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ORIGINAL ARTICLE

Involvement of *S*-nitrosothiols modulation by *S*-nitrosoglutathione reductase in defence responses of lettuce and wild *Lactuca* spp. to biotrophic mildews

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Abstract

Main conclusion **Resistant *Lactuca* spp. genotypes can efficiently modulate levels of *S*-nitrosothiols as reactive nitrogen species derived from nitric oxide in their defence mechanism against invading biotrophic pathogens including lettuce downy mildew.**

Abstract *S*-Nitrosylation belongs to principal signalling pathways of nitric oxide in plant development and stress responses. Protein *S*-nitrosylation is regulated by *S*-nitrosoglutathione reductase (GSNOR) as a key catabolic enzyme of *S*-nitrosoglutathione (GSNO), the major intracellular *S*-nitrosothiol. GSNOR expression, level and activity were studied in leaves of selected genotypes of lettuce (*Lactuca sativa*) and wild *Lactuca* spp. during interactions with biotrophic mildews, *Bremia lactucae* (lettuce downy mildew), *Golovinomyces cichoracearum* (lettuce powdery mildew) and non-pathogen *Pseudoidium neolycopersici* (tomato powdery mildew) during 168 h post inoculation (hpi). *GSNOR* expression was increased in all genotypes both in the early phase at 6 hpi and later phase at 72 hpi, with a high increase observed in *L. sativa* UCDM2 responses to all three pathogens. GSNOR protein also showed two-phase increase, with highest changes in *L. virosa–B. lactucae* and *L. sativa* cv. UCDM2–*G. cichoracearum* pathosystems, whereas *P. neolycopersici* induced GSNOR protein at 72 hpi in all genotypes. Similarly, a general pattern of modulated GSNOR activities in response to biotrophic mildews involves a two-phase increase at 6 and 72 hpi. Lettuce downy mildew infection caused GSNOR activity slightly increased only in resistant *L. saligna* and *L. virosa* genotypes; however, all genotypes showed increased GSNOR activity both at 6 and 72 hpi by lettuce powdery mildew. We observed GSNOR-mediated decrease of *S*-nitrosothiols as a general feature of *Lactuca* spp. response to mildew infection, which was also confirmed by immunohistochemical detection of GSNOR and GSNO in infected plant tissues. Our results demonstrate that GSNOR is differentially modulated in interactions of susceptible and resistant *Lactuca* spp. genotypes with fungal mildews and uncover the role of *S*-nitrosylation in molecular mechanisms of plant responses to biotrophic pathogens.

Keywords *Bremia lactucae* · *Golovinomyces cichoracearum* · Lettuce downy mildew · Lettuce powdery mildew · Nitric oxide · *Pseudoidium neolycopersici*

Abbreviations

GSNO	*S*-Nitrosoglutathione
GSNOR	*S*-Nitrosoglutathione reductase
hpi	Hours post inoculation
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

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Introduction

Bremia lactucae, the causative agent of lettuce downy mildew, threatens plants of *Lactuca* spp. by infections causing important economic losses to the lettuce crop yield worldwide. For its high genotypic and phenotypic variability, this pathosystem is a widely used model to study plant–biotrophic oomycete interactions from field to molecular level (Lebeda et al. 2008). The complex process of *B. lactucae* pathogenesis involves penetration, formation of primary and secondary vesicles in the epidermal cells and formation of intracellular hyphae and haustoria (Sedlářová et al. 2001). Although the manipulation of host metabolism by downy mildew is inconspicuous in any of these stages, pathogen recognition and induction of a hypersensitive response can be initiated based on the presence of *Dm*-resistance genes or quantitative trait loci in attacked plants (Lebeda et al. 2008; Parra et al. 2016). Increased resistance to pathogens belongs to the traits of crucial importance in lettuce breeding (Lebeda et al. 2014a). Lettuce powdery mildew, *Golovinomyces cichoracearum*, has emerged recently as another important biotrophic pathogen of lettuce and *Lactuca* spp. (Lebeda et al. 2012, 2013). *Pseudoidium neolycopersici* (tomato powdery mildew) represent a well-recognized dangerous pathogen of *Solanum* spp., however, a non-pathogen of *Lactuca* spp. (Lebeda et al. 2014b).

