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Research article

Differential modulation of S-nitrosoglutathione reductase and reactive nitrogen species in wild and cultivated tomato genotypes during development and powdery mildew infection

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ABSTRACT

Nitric oxide plays an important role in the pathogenesis of *Pseudoidium neolycopersici*, the causative agent of tomato powdery mildew. S-nitrosoglutathione reductase, the key enzyme of S-nitrosothiol homeostasis, was investigated during plant development and following infection in three genotypes of *Solanum* spp. differing in their resistance to *P. neolycopersici*. Levels and localization of reactive nitrogen species (RNS) including NO, S-nitrosoglutathione (GSNO) and peroxynitrite were studied together with protein nitration and the activity of nitrate reductase (NR). GSNOR expression profiles and enzyme activities were modulated during plant development and important differences among *Solanum* spp. genotypes were observed, accompanied by modulation of NO, GSNO, peroxynitrite and nitrated proteins levels. GSNOR was down-regulated in infected plants, with exception of resistant *S. habrochaites* early after inoculation. Modulations of GSNOR activities in response to pathogen infection were found also on the systemic level in leaves above and below the inoculation site. Infection strongly increased NR activity and gene expression in resistant *S. habrochaites* in contrast to susceptible *S. lycopersicum*. Obtained data confirm the key role of GSNOR and modulations of RNS during plant development under normal conditions and point to their involvement in molecular mechanisms of tomato responses to biotrophic pathogens on local and systemic levels.

1. Introduction

Nitric oxide (NO) is an important signalling molecule in a broad spectrum of physiological and developmental processes throughout the plant life, including germination, leaf expansion, root development, flowering, stomatal closure and senescence (Mur et al., 2013; Yu et al., 2014). NO is also involved in plant responses to abiotic stresses (Corpas and Barroso, 2015; Farnese et al., 2016) and pathogen infection (Mur et al., 2019). Regarding sources of NO, no protein with similarity to animal nitric oxide synthases (NOS) has been found in plants, although NOS-like activities were reported *in vivo* and *in vitro* (Corpas et al., 2009; Corpas and Barroso, 2014). In the plant kingdom, the NOS sequences have been found so far only in unicellular green algae species (Foresi et al., 2010). Results of a recent transcriptomic study on 1000 species of land plants and algae support the conclusion that plants produce NO by mechanism(s) different from animals (Jeandroz et al., 2016). Several oxidative and reductive pathways of NO production have been identified to operate in plants (reviewed in Gupta et al., 2011). Beside non-enzymatic NO production from nitrite, nitrate reductase (NR, EC 1.6.6.1) has been described as an important enzyme source of NO in higher plants (Yamasaki et al., 1999). NR catalyses the first step of the nitrogen assimilation, i.e. transfer of two electrons from NAD(P)H to

Abbreviations: ADH, alcohol dehydrogenase; APF, 3'-(*p*-aminophenyl)fluorescein; CLSM, confocal laser scanning microscopy; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; GSNO, S-nitrosoglutathione; GSNOR, S-nitrosoglutathione reductase; ID, index degree; NOS, nitric oxide synthase; NR, nitrate reductase; RNS, reactive nitrogen species; ROS, reactive oxygen species.

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nitrate to produce nitrite, whereas the production of NO is a result of a one-electron nitrite reduction (Kaiser et al., 2002). NR is known to be regulated by post-translational phosphorylation of conserved serine residue (Kaiser and Huber, 2001).

NO produced in plant cells can react with reactive oxygen species such as superoxide to produce peroxynitrite and other reactive nitrogen species, which are involved in covalent modifications of cellular biomolecules including proteins, lipids and nucleic acids (Kolbert et al., 2017; Mata-Pérez et al., 2017). Alternatively, NO can be efficiently scavenged within plant cell compartments by plant phytoglobins, or in mitochondria by alternative oxidase under normoxic conditions (Rubio et al., 2019; Vishwakarma et al., 2018). S-nitrosation belongs to important protein redox-based post-translational modifications and involves the binding of NO group to cysteine thiols (Lamotte et al., 2015). S-nitrosation is considered an integral part of NO-dependent signalling pathways in a variety of cellular processes (Lindermayr et al., 2005; Gaupels et al., 2011). S-nitrosoglutathione reductase (GSNOR) regulates S-nitrosothiol homeostasis through the irreversible conversion of S-nitrosoglutathione (GSNO) to glutathione sulphinamide (Jensen et al., 1998; Liu et al., 2001). GSNOR, formerly named also as S-(hydroxymethyl)glutathione dehydrogenase (EC 1.1.1.284), is a member of the medium-chain alcohol dehydrogenase class III (ADH3; EC 1.1.1.1) family highly conserved among all kingdoms (Xu et al., 2013). GSNOR is supposed to control transnitrosation equilibrium between GSNO and protein S-nitrosothiols and to play a key role in the overall metabolism of reactive nitrogen species (RNS) (Feechan et al., 2005; Frungillo et al., 2014; Jahnová et al., 2019). The GSNOR activity has been demonstrated in multiple plant species localized in the cytosol, although chloroplastic, mitochondrial, nuclear and peroxisomal localizations were also reported (Fernández et al., 2003; Barroso et al., 2013; Xu et al., 2013; Tichá et al., 2017a,b). GSNOR is involved in plant development (Lee et al., 2008; Xu et al., 2013), plant disease resistance (Feechan et al., 2005), plant cell death (Chen et al., 2009) and protection against nitrosative stress (Valderrama et al., 2007). GSNOR activity and expression are modulated by exposure to abiotic stresses as low and high temperatures, continuous light or darkness, wounding and heavy metals (Corpas et al., 2008; Chaki et al., 2011a; Airaki et al., 2012; Ziogas et al., 2013; Kubienová et al., 2014; Jahnová et al., 2019).

GSNOR is known to play a pivotal role in plant immunity. GSNOR is down-regulated by wounding and jasmonic acid and activated by salicylic acid (Martínez et al., 1996; Dolferus et al., 1997; Espunya et al., 2012). GSNOR expression in *A. thaliana* is regulated in response to signals associated with plant defence (Díaz et al., 2003). The modulation of S-nitrosothiols by GSNOR is involved in disease resistance of *A. thaliana* and GSNOR regulates the signalling network controlled by salicylic acid (Feechan et al., 2005). Transgenic plants of *A. thaliana* with decreased GSNOR using antisense strategy show enhanced resistance against *Peronospora parasitica*, whereas systemic acquired resistance is impaired in plants overexpressing GSNOR (Rusterucci et al., 2007). GSNOR is induced in the resistant cultivar of sunflower infected with *Plasmopara halstedii* (Chaki et al., 2009).

Tomato powdery mildew, caused by biotrophic fungus *Pseudoidium neolycopersici*, represents an important disease of cultivated tomatoes occurring worldwide mainly on glasshouse crops (Lebeda et al., 2014). Interactions between host plants of *Solanum* spp. and *P. neolycopersici* include both race-nonspecific and race-specific resistance shown to be associated mainly with the hypersensitive response (Mieslerová et al., 2000; Lebeda et al., 2014). Activation of defence responses in resistant genotypes *S. habrochaites* or *S. chmielewskii* includes increased production of reactive oxygen species (ROS), with the highest levels detected in genotypes exhibiting intensive hypersensitive response such as *S. chmielewskii* (Mlíčková et al., 2004; Tománková et al., 2006). The early events of pathogen recognition include induction of ROS and NO production in host cells which are observed both in susceptible and resistant *Solanum* genotypes 2–6 h post-inoculation (hpi), while ROS and RNS are produced also in pathogen cells during development of pathogen structures. In contrast, only resistant plants are capable to induce the accumulation of ROS and RNS in the later stages of the infection 24–96 hpi, connected with the initiation of plant cell death as a part of hypersensitive response (Piterková et al., 2009, 2011; Sedlářová et al., 2016).

In this work three *Solanum* spp. genotypes with different level of resistance to *P. neolycopersici* were used to investigate the GSNOR activity and expression both during plant development under normal conditions and during biotic stress conditions induced by biotrophic pathogen infection. Other parameters of RNS metabolism, including NO, GSNO and peroxynitrite were analysed together with protein nitration. Our study brings new insights into several components of the RNS metabolism, including key enzymes GSNOR and NR, and relevance of these parameters to the susceptibility and resistance of *Solanum* spp. plants to the tomato powdery mildew.

2. Material and methods

2.1. Plant material

Three genotypes of *Solanum* spp., differing in their susceptibility to tomato powdery mildew (*Pseudoidium neolycopersici*) were used: highly susceptible *S. lycopersicum* cv. Amateur (% max infection degree ID 100), moderately resistant *S. chmielewskii* (LA 2663) (% max ID 30; hypersensitive response, occasionally following by defoliation of infected leaves) and highly resistant *S. habrochaites* f. *glabratum* (LA 2128) (% max ID 0; hypersensitive response); both later mentioned genotypes are completely resistant in the field conditions (Mieslerová et al., 2004). Seeds were sown on perlite in plastic pots and after full development of primary leaves, seedlings were transferred to soil/peat mixture (2:1, v/v) and grown in a glasshouse. Plant samples were harvested at the age of 10, 30, 45 and 90 days. All plant samples were immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Pathogen inoculation

Pseudoidium neolycopersici L. Kiss (L. Kiss) (isolate C-2) from the Czech National Collection of Microorganisms (collection n. UPOC-FUN-127) was used for the experiments (Mieslerová et al., 2004). Plant inoculation was performed as described elsewhere (e.g. Piterková et al., 2009). The adaxial side of the 4th true leaf of each plant was inoculated by surface contact (dusting/tapping) with leaves of *S. lycopersicum* cv. Amateur covered by freshly sporulating *P. neolycopersici* mycelium. An average number of 65 ± 15 conidia were applied per mm^2 of the leaf surface. Inoculated plants were incubated in a growth chamber at 20 °C/18 °C and 12 h/12 h light/dark regime (with the light period starting at 6 a.m.) under a light intensity of 100 μmol $m^{-2}.s^{-1}$. All experiments were initiated at 8:30 am to follow the same circadian rhythm. Leaves from inoculated and control plants were collected separately at 4, 8, 24, 32, 48 and 72 h post-inoculation (hpi), immediately frozen in liquid nitrogen and stored at −80 °C. Plant growing and inoculation procedures were repeated in three independent experiments, and leaves from three plants were collected for each time interval.

