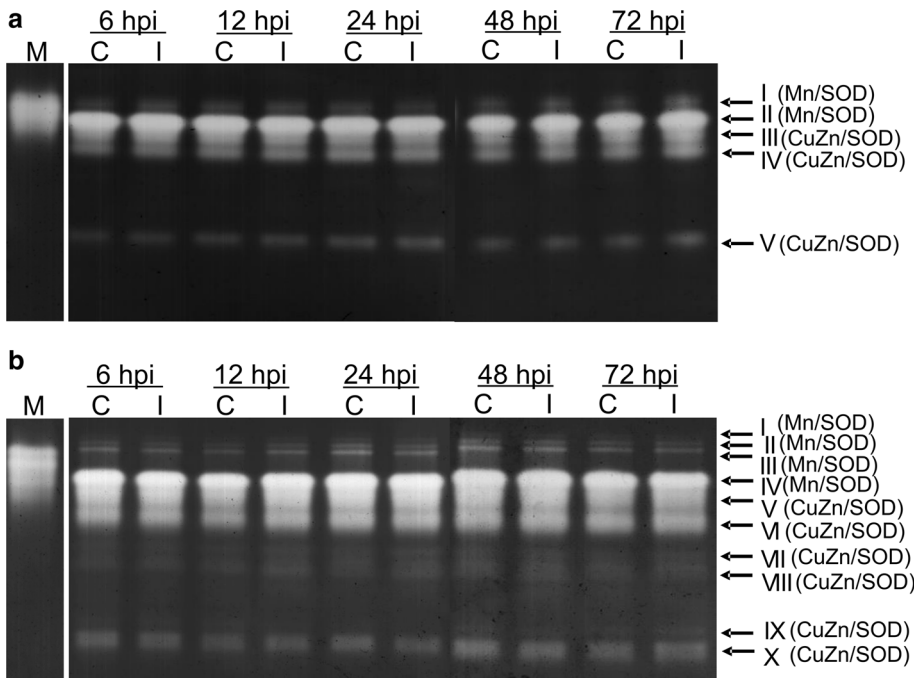

Fig. 4 Effects of *S. scitamineum* infection on the activity of superoxide dismutase (SOD). **a** Activity staining for SOD following non-denaturing PAGE from smut-susceptible genotypes over the time course from 6 to 72 hpi. **b** Activity staining for SOD following non-denaturing PAGE from smut-resistant genotypes over the time course from 6 to 72 hpi. The first lane is a bovine SOD standard, and *arrows* indicate sequentially numbered SOD bands for smut-susceptible (I–V) and smut-resistant (I–X) plants. *C* represents control buds and *I* represents inoculated buds

in the expression of the distinct isoenzymes in any of the genotypes in response to the inoculation with *S. scitamineum*. The five isoenzymes present in the susceptible genotype were also present in the resistant one (susceptible SOD II, III, IV, and V corresponding to resistant SOD IV, V, VI, and X), three of them (IAC66-6 II, III, and IV; SP8032-80 IV, V, and VI) accounting for most of the SOD activity.

CAT total activity was not altered in the susceptible genotype throughout the experiment (Fig. 5a). By contrast, for the resistant genotype, a decrease in CAT activity was observed at 12 hpi (67%) and at 72 hpi

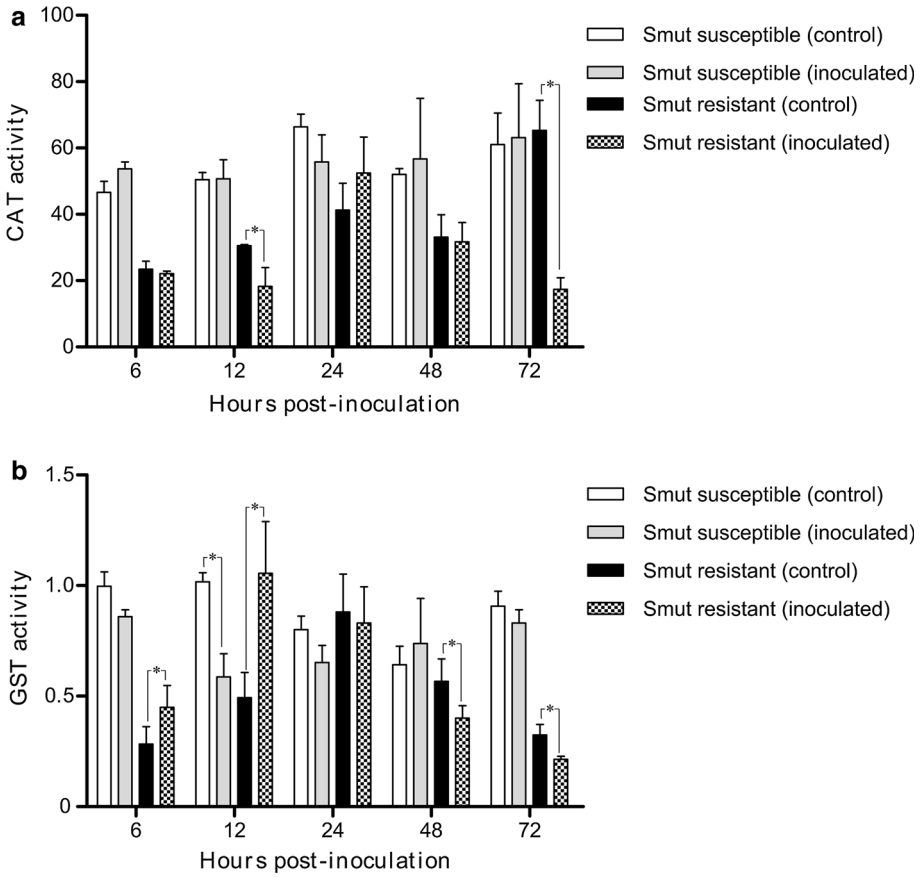

Fig. 5 Effects of *S. scitamineum* infection on the total specific activity of catalase (CAT) and glutathione S-transferase (GST) in susceptible- and resistant-sugarcane genotypes over the time course from 6 to 72 hpi. **a** Total specific activity of CAT (μmol min^{-1} mg^{-1} protein). **b** Total specific activity of GST (units min^{-1} mg^{-1} protein). Values represent the means from three independent biological replicates ±SD. *Asterisk* represents statistically significant differences ($P < 0.05$) between control (mock inoculated) buds and inoculated buds

(275% decrease) (Fig. 5a). CAT activity was consistently higher, especially at 6 and 12 hpi, in the susceptible genotype than in the resistant genotype independent of the inoculation (Fig. 5a). However, high CAT activity was also detected in the resistant non-inoculated plants at 72 hpi. At this time, the sugarcane bud is going through the germination process. In germinating seeds, active mitochondria are one of the major sources of ROS, generating superoxide, and subsequently H_2O_2. Chloroplasts can also generate ROS at the beginning of seed development as well as peroxisomes (El-Maarouf-Bouteau and Bailly 2008). Thus, we assume that the CAT activity in the resistant non-inoculated might be associated with the germination process of sugarcane bud.

Total GST activity exhibited contrasting results between the two genotypes. The susceptible genotype exhibited an early decrease (42% at 12 hpi) (Fig. 5b); however, the resistant genotype increased the activity even earlier (37% at 6 hpi), reaching its maximum value at 12 hpi (70% increase). These changes co-occurred with the teliospore initial germination and maximum germination rates, respectively (Fig. 2a-6, b-6). However, GST activity in the resistant genotype decreased 30 and 24% at 48 and 72 hpi, respectively (Fig. 5b).

Sugarcane proteins associated with oxidative burst are induced or repressed in response to *S. scitamineum* infection

Based on the results of protein identification and functional categorization (Barnabas et al. 2016), four proteins associated with oxidative burst were detected from a set containing 38 proteins (4 proteins from susceptible genotype and 34 proteins from resistant genotype) that were particularly present or absent between the two genotypes (Fig. 6; Table 1). The complete list of present or absent proteins consistently detected in replicates is presented in Table S2. Among these proteins, a cationic peroxidase spc4-like (peroxidase III class) was repressed in the susceptible genotype (Table S2). Three proteins were induced in response to infection in the resistant genotype: ascorbate peroxidase, thioredoxin h-type, and guanine nucleotide-binding protein subunit beta-like (Table S2).