2.3. Enzyme activity

For determination of GSNOR activity, frozen plant samples were processed as described previously (Kubienová et al., 2013). GSNOR activity was assayed in purified plant extracts spectrophotometrically at 25 °C by monitoring the absorbance of NADH at 340 nm. GSNOR activity was expressed as nmol of NADH oxidised per min and grams of fresh weight (FW).

For determination of NR activity, frozen plant leaves were homogenized in cold extraction buffer (250 mM Tris-HCl, pH 8.0, 1 mM EDTA Na^+, 1 μM Na_2MoO_4, 5 μM FAD, 14 mM β-mercaptoethanol and 500 mM Pefabloc). Homogenates were centrifuged at 12000×*g* for 15 min. One

hundred microliters of the supernatant were added to 900 μl of reaction buffer (50 mM potassium phosphate, pH 7.5, 10 mM KNO_3, 1 mM EDTA Na^+, 5 μM FAD and 1 mM NADH) and incubated at room temperature in dark. After 30 min, the reaction was stopped with 50 μl of 1 M zinc acetate and centrifuged at 12000×*g* for 10 min. Solutions of 1% sulfanilamide in 3 M HCl and 0.1% N-(1-naphthyl)ethylenediamine hydrochloride were added and nitrite concentration was measured by reading absorbance at λ = 546 nm and using calibration curve determined with sodium nitrite. GSNOR activity was expressed as nmol of nitrite produced per min per gram of FW.

The protein content in the plant extracts was measured by the Bradford method (Bradford, 1976).

2.4. RNA extraction and cDNA preparation

Total RNA from 100 mg of plant tissues was extracted using RNAsy Plant Mini Kit (Macherey-Nagel, Germany) and treated with Turbo DNAse (Applied Biosystems, USA). The cDNA was synthesized from 1 μg of total RNA using oligo$(dT)_{15}$ primer and AMV transcriptase (2 units, Promega, USA). The reaction mixture was then incubated at 42 °C for 60 min followed by heat inactivation of reverse transcriptase at 70 °C for 5 min.

2.5. Quantitative PCR

Gene sequences of *GSNOR* from *S. lycopersicum* (GU296438), *S. chmielewskii* and *S. habrochaites*, and of nitrate reductase from *S. lycopersicum* cv. Amateur (HQ616893) are available in the GenBank database (http://www.ncbi.nlm.nih.gov/) or TIGR (http://www.tigr.org). Primer pairs for qPCR were designed using Primer3 software (Supplementary Table S1). The qPCR was performed in 25 μl reaction mixtures using an ABsolute SYBR Green ROX Kit (ABgene Ltd, UK). Amplification of target genes and real-time detection of amplicon production were monitored on an Mx3000P analyser (Stratagene, USA). The SYBR Green fluorescent signal was standardized with an internal passive reference dye (1 mM ROX) included in the SYBR Green PCR mix. The following program was applied: initial DNA polymerase activation 95 °C for 15 min, then 40 cycles at 95 °C for 30 s, X°C (X°C means different annealing temperatures (T_m) for each primer pairs; see Supplementary Table S1) for 30 s and 72 °C for 30 s. The specificity of the PCR amplification was checked with a melting curve program 55–95 °C following the final cycle of the PCR. PCR conditions were optimized for high amplification efficiency >95% for each used primer pair and negative controls in the absence of template were also performed. All samples were run in triplicates simultaneously with negative controls. Relative quantification of gene expression was normalized to the housekeeping genes *EF1α* and *GAPDH* using the Pfaffl's method (Pfaffl, 2001).

2.6. Detection of NO, hROS and O_2^-

NO, hROS and O_2^- were detected in cross-sections of leaves of 30 days old plants. Leaf transversal sections were embedded in 4% agarose and 80 μm sections were cut on a vibratome (BangCo 1000Plus, Brno, Czech Republic). Superoxide anion radical was detected by incubation in 20 μM dihydroethidium in 10 mM Tris/HCl, pH 7.4 for 30 min. Control samples were pre-incubated for 20 min with 1 mM superoxide scavenger, 2,2,6,6-tetramethylpiperidin-1-oxyl (Valderrama et al., 2007). Nitric oxide (·NO) was detected using 4-amino-5-methylamino-2′, 7′-difluorofluorescein diacetate (DAF-FM DA). The sections were incubated with 20 μM DAF-FM DA for 30 min and washed three times with 10 mM Tris-HCl buffer, pH 7.4. Control samples were pre-incubated for 20 min with 200 μM NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) (Sedlářová et al., 2011). Highly reactive ROS (hROS) including peroxynitrite ($ONOO^-$), hydroxyl radical and hypochlorite were detected using 3'-(*p*-aminophenyl) fluorescein (APF). The sections were incubated with 20 μM APF for 30 min and washed three times with 10 mM Tris-HCl buffer, pH 7.4. Sections pre-incubated for 20 min with 20 μM ebselen as a peroxynitrite scavenger were used as controls. All incubations were performed at room temperature in darkness.

After the last incubation step, leaf sections were mounted on glass slides in 50% glycerol in 10 mM Tris-HCl, pH 7.4 (1:1, v/v) and examined by microscope IX81 attached to confocal laser scanning unit FV1000 (Olympus Czech Group, Prague, Czech Republic). The transmission light images were obtained by a 405 nm excitation using a near-ultraviolet diode laser, in single images combined with Nomarski DIC filters. Simultaneously, the fluorescence channel corresponding to NO and hROS was visualized by excitation with a 488 nm line of an argon laser and signal detection by 505–525 nm emission filter. For detection of O_2^- signal, the emission filter was modified to a range of 505–570 nm.

The evaluation of fluorescence signal intensity in selected regions of plant tissue within the microscopic images was performed by ImageJ 1.33 software (National Institute of Health, USA). Three sections per experimental setting were analysed and fluorescence signal intensities were normalized to the pixel areas of each leaf section.

2.7. Immunolocalization of GSNO and GSNOR

Leaves of tomato cut into 5 × 5 mm pieces were fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 3 h at room temperature. Serial 80 μm sections were obtained by the vibratome (BangCo 1000Plus, Brno, Czech Republic). After three washes for 10 min in washing buffer (5 mM Tris-HCl buffer, pH 7.6, 0.9% (w/v) NaCl, 0.05% (w/v) sodium azide, 0.1% (w/v) bovine serum albumin, 0.1% (v/v) Triton X-100), sections were incubated overnight at 4 °C with rabbit polyclonal antibody against tomato GSNOR (1:500 dilution, Kubienová et al., 2013) or rat polyclonal antibody against GSNO (1:2000 dilution, Agrisera, Sweden) in washing buffer. After several washes with washing buffer, sections were incubated with goat anti-rabbit IgG (H + L) DyLight® 488 conjugated (Pierce) for SlGSNOR or with rabbit anti-rat IgG (H + L) DyLight® 488 conjugated (Pierce) for GSNO, diluted 1:1000 in washing buffer, for 1 h at room temperature. After incubation sections were washed in washing buffer and mounted in 50% glycerol in 10 mM Tris-HCl, pH 7.4 on glass slides and observed by confocal laser scanning microscopy (CLSM) using excitation by 488 nm line of an argon laser and the emission of 505-525 nm. Controls for background fluorescence were performed omitting the primary antibody.

2.8. Quantification of GSNOR and nitrated proteins by western blotting

Denaturing SDS-PAGE was carried out in 12% polyacrylamide gels. Proteins were transferred onto a nitrocellulose membrane with a semi-dry Trans-Blot cell (Bio-Rad, Hercules, CA, USA) and probed with anti-SlGSNOR polyclonal rabbit antibody or a mouse monoclonal antibody against 3-nitrotyrosine (clone 2A12, Abcam, UK), both diluted 1:1000 (Valderrama et al., 2007). For immunodetection, a goat anti-rabbit IgG horseradish peroxidase conjugate (diluted 1:10 000; Sigma-Aldrich) or goat anti-(mouse IgG)-horseradish peroxidase conjugate (diluted 1:10 000; Pierce, Thermo Fisher Scientific) were used. After washing in TBS and 0.1% Tween 20 for 2 h, the membrane was incubated with Western Blotting Luminol Reagent (Santa Cruz Biotechnology, USA) for 5 min. The chemiluminescence was detected with a sensitive photographic film (Hyperfilm ECL, GE Healthcare, UK) (Fig. 3) or a chemiluminescence scanner (C-digit, LI-COR, USA) (Fig. 8).