Gene expression analysis

The ROS-related marker genes were selected based on previously obtained RNAseq data (Schaker et al. 2016) and on the protein sequences induced and repressed by the

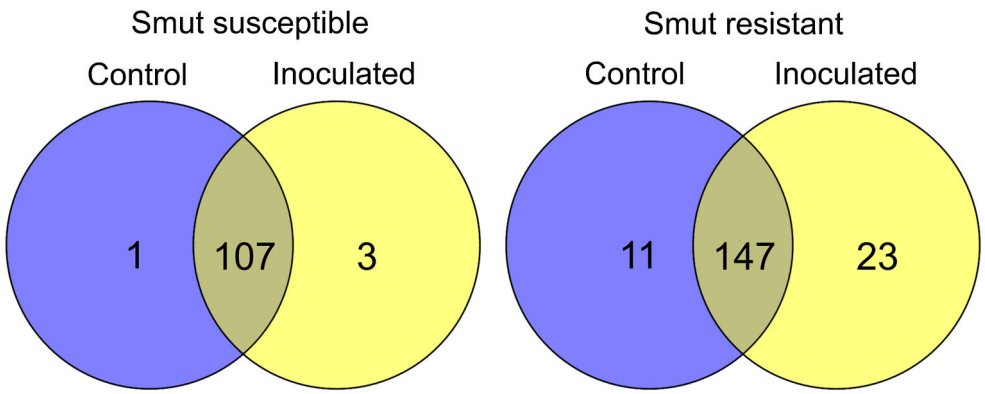

Fig. 6 Venn diagram of induced or repressed proteins in susceptible- and resistant-sugarcane genotypes inoculated with *S. scitamineum* (72 hpi)

Table 1 Induced or repressed proteins related to ROS metabolism at 72 hpi

Treatment[a]	Protein description[b]	Protein threshold[c]	% Sequence coverage[d]					
			C1	C2	C3	Inoc1	Inoc2	Inoc3
Smut resistant (inoculated)	Ascorbate peroxidase	0.9%	0	0	0	10.00%	10.00%	0
Smut resistant (inoculated)	Guanine nucleotide-binding protein subunit beta-like protein a	0.9%	0	0	0	18.09%	15.09%	
Smut resistant (inoculated)	Thioredoxin h-type	1%	0	0	0	25.4%	25.4%	31.5%
Smut susceptible (mock inoculated)	Class III peroxidase	1%	14.5%	14.5%	9.44%	0	0	0

[a] Corresponds to treatment that protein is present

[b] Protein description based on UniProt Knowledgebase

[c] Protein threshold corresponds to false discovery rate (FDR)

[d] % Sequence coverage represents the percentage of all the amino acids in the protein sequence that were covered by identified peptides detected in the sample. C1, C2, and C3 represent control 1, 2, and 3, respectively. Inoc1, Inoc2, and Inoc3 represent inoculated 1, 2, and 3, respectively

fungus identified in this work. A total of ten genes were analysed at two time points: 24 and 72 hpi (Fig. 7). These two time points were chosen, because 24 hpi coincided with appressorium formation in the susceptible genotype, and 72 hpi coincided with the increase in H_2O_2 and lipid peroxidation concentration in the resistant genotype. At 24 hpi (Fig. 7a), the genes encoding SOD and the two GSTs exhibited a similar regulation pattern in both genotypes, in which the genes of SOD and GSTs were downregulated. The genes encoding GDP and PRX4 were significantly upregulated only in the resistant genotype. By contrast, the *cat* genes along with the genes coding for thioredoxin and POX5 peroxidases showed opposite regulation. They were all upregulated in the resistant genotype but were downregulated in the susceptible one. At 72 hpi (Fig. 7b), genes encoding SOD remained downregulated for both genotypes, and genes encoding PRX4 remained upregulated for the resistant genotype. However, the CAT genes and genes for peroxidases POX5 and TRX h had their expression regulation inverted. They were all downregulated in the resistant genotype, but were upregulated in the susceptible one. The gene for GDP was significantly downregulated at 72 hpi in the susceptible genotype (Fig. 7b).

Discussion

To characterize the sugarcane responses in the early stages of interaction, we performed histochemical and biochemical studies and assessed the gene expression profile and protein identification associated with the antioxidant system of sugarcane genotypes susceptible and resistant to smut. Similar to other smut species, *S. scitamineum* is a biotrophic fungus that during the early stage of infection penetrates plant tissues colonizing the primary meristem (Sundar et al. 2012). To depict the events related to ROS, we used a resistant SP80-3280 genotype, which is considered highly resistant to smut and is largely cultivated in Brazil, and the IAC66-6 genotype, which is highly susceptible to smut and is maintained only for research purposes (Carvalho et al. 2016).