2.9. Statistical analysis

Results are presented as means ± SD of samples from at least three independent experiments. Statistical analyses were done by unpaired *t*-test or one-way ANOVA followed by Bonferroni's multiple comparison test using GraphPad Prism 5.0. Asterisks were used to denote significant

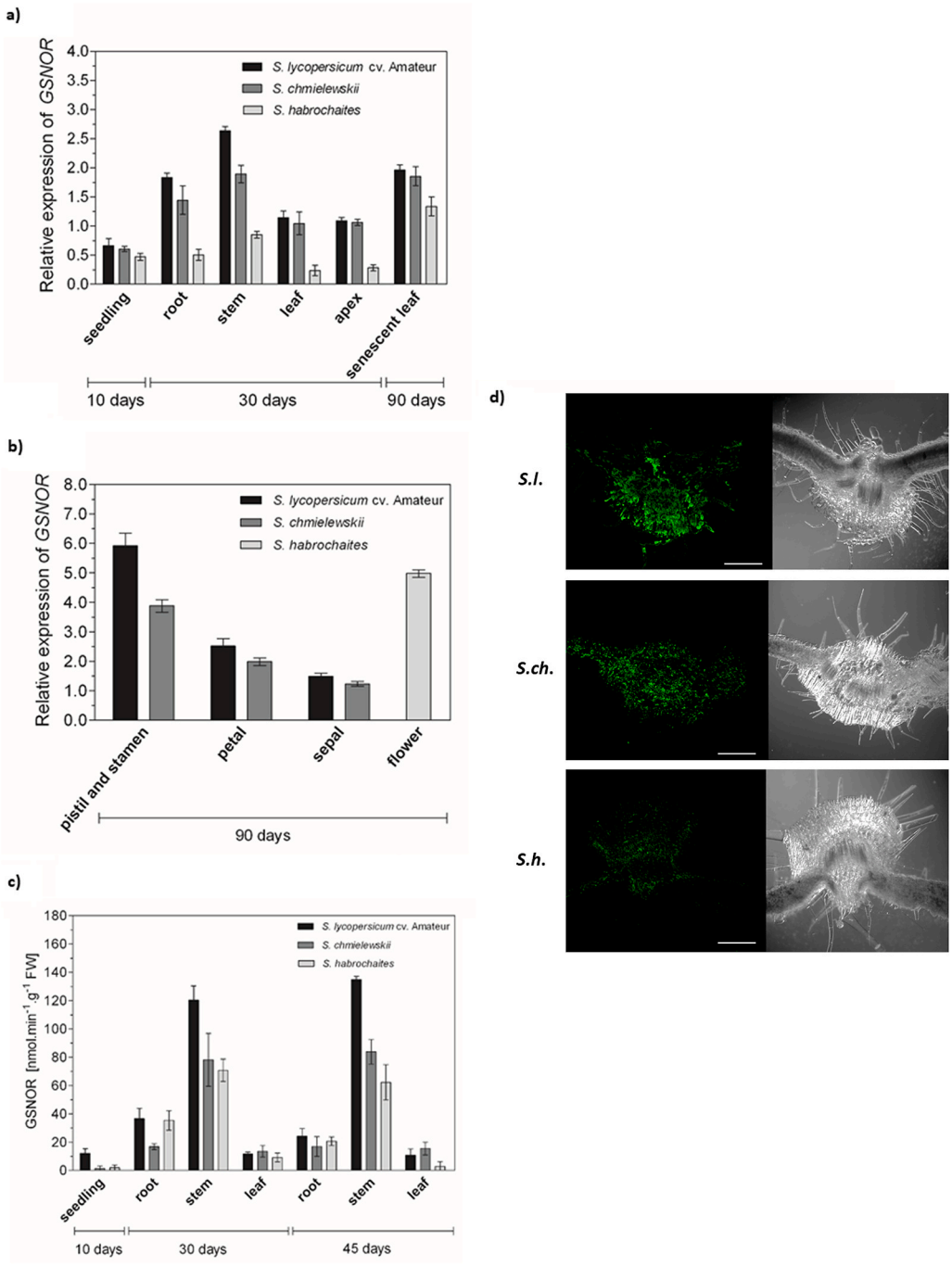

Fig. 1. GSNOR expression and activity during development of *Solanum* spp. genotypes.
GSNOR expression in seedlings, roots, stems, leaves, shoot apices **(a)** and different parts of flowers **(b)** collected from 10, 30 and 90 days old plants of *S. lycopersicum* cv. Amateur, *S. chmielewskii* and *S. habrochaites*. The *GSNOR* gene expression is shown in relative values to the *GSNOR* expression in *S. lycopersicum*. **(c)** GSNOR activity in roots, stems and leaves from 10, 30 and 45 days old plants of *S. lycopersicum* cv. Amateur, *S. chmielewskii* and *S. habrochaites*. Data represent means ± SD ($n \geq 3$). **(d)** Immunolocalization of GSNOR (green fluorescence signal) in cross-sections of leaves of 30 days old plants. Scale bar = 200 μm. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

differences in mean values at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

3. Results

3.1. Powdery-mildew resistant Solanum spp. genotype shows lower GSNOR expression and activity during plant development

The expression of *gsnor* gene was quantified by qPCR in 10 days old seedlings and various organs of 30 and 90 days old plants, including senescent leaves and different part of flowers (Fig. 1a and b) whereas GSNOR activities were evaluated by spectrophotometric measurements of NADH-dependent reductase activity (Fig. 1c). The studied *Solanum* spp. genotypes show a similar growth rate of vegetative tissues, whereas a slower rate of reproductive organs development is distinctive in *S. habrochaites*. For this reason, only entire flowers of this genotype were used for the *GSNOR* gene expression analysis. On the other hand, the small amount of reproductive organ samples did not permit to perform determination of GSNOR enzyme activity. As a general trend, both *GSNOR* expression and activity were lower in the resistant genotype *S. habrochaites* compared to the susceptible tomato genotype *S. lycopersicum* cv. Amateur or moderately resistant *S. chmielewskii*. In 30 days old plants, *GSNOR* expression was higher in the root and stem compared to leaves and shoot apex, as well as it was higher in senescent leaves of 90-days old plants compared to leaves of 30 days old plants. High *GSNOR* expression was also detected in the inflorescence, namely in the pistil and stamens. Similarly, GSNOR activity expressed per grams of leaf FW was found higher in older plants: 10 days old seedlings

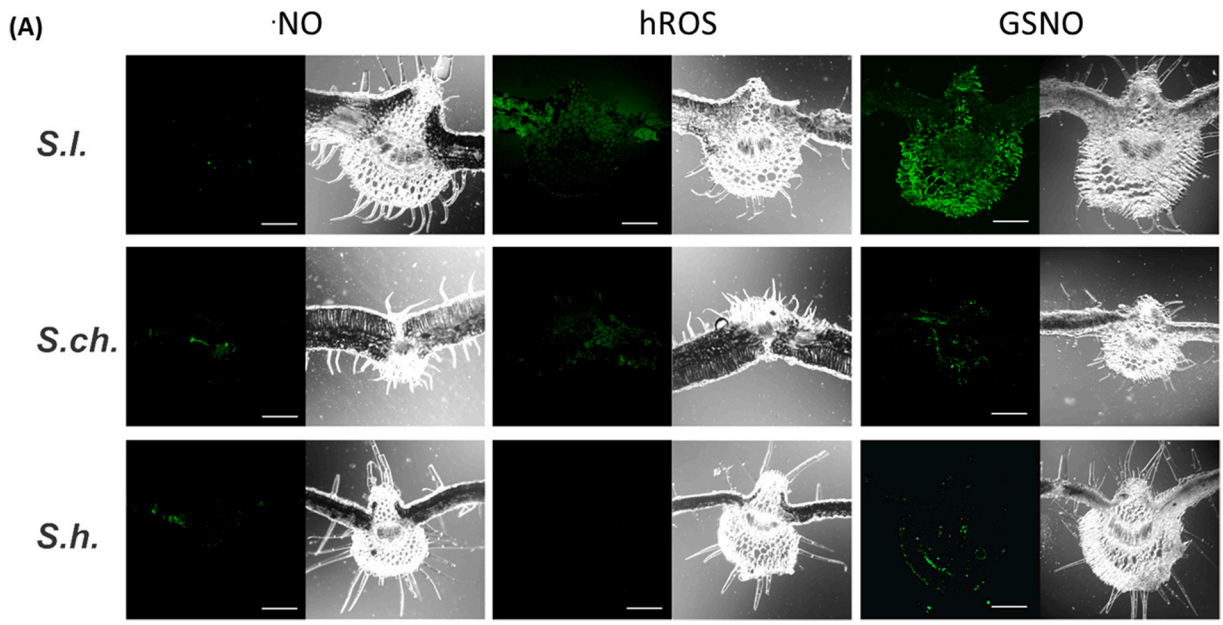

Fig. 2. Levels of NO, hROS and GSNO in non-infected plants of *Solanum* spp.
NO and hROS were detected in leaf cross-sections of 30 days old plants using fluorescent probes DAF-FM DA and APF in 20 μM concentrations, respectively, whereas detection of GSNO was performed immunohistochemically using a commercial anti-GSNO primary antibody. Images obtained in the fluorescence channel (left column) are combined with the Nomarski DIC channel (right column) and shown for each sample. Green fluorescence detected by confocal laser microscopy fluorescence channel corresponds to NO, hROS, and GSNO respectively. Scale bar = 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

showed very low activity of GSNOR compared to 30 and 45 days old plants (Fig. 1c). In later stages of plant development, the highest activity was detected in stems, whereas the lowest activity in leaves. In general, the susceptible genotype *S. lycopersicum* cv. Amateur showed higher GSNOR activity compared to two other genotypes, except for leaves where no significant differences were observed among the studied genotypes.