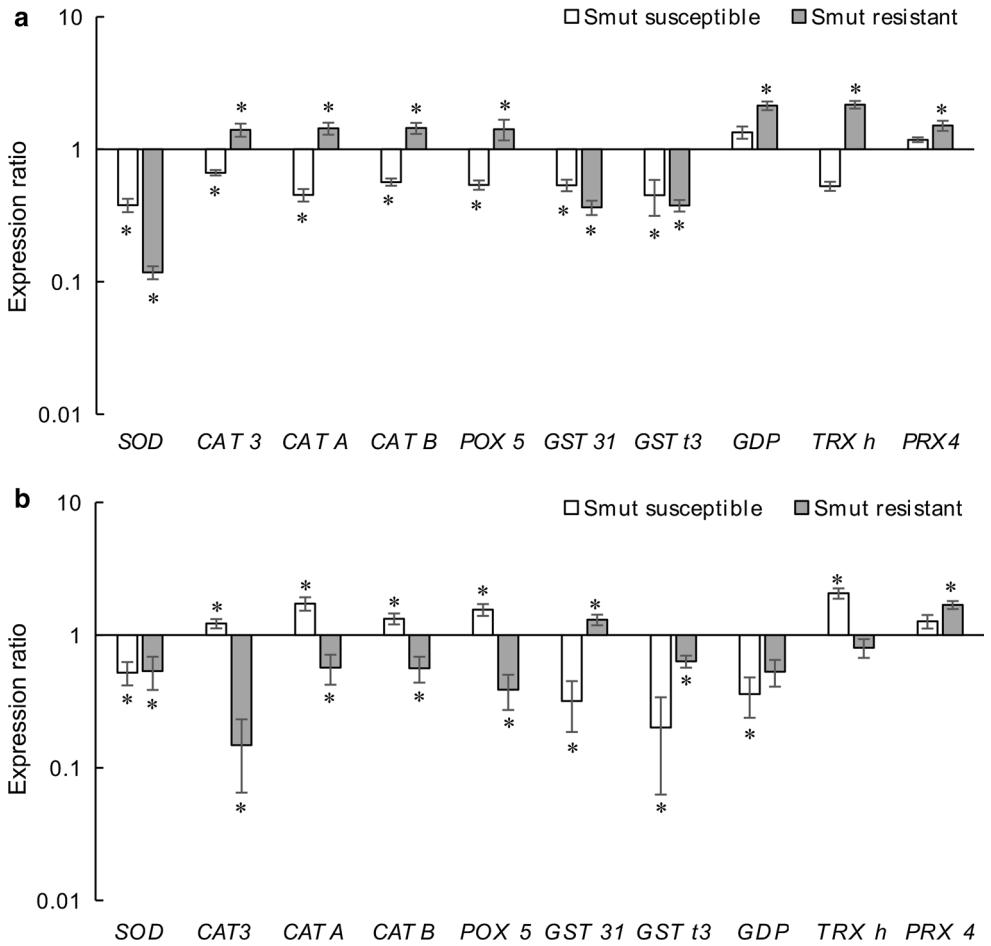

Fig. 7 Expression profiles of superoxide dismutase (SOD-*comp186491_c0_seq 1*), catalase 3 (CAT3-*comp189288_c1_seq 1*), catalase A (CATA-*comp189288_c0_seq 1*), catalase B (CATB-*comp191235_c0_seq 1*), peroxidase 5-like (POX5-*comp127311_c0_seq 1*), glutathione S-transferase 31 (GST31-*comp179663_c0_seq 1*), glutathione S-transferase t3 (GST t3-comp198747_c0_seq 1), guanine nucleotide-binding protein a (GDP-*Sb09g027690.1|PACid:1981757*), thioredoxin h like (TRX h-*evm.model.scga7_unitig_341686.1*), and peroxidase III class (PRX4-*evm.model.scga7_uti_cns_0172034.2*) genes associated with the antioxidant system in smut-susceptible and -resistant genotypes by RT-qPCR analysis. **a** Gene expression at 24 hpi. **b** Gene expression at 72 hpi. Statistical analysis was performed using the REST® software. *Asterisk* represents genes differentially expressed by RT-qPCR ($P < 0.05$)

Buds of these two genotypes were clearly distinguished by the format, hardness, and pigmentation (Fig. S1a). Our data revealed that teliospore germination was delayed in the resistant genotype; however, it still proceeded, reaching its maximum at 12 hpi. It is noteworthy that fungal colonization was achieved for both genotypes using the inoculation method that did not injure sugarcane tissues. Following the germination results, appressorium formation was also delayed in resistant plants but was not completely impaired.

ROS production causes a direct toxic effect to the pathogen along with localized injuries to the plant cell membrane (Torres 2010), which delays or impairs fungal colonization. Although some previous studies (LaO et al. 2008; Song et al. 2013; Su et al. 2014) have reported the relevance of the oxidative burst to counteract fungal colonization, we were able to relate each fungal developmental stage to the variation of the plant responses. Regarding histochemical studies, our results demonstrated that *S. scitamineum* markedly induced H_2O_2 accumulation at 6, 48, and 72 hpi in the inoculated buds of the resistant genotype. These three time points were related to the three phases of fungal development: phase I, coinciding with initial spore germination at 6 hpi (beginning of promycelium formation); phase II: proceeding at 48 hpi, at the moment of the accumulation of H_2O_2 surrounding the appressorium; and the third phase at 72 hpi when an extensive net of fungal hyphae was observed and DAB staining revealed H_2O_2 spread over the epidermal cells of the bud surface (H_2O_2 increased by 70% compared with the control). This triphasic type of H_2O_2 accumulation in incompatible interactions has been described for other

monocots involving interactions such as those of *Blumeria graminis* f. sp. *hordei*-infected barley (Hückelhoven and Kogel 2003) and *Septoria tritici*-infected wheat (Shetty et al. 2007). Other pathosystems seem to rely on two phases of an ROS-associated response to pathogens (Lamb and Dixon 1997; Torres 2010). These differences presumably are determined by the host genotype, as well as by the pathogen infection process (Shetty et al. 2007). In our study, for the susceptible genotype, H_2O_2 accumulation was first detected only at 72 hpi, suggesting that, in this genotype, the recognition of the pathogen and oxidative burst is more delayed and weaker.

Similar to ROS production, lipid peroxidation is a biochemical marker of oxidative stress (Gratão et al. 2005). It has been proposed that lipid peroxidation is a key process for membrane alteration in plants (Lamb and Dixon 1997), and in many cases, this response is efficient against biotrophic pathogens that depend on living cells to survive (Koeck et al. 2011). In our study, the smut-resistant genotype showed increased levels of MDA content at 72 hpi, a finding that was not detected for the susceptible genotype. Interestingly, the reduced GST antioxidant enzyme activity and particularly the CAT activity, probably contributed to the increased levels of H_2O_2, leading consequently to this increased lipid peroxidation. CAT is referred as a key H_2O_2-scavenging enzyme in plants and is generally localized in the peroxisomes, where most of the cellular H_2O_2 is produced as well as several signalling molecules derived from β-oxidation, including salicylic acid (SA) (del-Río and López-Huertas 2016). For instance, CAT activity may be inhibited by the action of SA during HR, elevating H_2O_2 levels, and inducing the expression of defence genes in response to pathogens (Mittler et al. 1999). In addition, inhibition of CAT can convert SA into a free radical, which can also initiate lipid peroxidation (Durner and Klessig 1996). It was also described that SA signalling is the major pathway activated by biotrophic pathogens (Glazebrook 2005) associated with the early defence response of sugarcane to *S. scitamineum* (Chen et al. 2012). These results lead us to propose a key role of H_2O_2 in the sugarcane early defence response to smut, probably in association with SA. In addition, the MDA data showed that the constitutive antioxidant system between the two sugarcane genotypes was notably different. In all treatments, the smut resistant exhibited 70% lower MDA content that of the in smut-susceptible genotype independent of conditions applied.