Tissue localization of GSNOR in leaf sections was analysed by CLSM (Fig. 1d). Green fluorescence signals attributable to GSNOR localized mainly in the vascular tissue and epidermal cells of the leaf sections. The highest signal of GSNOR was observed in the tissue of susceptible genotype *S. lycopersicum*. In contrast, in case of resistant genotype

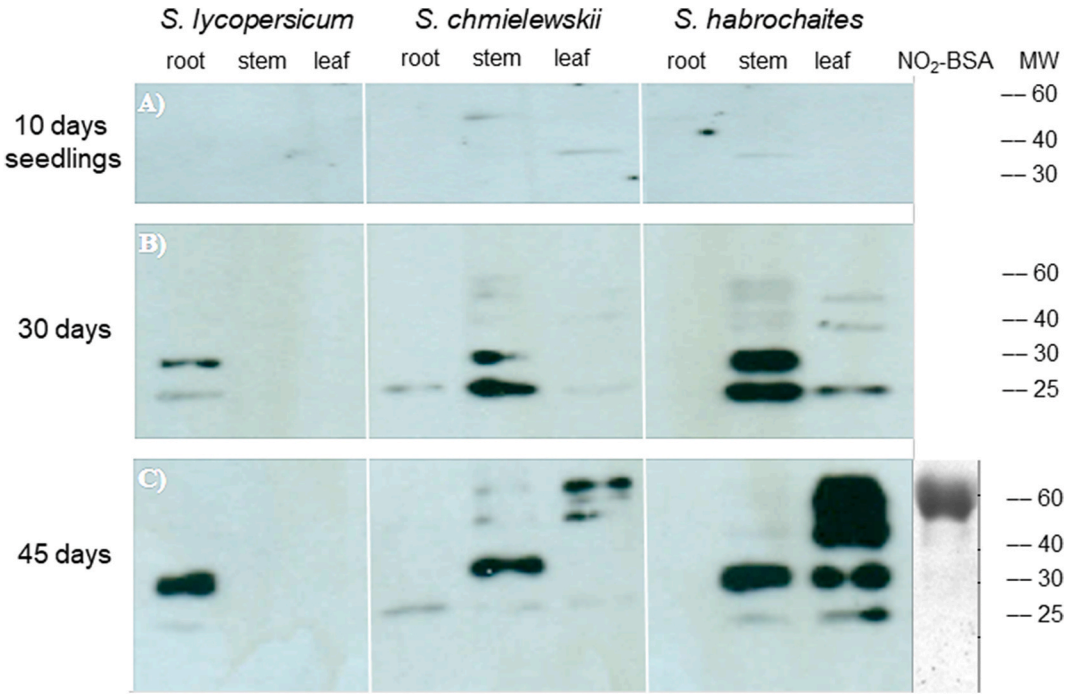

Fig. 3. Protein nitration during development of non-infected plants of *Solanum* spp.
Protein nitration in extracts of root, stem and leaf samples collected from 10, 30 and 45 days old plants was analysed by SDS-PAGE and Western blot using rabbit polyclonal antibody against 3-nitrotyrosine (15 μg of protein was loaded in each lane). Nitrated BSA was used as a positive control (10 μg of protein per lane).

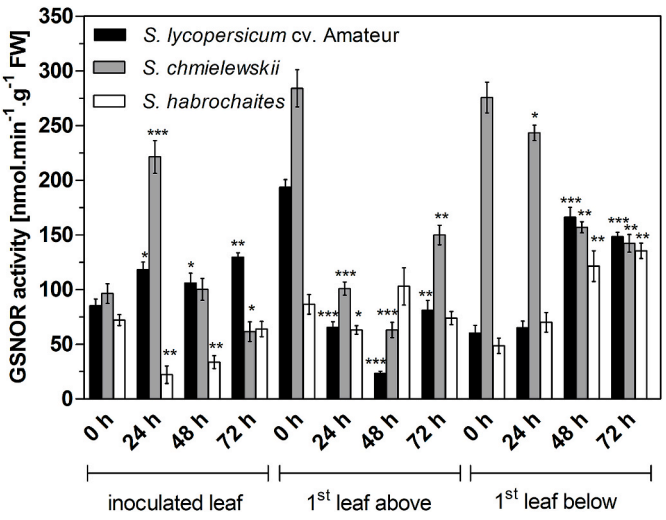

Fig. 4. Modulation of leaf GSNOR activity in *Solanum* spp. genotypes infected with *P. neolycopersici*.
GSNOR activity was determined spectrophotometrically at 25 °C by monitoring a decrease in NADH absorbance at 340 nm. GSNOR activity was analysed in extracts of samples of the inoculated leaf, 1st leaf above and 1st leaf below the inoculated leaf were collected from each plant. Data are shown as means ± SD ($n \geq 3$). Asterisks were used to denote significant differences of the values compared to respective values of each genotype prior to inoculation at 0 hpi evaluated by unpaired *t*-test at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

S. habrochaites, the green fluorescence was strongly reduced compared to susceptible genotype, in accordance with results on GSNOR expression and activity. When the primary antibody against GSNOR was omitted in the control experiments, no fluorescence signal was detected (images not shown).

3.2. Levels of reactive nitrogen species and protein nitration differ in Solanum spp. genotypes during plant development

The cellular localization of RNS and hROS was studied in leaf cross-sections by CLSM using fluorescent probes 4-aminomethyl-2′,7′-difluorofluorescein diacetate (DAF-FM DA) and 3'-(*p*-aminophenyl) fluorescein (APF) for NO and hROS staining, respectively, or by an immunohistochemical method using a commercial antibody against GSNO (Fig. 2). The green fluorescence signals corresponding to NO or hROS were localized mainly in the vascular tissue and palisade mesophyll. Very low levels of NO were found in the susceptible genotype, which on the contrary showed the highest signal of hROS and GSNO. Interestingly, no hROS fluorescence signal was observed in the resistant genotype *S. habrochaites*, which is associated with intensive protein tyrosine nitration. When leaf sections were pre-incubated with a NO scavenger (200 mM cPTIO) or with a peroxynitrite and hydrogen peroxide scavenger (20 μM ebselen, Masumoto and Sies, 1996), the green fluorescence signals were strongly reduced mainly in vascular tissues (images not shown).

The protein tyrosine nitration was analysed in plant extracts by immunoblot analysis, using a primary antibody against 3-nitrotyrosine (Fig. 3). While only a limited number of immunoreactive bands was detected in extracts of 10-days old seedlings, the intensity of protein nitration increased with plant age in all genotypes. Distinct patterns of pronounced immunoreactive bands with molecular masses of 50, 40, 30 and 28 kDa were observed in various organs of 30 and 45-days old plants. In *S. lycopersicum* cv. Amateur, low tyrosine nitration was observed only in roots, with no immunoreactive bands in stems and leaves, whereas the most intensive nitration was observed in stems and leaves of *S. habrochaites*. The pattern of immunoreactive bands in plants of *S. chmielewskii* followed that of *S. habrochaites*, but in general with lower band intensity.

3.3. GSNOR expression and activity in Solanum spp. genotypes are differentially modulated by pathogen infection

We analysed changes in GSNOR activity in infected plants both as local response in the inoculated leaves and systemic responses in the leaves above and below the site of inoculation (Fig. 4). GSNOR activity in inoculated leaves of susceptible genotype *S. lycopersicum* was slightly increased, whereas it showed a high increase in inoculated leaves of moderately resistant genotype *S. chmielewskii* namely 24 hpi. In contrast, enzyme activity was found strongly suppressed in the inoculated leaves of highly resistant genotype *S. habrochaites* during the interval 24–72 hpi. In case of the 1st leaf above the inoculation site, GSNOR activity in both susceptible and moderately resistant genotypes strongly decreased while remained generally unchanged in the resistant genotype. Interestingly, in case of 1st leaf below the infection site, GSNOR activity in moderately resistant genotype showed similar decreasing trend as in the 1st leaf above, whereas GSNOR activities in the susceptible and resistant genotypes increased after the inoculation.

Following powdery mildew infection, the levels of *GSNOR* transcripts showed a slight significant decrease in leaves of susceptible *S. lycopersicum* as well as of moderately resistant *S. chmielewskii* 72 hpi (Fig. 5a and b). Conversely, in highly resistant *S. habrochaites* the *GSNOR* gene expression was found induced in the infected leaves 24 hpi but reduced later at 72 hpi (Fig. 5c). The observed changes of *GSNOR* expression were in accordance with changes of GSNOR protein levels determined by Western blot analysis of leaf extracts (Fig. 5d).

Localization of GSNOR in transversal sections of inoculated leaves was analysed by CLSM (Fig. 5e). No immunofluorescence was detected in negative controls when the primary antibody was omitted (data not shown). GSNOR signal was more prominent in vascular bundles, parenchyma and epidermal cells of control plants compared to infected plants of susceptible and moderately resistant genotypes. The most pronounced decrease of GSNOR signal was recorded in the resistant genotype *S. habrochaites* 72 hpi.