The ROS-scavenging systems play an important role in managing ROS generated in the plant–pathogen interaction (Torres 2010). SOD catalyses the dismutation of superoxide anion to H_2O_2 and O_2, and represents the first line of defence against ROS (Gratão et al. 2005). In the present study, SOD activity did not exhibit any major alterations that could be due to the inoculation in either the resistant or susceptible genotype. No major alterations in the band intensity or specific induction or repression of isoenzymes were observed between the control and inoculated plants. Conversely, the two genotypes exhibited a different number of isoenzymes, explained by the genetic background, but not the infection with *S. scitamineum* or resistance/susceptibility. The isoenzyme pattern observed for the SP80-3280-resistant genotype agrees with the previous report by Fornazier et al. (2002) who used the same genotype to study cadmium-induced stress in sugarcane. These authors also identified a large number of SOD isoenzymes with the same ones also accounting for most of the SOD activity observed. Because SOD dismutates the superoxide radicals into H_2O_2 and O_2, the results suggest that, at least under the conditions tested, the changes in the H_2O_2 accumulation observed (Fig. 2b-6, b-9, 2b-10) cannot be explained by changes in SOD activity.

The increase in CAT activity in the buds of resistant-sugarcane plants (Yacheng 05–179) infected with *S. scitamineum* in the early stages of interaction (6 and 24 hpi) was previously detected, whereas the susceptible genotype (Liucheng 03–182) did not alter the expression levels during the analysis (Su et al. 2014). According to the authors, there is a positive correlation between the CAT activity and sugarcane resistance to smut. Our data corroborate these previous results regarding the susceptible genotype. However, CAT activity decreased at 12 and 72 hpi in the smut-resistant genotype used here, suggesting that resistant genotypes may respond differently than previously suggested to *S. scitamineum* infection. Our work revealed that, particularly at 72 hpi, the infected buds had an increased H_2O_2 content and lipid peroxidation and a decreased CAT activity.

Likewise, GSTs are responsible for antioxidant activity, reducing damage caused by pathogens through the removal of lipid hydroperoxides produced by the peroxidation of membranes (Dean et al. 2005; Ghelfi et al. 2011). In the present study, GST activity was increased in the infected resistant genotype at 6 and 12 hpi probably contributing to the inhibition of lipid peroxidation. We conclude that this increased GST activity may be associated with smut resistance, because we did not detect alterations in the susceptible genotypes, except at 12 hpi, in which GST activity was decreased. In other pathosystems, the GST increased activity has also been associated with pathogen resistance (Debona et al. 2012; Fortunato et al. 2015).

We also analysed susceptible and resistant sugarcane plants at 72 hpi at the protein level. Thirty-nine proteins were detected as induced or inhibited in sugarcane in response to *S. scitamineum* infection, and four of these were oxidative-stress related. In this first proteomic attempt, we did not use a quantitative approach; instead,

proteins were identified as particularly present in only one of the two genotypes tested upon infection. The protein cationic peroxidase spc4-like (*evm.model.sc-ga7_uti_cns_0172034.2*) (classified as class III peroxidase) was inhibited in susceptible buds. In addition, this same protein-encoding gene tested via RT-qPCR was upregulated in the smut-resistant genotype at 24 and 72 hpi; however, in the smut-susceptible genotype, its expression did not change throughout the time points analysed. We speculate that *S. scitamineum* caused the inhibition of this peroxidase probably through the action of effectors. The pathosystem involving maize and the smut fungus *Ustilago maydis* expressed the PEP1 effector that inhibits this same peroxidase (identity 86%) in susceptible plants, leading to

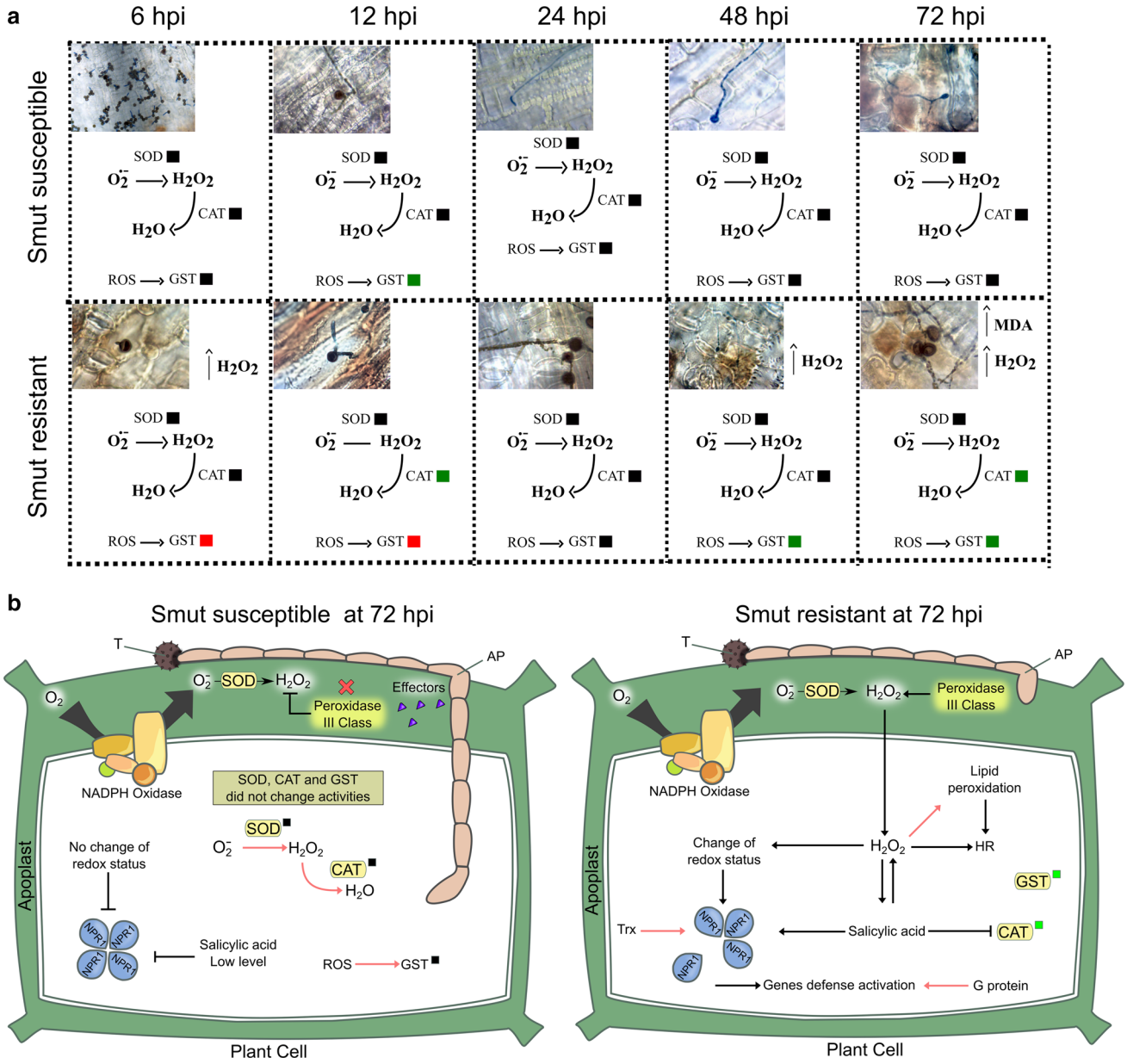

Fig. 8 General view of ROS metabolism of sugarcane buds inoculated with *S. scitamineum*. **a** Summary of the antioxidant system, oxidative-stress markers, and infection process of *S. scitamineum* on sugarcane bud scales in a time-course experiment (6, 12, 24, 48, and 72 hpi) based on the data obtained in the present study. Superoxide dismutase (SOD); catalase (CAT); glutathione S-transferase (GST); lipid peroxidation (MDA); H_2O_2; thioredoxin enzyme (Trx); nonexpressor of pathogenesis-related 1 oligomeric complex (NPR1); hypersensitive response (HR). *Green squares* indicate decreases in enzymatic activity; *red squares* indicate increases in enzymatic activity; *black squares* indicate no alterations. **b** Overview of the mechanisms associated with ROS and antioxidant enzymes of susceptible and resistant sugarcane inoculated with *S. scitaminum* at 72 hpi. *Red arrows* represent results from this study. *Green squares* indicate decreases in enzymatic activity; *black squares* indicate no alterations. *Symbol* indicates "x" repression (only in smut-susceptible plants). *T* teliospore, *Ap* appressorium. All changes are relative to the mock control