3.4. NR expression and activity are down-regulated in susceptible but induced in resistant genotype by pathogen infection

Our previous results suggested that host-pathogen interactions in the pathosystem of *Solanum* spp. – *P. neolycopersici* involved an increased NO production, at least partly originated from plant NOS-like activity (Piterková et al., 2009). Here we investigated the modulation of NR activity and expression in studied *Solanum* spp. genotypes following their infection by the powdery mildew (Fig. 6). The modulation of *NR* gene expression following circadian rhythms was clearly observed in control non-infected plants of *S. lycopersicum*, including a decline at 4 hpi (corresponding to the time of sample collection at 12:30 a.m.), an increase at 8 hpi (4:30 p.m.), and identical levels of expression 0 and 24 h after the start of the experiment (at 8:30 a.m.) (Fig. 6a). However, the changes of *NR* expression during the day did not follow the same trend in resistant genotypes. In the susceptible genotype *S. lycopersicum*, the inoculation with *P. neolycopersici* decreased *NR* gene expression but slightly increased its enzyme activity (Fig. 6a, d). Surprisingly, low gene expression was observed 32 and 72 hpi in the infected leaves although the enzyme activity did not change significantly after the infection. In moderately resistant *S. chmielewskii* both *NR* expression and activity were decreased by infection (Fig. 6b, e). The decrease started already at 4 hpi and the lowest *NR* gene expression and lowest activity were detected at 8 and 24 hpi, respectively. Later, the measured parameters in infected samples slightly increased but still reached only 30–60% of the values in control non-inoculated plants. In resistant *S. habrochaites*, the initial decrease of *NR* transcripts during 24 hpi was followed by a steep increase of both *NR* expression and activity up to 72 hpi (Fig. 6c, f).

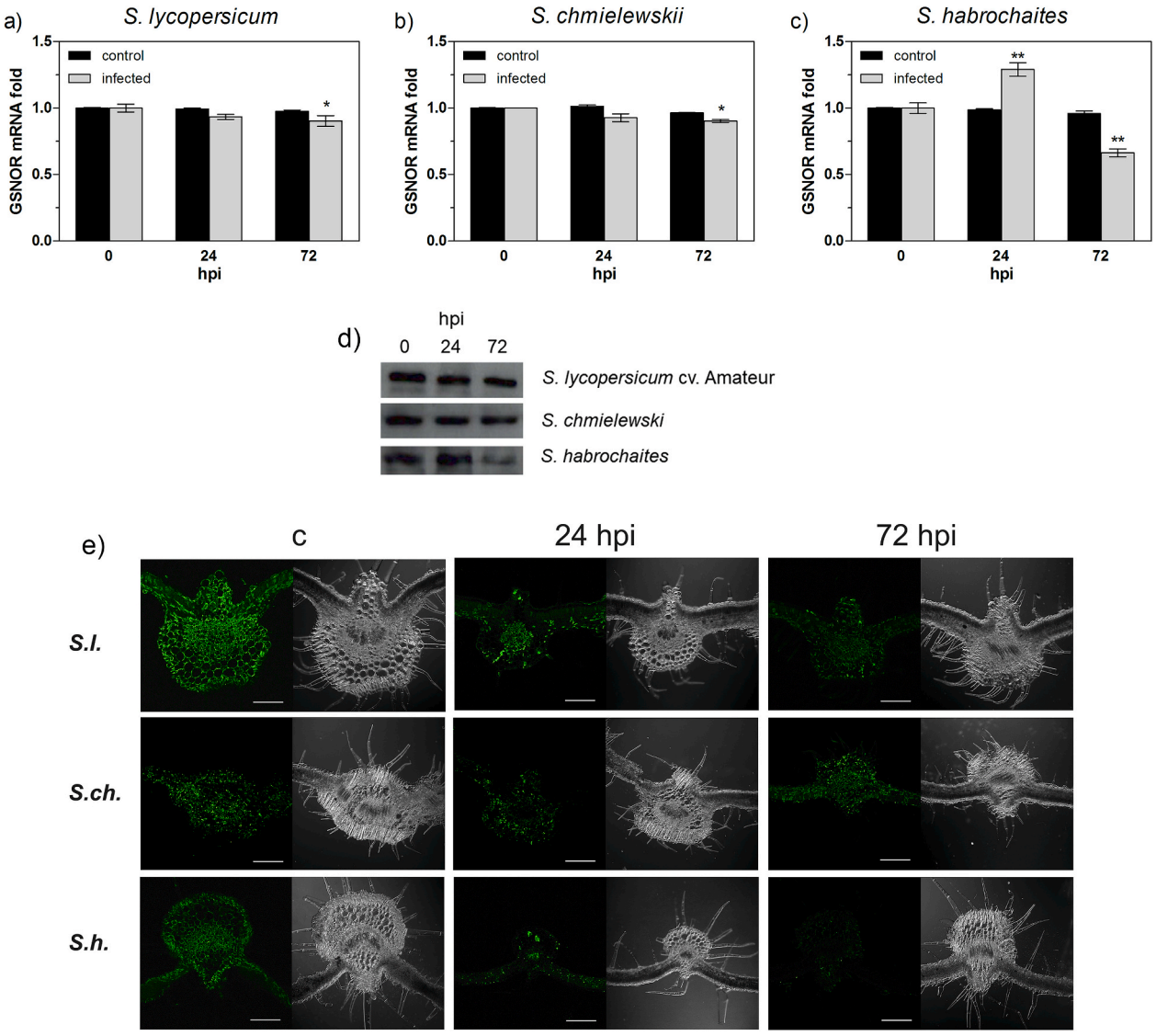

Fig. 5. Modulation of GSNOR expression and level in *Solanum* spp. genotypes infected with *P. neolycopersici*. Expression of *GSNOR* gene in *S. lycopersicum* cv. Amateur (**a**), *S. chmielewskii* (**b**) and *S. habrochaites* (**c**) was determined in inoculated leaves by qPCR at time intervals 0, 24 and 72 hpi. Data are shown as means ± SD ($n \geq 3$), statistical differences between values of infected and control plants of each genotype prior to inoculation at 0 hpi were evaluated by the unpaired *t*-test at $p < 0.05$ (*) and $p < 0.01$ (**). (**d**) Protein levels of GSNOR in leaf extracts were assayed by immunoblotting using anti-SlGSNOR polyclonal rabbit antibody. **(e)** GSNOR was localized by CLSM in leaf cross-sections from control and infected plants 24 and 72 hpi. Scale bar = 250 μm.

3.5. Changes in RNS and protein nitration in response to pathogen infection

Localization of RNS in leaf transversal sections was studied by CLSM using specific fluorescence probes (Fig. 7a–c). Production of endogenous NO was analysed using the fluorescent probe DAF-FM DA (Fig. 7a). The NO signal was localized mainly in the tissues surrounding the vascular bundles, in some epidermal cells or distributed in cells of the palisade and spongy mesophyll. Increased levels of NO was found after *P. neolycopersici* infection, where the strongest signal was observed in the moderately resistant genotype *S. chmielewskii* at 72 hpi, whereas lower levels of NO were found both at 24 and 72 hpi in the susceptible *S. lycopersicum*, and very weak NO signal in resistant *S. habrochaites* (Fig. 7a).

Highly reactive ROS (hROS) were localized in leaf section by CLSM using the fluorescent probe APF. The hROS signal was detected mainly in the mesophyll and vascular bundles (Fig. 7b), similarly to the localization of NO signal. In resistant genotypes, the hROS signal was higher in infected plants 24 hpi compared to controls, whereas at 72 hpi hROS were strongly decreased in *S. chmielewskii* but remained increased in *S. habrochaites*. CLSM observations revealed low GSNO levels in control plants which were increased following pathogen infection (Fig. 7c). The lowest signal detected at time 0 h in control plants of *S. chmielewskii* corresponds with the results for GSNOR analysis where a decreased signal was recorded in *S. habrochaites* at 72 hpi (Fig. 5e). The production of superoxide radical in plant leaves was analysed using the fluorescence probe dihydroethidium (Supplementary data, Fig. S1). The $O_2^{\cdot-}$ fluorescence signal was present mainly in the vascular bundles, but no significant changes of signal intensity were recorded among the studied genotypes or after the pathogen infection.

Protein nitration as an indicator of the nitrosative stress was studied in plant leaf extracts by immunoblot analysis (Fig. 8). In control plants as well as those infected with the powdery mildew, a pattern of seven pronounced immunoreactive bands was found with molecular masses of 20, 25, 30, 35, 40, 52 and 68 kDa. The bands of 35 and 30 kDa were the most prominent but in the case of 35 kDa band, no changes were observed after the infection. A slight decrease of protein nitration was observed in infected plants, except for the highly resistant genotype

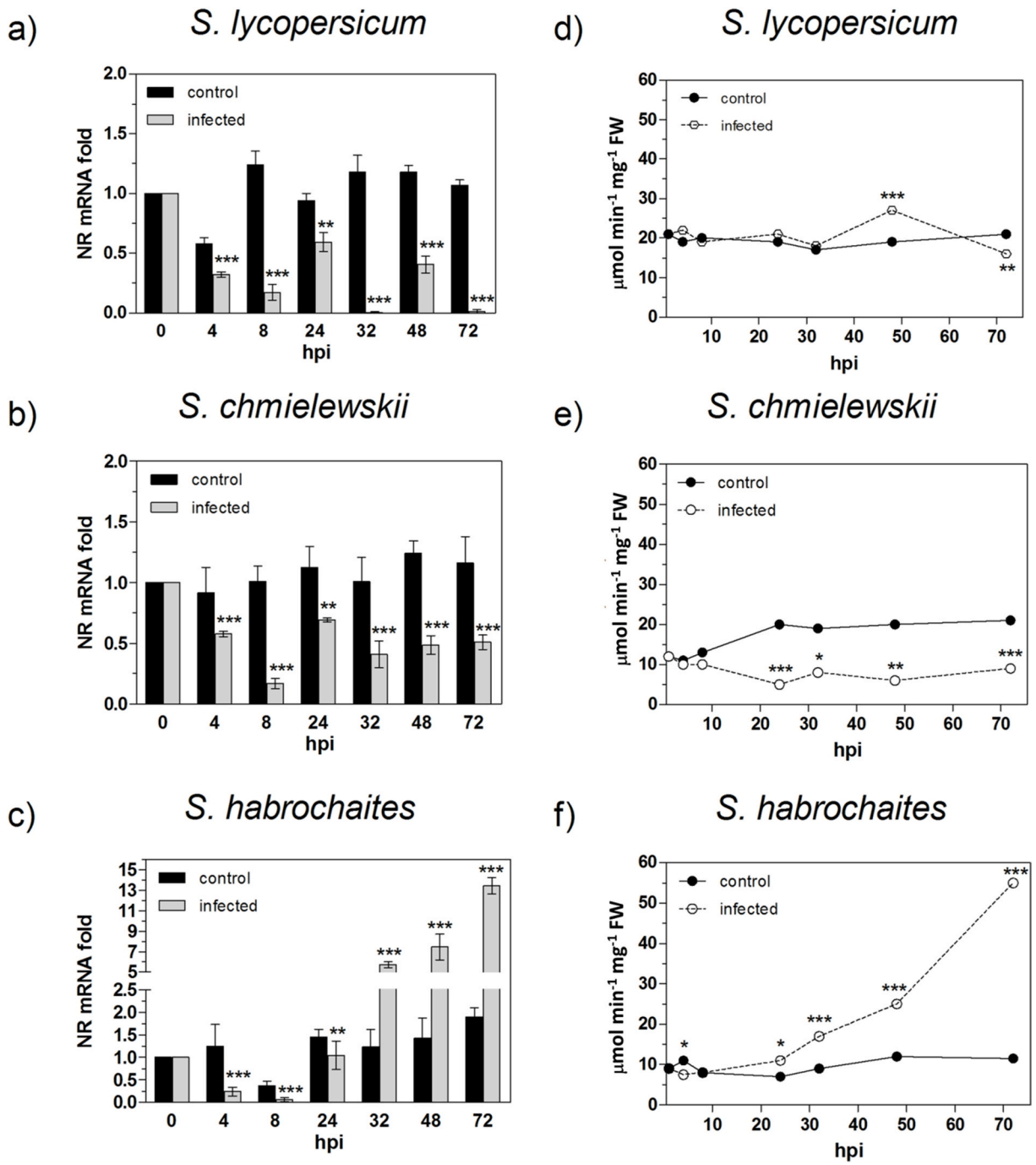

Fig. 6. Gene expression and activity of NR in leaves of *Solanum* spp. genotypes infected with *P. neolycopersici*.
NR gene expression (**a-c**) and NR enzyme activity (**d-f**) in plants of *S. lycopersicum* cv. Amateur (**a,d**), *S. chmielewskii* (**b,e**), and *S. habrochaites* (**c,f**) inoculated with *P. neolycopersici* were determined 0, 4, 8, 24, 32, 48 and 72 hpi. Data are presented as fold induction compared to control samples from non-infected plants. Data are shown as means ± SD (n ≥ 3). Asterisks were used to denote significant differences of the values of infected compared to corresponding control plants prior to inoculation at 0 hpi evaluated by unpaired *t*-test at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

S. habrochaites early after infection. Besides, an attenuation of the 68 kDa band was also detected in infected plants of *S. habrochaites*.

4. Discussion

4.1. Different levels of GSNOR are associated with different levels of RNS and protein nitration during plant development

In plants as well in other organisms, GSNO is considered to be a relatively stable and mobile reservoir of reactive NO (Sakamoto et al., 2002; Yun et al., 2016). Intracellular levels of GSNO are controlled by the rate of NO production and its nitrosative reaction with GSH, and by its irreversible catabolism mediated by GSNOR, by which this enzyme indirectly controls the transnitrosation balance between GSNO and protein S-nitrosothiols (Feechan et al., 2005; Foster et al., 2009). Previously we have characterised GSNOR from *S. lycopersicum* cv. Amateur, including changes in *GSNOR* gene expression during plant ontogenesis, kinetic properties and protein crystal structure (Kubienová et al., 2013). Study of *GSNOR* expression during the development of *Solanum* spp. genotypes showed it was higher in roots and stems compared to leaves

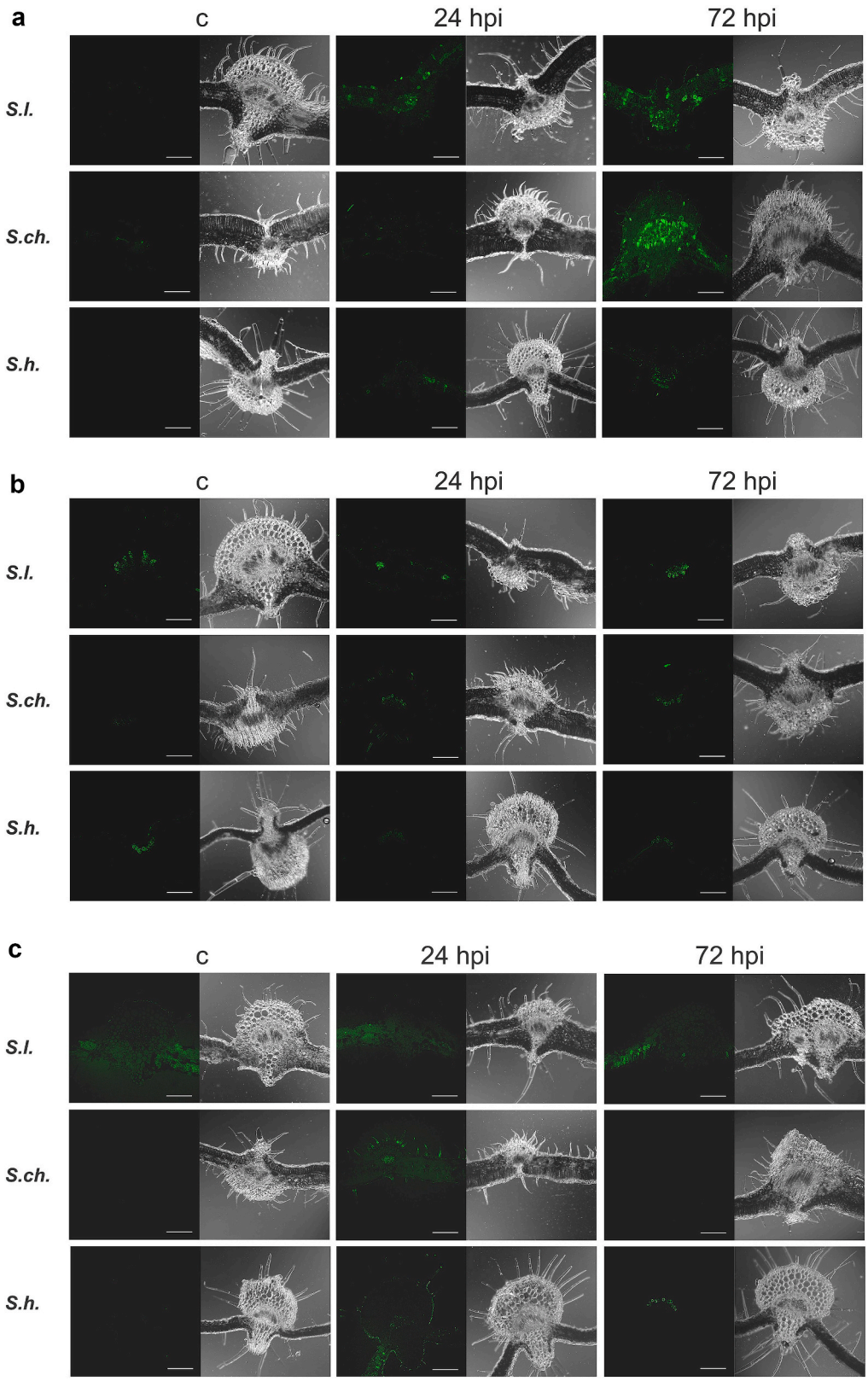

Fig. 7. Detection of NO, hROS and GSNO levels in leaves of *Solanum* spp. genotypes infected with *P. neolycopersici*.
(**a**)NO, (**b**) hROS and (**c**) GSNO were detected in transversal sections of leaves 0, 24 and 72 hpi with *P. neolycopersici*, using fluorescence probes (NO and hROS) or immunolocalization using a specific primary antibody (GSNO). The green fluorescence channel is shown separately (left) and complemented with Nomarsky DIC channel of each image (right). Bar scale = 250 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