blockade of the oxidative burst and suppression of the early immune responses of maize (Hemetsberger et al. 2012). The gene encoding an orthologue of PEP1 is present in the *S. scitamineum* genome (Hemetsberger et al. 2015). We also identified in the infected sugarcane-resistant genotype proteins, such as thioredoxin h-type (evm.model.sc-ga7_unitig_341686.1), guanine nucleotide-binding protein, subunit beta-like protein a (*Sb09g027690.1|PACid:1981757* classified as G protein), and ascorbate peroxidase (*Sb02g044060.1|PACid:1959988*), which were not detected in the susceptible genotypes. Thioredoxins can regulate the redox status of proteins through thiol-disulphide exchange reactions (Sevilla et al. 2015). In plant–pathogen interactions, thioredoxins are required to catalyse the conversion of the SA-induced nonexpressor of pathogenesis-related (PR) genes 1 (*NPR1*) into a monomer and to activate defence responses (Tada et al. 2008). Previous data have shown that sugarcane plants infected with *S. scitamineum* upregulated *NPR1* (Chen et al. 2012). In addition, G proteins are associated with defence signalling in plants (Liu et al. 2013), in which the activation of these proteins (Gβ) occurs in response to pathogen elicitors, leading to ROS increase (Torres et al. 2013) and the synthesis-related (PR) proteins (Beffa et al. 1995).

We further investigated the expression of genes at 24 and 72 hpi that were identified by histo-biochemical and proteomic data regarding the processes of oxidative burst and antioxidative defence. These two points were relevant and markedly different considering the two genotypes analysed. The transcription profiles of marker genes of the antioxidant system followed the pattern of ROS management discussed here between sugarcane-susceptible and -resistant genotypes. The response of the smut-resistant genotype was earlier (at 24 hpi), showing high transcript accumulation after infection for several of the analysed genes; however, in the smut-susceptible genotype, these same genes were downregulated. Conversely, these same genes were upregulated in the susceptible genotype later at 72 hpi. Previous RNAseq data from sugarcane cultivars resistant (Yacheng 05-179) and susceptible ("ROC"22) to *S. scitamineum* revealed that the genes associated with resistance in the resistant cultivar were earlier expressed (24–48 hpi) than those detected for smut-susceptible cultivars (28–120 hpi) (Que et al. 2014). Other regulatory processes may be involved, because the expression of genes encoding the antioxidant enzymes did not necessarily coincide with the enzyme response. Moreover, other antioxidant enzymes may also be involved in the stress response and must be considered in future studies. In particular, we suggest that special attention should be given to apoplastic class III peroxidases and the enzymes of the ascorbate–glutathione cycle.

In conclusion, we described the sugarcane response regarding the oxidative burst induced by *S. scitamineum* using a combination of molecular and biochemical tools to identify candidate genes that allow relevant information on resistance to smut. At the initial step of smut infection, ROS production and antioxidant enzymes have different outcomes between the susceptible- and resistant-sugarcane genotypes. Our results demonstrated that fungal developmental stages within sugarcane tissues (smut resistant) impose an earlier oxidative-burst response by reducing the activity of antioxidant enzymes and significantly increasing H_2O_2 accumulation, resulting in severe lipid peroxidation (Fig. 8a, b). This timely response in the sugarcane-resistant genotype is stronger at 72 hpi. The fungal hyphae upon exposure to the plant response accumulated H_2O_2 in vesicles throughout its extension. This is an effect that needs to be further explored. It is also clear that the different enzymes analysed may respond differently depending on the period tested, inoculation and genotype. However, regardless of the genotypes, fungal colonization is achieved for both genotypes at 72 hpi.

Author contribution statement Conceived and designed the experiments: CBMV LPP GC RAA. Performed the experiments: LPP MBV. Analyzed the data: CBMV LPP RAA. Contributed reagents/materials/analysis tools: CBMV RAA SC. Wrote the paper: LPP GC RAA SC CBMV. Provided expertise and editing: CBMV RAA. All authors read and approved the manuscript.

Acknowledgments The authors thank the Centro Nacional de Pesquisa em Energia e Materiais (CNPEM) for performing the mass spectrometry analysis. The authors also thank the technical support of Elaine Vidotto.

Compliance with ethical standards

Financial support The authors acknowledge the support of the Brazilian institutions FAPESP and CNPq (CNPq 443827/2014-1; FAPESP 2015/07112-4 [CBM-V], FAPESP 2009/54676-0 [RAA] and fellowships to LPP (2013/15014-7), GC (CNPq 159973/2012-0), CBMV (CNPq 303965/2015-0), RAA (CNPq 302540/2011-3), and MBV (CNPq 142736/2011-2).

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