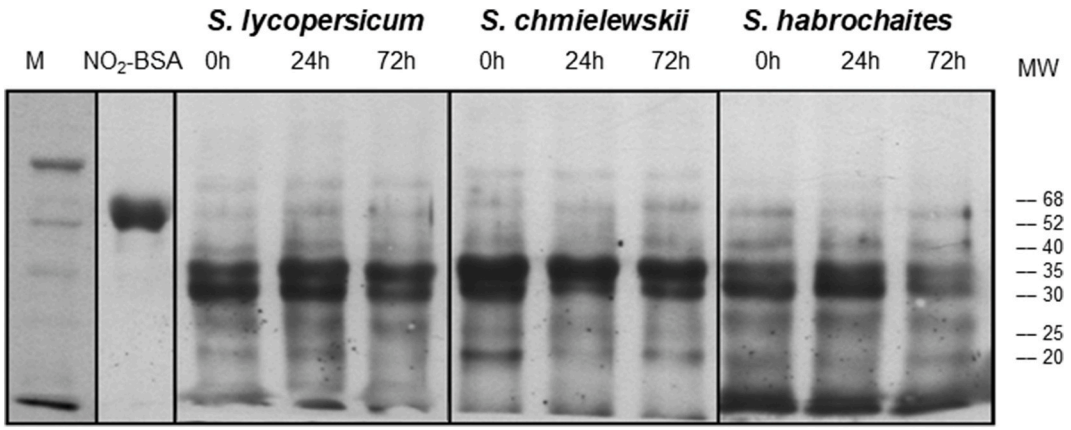

Fig. 8. Protein nitration in leaves of *Solanum* spp. genotypes infected with *P. neolycopersici*.
Levels of nitrated proteins were analysed in leaf extracts (100 μg of protein per lane) from plants of *Solanum* spp. infected with *P. neolycopersici* collected 0, 24 and 72 hpi. Extracted proteins were subjected to SDS-PAGE and Western blot analysis using a mouse polyclonal antibody against 3-nitrotyrosine. Commercial nitrated BSA (NO_2-BSA, 10 μg), served as the positive control; M, molecular weight markers.

and that it was also expressed in the pistil, stamens and fruits during ripening. High expression of *GSNOR* in reproductive organs can be related to the importance of GSNOR for floral development and fertility (Kubienová et al., 2013). *Arabidopsis* plants also show high *GSNOR* expression in roots and flowers (Espunya et al., 2006; Xu et al., 2013). Here we observed that non-infected *Solanum* spp. genotypes with different level of resistance to powdery mildew differ in their basal levels of *GSNOR* expression, the lowest found in highly resistant *S. habrochaites*. In later stages of development, higher expression was found in the root and stem as compared with decreased expression in leaves and the shoot apex. Search results obtained by Genevestigator (Zimmermann et al., 2004) using tomato (*S. lycopersicum*) genome array (LE_10K) also point to higher *GSNOR* expression in roots and fruits of mature plants.

The levels and organ localization of the *GSNOR* transcripts were observed in agreement with GSNOR activity measurements. The lower activity of GSNOR found in resistant plants may be related to diverse mechanisms of how defence reactions are triggered and how GSNOR is involved in the resistance signalling networks (Rusterucci et al., 2007). Interestingly, higher GSNOR activity was found in leaves of non-infected plants of a *Cucumis* spp. genotype susceptible to biotrophic pathogen *Golovinomyces cichoracearum* compared to resistant genotypes (Kubienová et al., 2014). Similarly, higher GSNOR activities were found in non-infected plants of susceptible *Lactuca sativa* UCDM2 and *L. serriola* genotypes in contrast to genotypes resistant to the biotrophic pathogen *Bremia lactucae* (Tichá et al., 2017a,b).

The content of GSNO in plant leaves was negatively correlated with GSNOR activity. These results are in agreement with previous findings in *A. thaliana* leaves and pepper plant organs (Airaki et al., 2011). Our data suggest the possible involvement of GSNOR in the regulation of protein nitration during plant development. Low levels of protein nitration were observed in *S. lycopersicum* cv. Amateur, which showed the highest GSNOR activity; conversely, intensive protein nitration found in *S. habrochaites* corresponds to low GSNOR activity. Protein nitration was previously found increasing with the age and namely during senescence of pea plants; however, the involvement of GSNOR and S-nitrosothiols in this process has not been studied and requires further investigation (Begara-Morales et al., 2013).

Recently, reduction of GSNOR expression by RNAi was found to impact a variety of developmental processes in tomato, including fruit formation and yield and seed development, whereas, unlike in Arabidopsis *gsnor* mutants, the root growth was not influenced (Hussain et al., 2019). However, this study used tomato dwarf cultivar Micro-Tom, which bears several mutant alleles responsible for its small plant size and determinate growth habit. Collectively, accumulated evidence suggests that GSNOR regulate development processes both in the model plant *A. thaliana* and tomato as an important crop species.

4.2. GSNOR and NR in Solanum spp. genotypes differing in pathogen resistance are differentially modulated by pathogen infection

The expression and activity of GSNOR are modulated by plant exposure to diverse stress stimuli. *GSNOR* expression in general decreases under abiotic stress, as shown for mechanical injury or heat stress in sunflower (Chaki et al., 2011a, 2011b), exposure to cadmium in pea (Barroso et al., 2006) or low temperatures in pepper plants (Airaki et al., 2012). However, we previously reported increased GSNOR activity in response to abiotic stress stimuli in pea and cucumber (Kubienová et al., 2014). Recently, overexpression of *GSNOR* was found associated with increased ROS scavenging and increasing tolerance to alkaline stress in tomato (Gong et al., 2014). Research on GSNOR role in plant responses to biotic stress with various pathosystems has resulted in divergent conclusions (Petřivalský et al., 2015; Jahnová et al., 2019). Reduced activity of GSNOR and subsequent increase in *S*-nitrosothiols were proposed to reduce basic and non-host plant resistance in *A. thaliana* infected with *Pseudomonas syringae* pv. *tomato* (Feechan et al., 2005) or in sunflower genotype resistant to *Plasmopara halstedii*, where higher S-nitrosothiol content, with concomitant low levels of NO, might prevent the initial pathogen spread (Chaki et al., 2009). Mutation of *atgsnor1* modulates the extent of cellular S-nitrosothiol formation and turnover, which controls multiple modes of plant disease resistance (Feechan et al., 2005). Transgenic *A. thaliana* plants with decreased *GSNOR* expression using antisense strategy showed increased levels of intracellular S-nitrosothiols and higher resistance to *Peronospora parasitica* (Rusterucci et al., 2007). This is in agreement with the present study on the tomato powdery mildew pathosystem, where observed decreased GSNOR and higher GSNO content seems to be involved in resistance mechanisms. However, reduction of GSNOR transcript abundance in the tomato dwarf cultivar Micro-Tom compromised plant immune response to bacterial infection by *P. syringae* pv. *tomato* DC3000 (Hussain et al., 2019), whereas GSNOR overexpression promoted tomato resistance in agreement with the previous report on Arabidopsis (Feechan et al., 2005). The discrepancies in the result might by caused by different used tomato genotypes or bacterial and fungal pathogens. Micro-Tom was previously reported to be susceptible to most of the important tomato pathogens showing typical infection symptoms, but some pathogens are restricted by either hypersensitive resistance or nonhost resistance, including *P. syringae* pv. *tomato* (Takahashi et al., 2005).

Interestingly, even at the start of the infection experiment, different

Solanum spp. genotypes showed high differences in measured GSNOR activities among the inoculated leaf and the leaves above and below the inoculation site (Fig. 4). These observations, in accordance with our previous results on the changes in NO production in the same tomato-powdery mildew pathosystem (Piterková et al., 2009) suggest the importance of the tomato plant architecture and phyllotaxy in differential capacities of individual leaves to activate responses to pathogen challenge. This potentially important factor has been largely unexplored in plants with leaf rosette architecture like Arabidopsis or lettuce (Tichá et al., 2018) but surely deserves further investigation in Solanaceae plants.

The comparison of changes observed on GSNOR mRNA and protein levels induced by the pathogen infection in our study support the potentially important role of enzyme regulation at the post-translational level. As reported previously, the changes of mitochondrial GSNOR activities were not correlated to changes in GSNOR gene expression but rather related to the ratio of NO and S-nitrosothiol content (Frungillo et al., 2013). The outcomes of a detailed study on AtGSNOR regulation by reversible inhibition through S-nitrosation of critical cysteines in GSNOR protein pointed to a molecular mechanism of reversible GSNOR down-regulation during NO bursts (Guerra et al., 2016). The suggested dynamic regulation of GSNOR by modifications of conserved cysteines may be involved in the control of intracellular RNS following perception of an NO signal. Our previous studies on the role of NO in *Solanum* spp. - *P. neolycopersici* interactions concluded that, at least in part, both local and systemic increase in NO production in later phases of the infection originated from the oxidative L-arginine-dependent pathway mediated by NOS-like enzyme activity (Piterková et al., 2009; Piterková et al., 2011). Here we addressed the involvement of NR, an enzyme known to contribute to NO production in plants (Yamasaki et al., 1999; Rockel et al., 2002). *NR* expression and activity is regulated by reversible phosphorylation affected by multiple factors such as light conditions, hormones or growth stage (Crawford, 2006; Lea et al., 2006). Pathogen-induced expression of NR genes in potato previously indicated involvement of NR in NO production observed during the pathogenesis of *Phytophthora infestans*, where the induction of *NR* gene peaked 6 h after inoculation with the incompatible, but not the compatible race of *P. infestans* (Yamamoto et al., 2003). Similarly, oligogalacturonides induced an accumulation of *NR* transcripts as well as NR activity in *Arabidopsis* together with increased NR-dependent NO production (Rasul et al., 2012). In tomato, NR mRNA, protein level and enzyme activity show robust circadian rhythms, with the highest activity in the morning and the lowest in the night (Lillo et al., 2004). Beside the circadian modulation of *NR* expression, we found strong down-regulation of *NR* gene following *P. neolycopersici* inoculation in susceptible *S. lycopersicum* cv. Amateur genotype and in a lesser extent also in moderately resistant *S. chmielewskii*, in contrast to high induction of *NR* expression and activity in the highly resistant *S. habrochaites* genotype. Surprisingly, observed high increase of NR activity in *S. habrochaites* was not accompanied by increased levels of NO evaluated in transversal sections of infected leaves of this genotype. However, previously we observed that preincubation of infected *S. habrochaites* leaves with NR inhibitor tungstate did not influence increased NO levels detected by confocal microscopy (Piterková et al., 2009; J. Piterková, personal communication). Accumulated data suggest an important role for NR in NO production in plant cells under physiological and stress conditions (Chamizo-Ampudia et al., 2017; Fagard et al., 2014). However, this is not supported by the observed low rate of NR-catalyzed NO production and the ambiguous correlation between NR activity and NO production efficiency (Fu et al., 2018). It was also suggested, that alternatively NR activity could be a source of substrates for NO production from L-Arg and nitrite in plant mitochondria (Salgado et al., 2006). Although NO, as a key component of plant-pathogen interactions responses, is strongly linked to the plant nitrogen metabolism, the exact mechanisms and roles of NR in NO production within nitrogen uptake and fate during plant pathogenesis are still largely unknown (reviewed in Fagard et al., 2014).

Previously, NO derived from nitrate assimilation was suggested to suppress GSNOR activity and hence the catabolism of GSNO in Arabidopsis (Frungillo et al., 2014). By these mechanisms including reversible GSNOR nitrosation, S-nitrosothiols might exert feed-back control of their production and scavenging. Moreover, NR might be also a target of S-nitrosation, as observed in poplar exposed to chilling stress, where stress-induced GSNOR inhibition promoted RNS accumulation and increased S-nitrosation of NR protein, suggesting a novel mechanism of posttranscriptional regulation of NR (Cheng et al., 2015).

Superoxide belongs to key reaction partners of NO because of their very fast reaction to produce $ONOO^-$ (Romero-Puertas et al., 2004). In this study, only a weak signal of intracellular production of $O_2^{\cdot-}$ was detected by CLSM mainly in vascular bundles of all genotypes, which was not influenced by *P. neolycopersici* infection 24 and 72 hpi. In the previous study on this pathosystem, superoxide generation was observed by NBT staining during the first few hours after pathogen infection only in the susceptible genotype *S. lycopersicum* cv. Amateur, which may be linked to the suppression of cell death or other host defences (Mlíčková et al., 2004). Based on our data on actual superoxide levels detected by a histochemical approach, we are not able to evaluate the rate of superoxide production. Two important pathways of superoxide conversion are supposed to operate in plant cells: superoxide dismutase-catalyzed decomposition to hydrogen peroxide or a fast reaction with NO to form peroxynitrite. As observed in previous studies on this pathosystem, increased levels of superoxide were observed in susceptible *S. lycopersicum* cv. Amateur, whereas the resistant genotypes presented increased DAB staining of H_2O_2, possibly related to cell death as a defence mechanism (Mlíčková et al., 2004).

NO and RNS play multiple roles in plant interactions with biotrophic pathogens, including pathogen growth and development, recognition and expression of plant defence mechanisms (Sedlářová et al., 2016). The previous histochemical study revealed NO production was specifically increased in leaf cells of resistant genotypes *S. chmielewskii* and *S. habrochaites*, which were in close contact or penetrated by invading pathogen structures (Piterková et al., 2011). Protein nitration is regarded as a marker of nitrosative stress in plants under environmental stresses such as salinity, mechanical wounding, low and high temperatures (Valderrama et al., 2007; Chaki et al., 2011a; Airaki et al., 2012) as well as in sunflower-downy mildew interactions (Chaki et al., 2009). Production of peroxynitrite ($ONOO^-$) involved in the process of protein nitration is dependent on the intensity and localization of NO and $O_2^{\cdot-}$ production (Corpas et al., 2007). In our study, changes in the representation of nitrated proteins were significant in resistant *Solanum* spp. genotype at 24 hpi, which correlates with expected $ONOO^-$ production during the pathogenesis of this genotype (Piterková et al., 2009). Our observation is in accordance with higher production of NO established by histochemical localization and detection of significantly increased NR activity of resistant genotype *S. habrochaites*. S-nitrosothiols might represent another source of RNS whose levels during pathogenesis is increased in resistant genotype due to decreased GSNOR activity. In pea plants exposed to low temperatures, activation of NOS-like activity was accompanied by an increase of S-nitrosothiol levels and intensification of protein nitration in the range of 29–59 kDa (Corpas et al., 2008). Similarly in the case of *Solanum* spp. changes in protein nitration were mainly in the range 20–68 kDa. Induction of protein nitration in 20–50 kDa range was observed in tobacco BY-2 suspension cells treated with a fungal elicitin INF1 from *Phytophthora infestans* (Saito et al., 2006) similarly to increased nitrotyrosine immunoreactivity during the hypersensitive response in *A. thaliana* (Romero-Puertas and Delledonne, 2007). Findings on interactions of sunflower with downy mildew *P. halstedii* suggest that the nitrosative stress induced under biotic stress conditions is supported by the induction of S-nitrosothiols content (Chaki et al., 2009). Protein nitration increased in the susceptible sunflower cultivar after infection, with its intracellular distribution coincident with that of S-nitrosothiols. Further study on nitro-oxidative stress

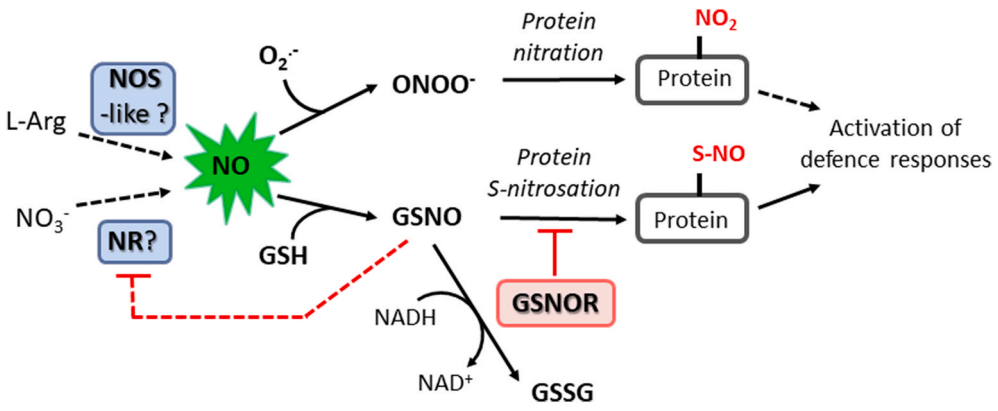

Fig. 9. Schematic model of the role of S-nitrosoglutathione reductase in the regulation of reactive nitrogen species metabolism in *Solanum* spp. GSH, L-glutathione; GSNO, S-nitrosoglutathione; GSNOR, S-nitrosoglutathione reductase; GSSG, oxidised glutathione; NOS, nitric oxide synthase; NR, nitrate reductase; $ONOO^-$, peroxynitrite.

induced by increased salinity in pea plants showed that monodehydroascorbate reductase was deactivated by post-translational modifications such as nitration and S-nitrosation, whereas glutathione reductase was not affected (Begara-Morales et al., 2015). This study concluded that the modulation of key enzymes of the ascorbate–glutathione cycle by reactive nitrogen species indicates an involvement of NO in antioxidant defence against nitro-oxidative stress in plants.

In summary, important modulations of GSNOR activity and expression during the development of *Solanum* spp. plants under physiological conditions were demonstrated. Different levels of the GSNOR were detected in plants varying in the resistance to a biotrophic pathogen as well as in the organ localization of the GSNOR enzyme activity and expression. Obtained results contribute to the understanding of the GSNOR key role in the regulation of not only S-nitrosothiol levels but of RNS metabolism in general during plant development under normal conditions and its involvement in molecular mechanisms of tomato resistance to biotrophic pathogens (Fig. 9). The importance of RNS for the resistance of *S. habrochaites* to powdery mildew was described together with the strong induction of NR expression and activity in later phases of plant infection. Studies on S-nitrosothiol status and identification of proteins S-nitrosated specifically in non-infected and infected plants are underway in our laboratory.

Author contribution

JB, LL, MP conception and design of the study; JB, LL, MP funding acquisition, project coordination; BM, JS, MS, TJ plant growth and pathogen application; BM, JJ, LC, MS, TJ sampling of plant material; JJ, JS, LC, MS, TJ, samples preparation, laboratory work and analyses; JB, LL, MP supervision of the laboratory work and analyses; JJ, LC, LL, MP data analysis and interpretation, manuscript writing, revision and editing; JB, JJ, LC, LL, MP, MS and TJ contributed to the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.06.039.

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