Nitric oxide (NO) has been recognized as an intra- and intercellular signalling molecule with multiple functions in plant metabolism, development and stress responses (Mur et al. 2013; Domingos et al. 2015). NO plays important roles in seed germination, primary and lateral root growth, stomata closure, flowering, fruit ripening and senescence. Signalling pathways of NO in plants include cross-talk with other plant hormones and interactions with reactive oxygen species (ROS), hemoproteins and thiols, involved in the activation of ion channels, protein kinases and gene expression (Yu et al. 2014). NO-dependent protein modifications, such as cysteine S-nitrosation and tyrosine nitration, serve as redox signalling switches that regulate protein structure functions (Lamotte et al. 2015). Under abiotic or biotic stress conditions, NO belongs to key regulators of plant defence responses (Yun et al. 2016). Increased production and subsequent accumulation of NO and derived reactive nitrogen species (RNS) in specific plant cell compartments participate together with increased ROS levels in the initiation of plant hypersensitive response (Hong et al. 2008). In some instances, NO and ROS are also produced by pathogen structures as necessary prerequisites for development, virulence and host invasion; moreover, eukaryotic phytopathogens belonging to oomycetes and fungi possess their own machinery for NO turnover which enables effective balancing of the local NO concentrations and thus to associate with and benefit from their hosts (reviewed by Arasimowicz-Jelonek and Floryszak-Wieczorek 2016; Sedlářová et al. 2016).

NO and reactive nitrogen species can efficiently react with major cellular thiol glutathione to form *S*-nitrosoglutathione (GSNO), which as prevailing low-molecular cellular *S*-nitrosothiol can serve as relatively stable reservoir of NO in vivo and participate in NO systemic transport (Corpas et al. 2013). GSNO also participates in *trans*-nitrosylation of cellular proteins, NO release and binding to the relevant cysteine residues of proteins. This post-translational modification known as *S*-nitrosylation affects functions of a wide spectrum of target proteins in plants (Lamotte et al. 2015). Intracellular homeostasis of GSNO is regulated by NADH-dependent de-nitrosylation activity of *S*-nitrosoglutathione reductase (GSNOR, EC 1.1.1.284), a member of class III alcohol dehydrogenase family, which metabolizes GSNOR irreversibly to glutathione disulphide (GSSG) and ammonia (NH_3) (Liu et al. 2001). *S*-nitrosothiols and GSNOR occupy prominent positions in NO-dependent signalling pathways of plants exposed to stress conditions (Letterier et al. 2011; Frungillo et al. 2014; Yun et al. 2016). Biotic stress stimuli predominantly activates *GSNOR* expression, e.g. in *Arabidopsis thaliana* treated with salicylic acid (Diaz et al. 2003), while abiotic stresses reduces *GSNOR* expression, e.g. mechanical damage in sunflower (Chaki et al. 2011), heat stress in *Arabidopsis* (Lee et al. 2008) or exposure to cadmium in pea plants and *A. thaliana* seedlings (Barroso et al. 2006). On the other hand, upregulation of GSNOR was reported in *A. thaliana* seedlings exposed to arsenic (Leterrier et al. 2012), in cucurbits and pea exposed to injury or darkness (Kubienová et al. 2014), and *GSNOR* overexpression in tomato was attributed to tolerance of sodic alkaline stress (Gong et al. 2015). GSNOR can also partly mediate jasmonate-inducible responses in plant–herbivore interactions (Wunsche et al. 2011).

Changes in *S*-nitrosothiols levels controlled by GSNOR are indispensable for plant responses to pathogens similarly to animal immune system (Malik et al. 2011). Transgenic *A. thaliana* plants with partially reduced GSNOR levels by RNAi technique and simultaneously higher levels of *S*-nitrosothiols showed increased basal resistance against downy mildew *Hyaloperonospora parasitica*, whereas *GSNOR* overexpression led to weakening of systemic acquired resistance (Rusterucci et al. 2007). A hypothetical model proposed for defence mechanisms in *Arabidopsis*, where NO and GSNO play roles as positive regulators of plant resistance, hypersensitive response and the activation of defence genes, was later suggested also for abiotic stress (Espunya et al. 2012). However, research using *Arabidopsis* GSNOR knock-out mutants and of other pathosystems has led to contradictory conclusions when disease susceptibility

was promoted by the loss of GSNOR function. Decreased GSNOR activity and increased *S*-nitrosothiols resulted in compromised basal and non-host resistance in *A. thaliana* infected with bacteria (*P. syringae* pv. *tomato* DC3000), powdery mildew (*Blumeria graminis*) and downy mildew (*Hyaloperonospora parasitica*) (Feechan et al. 2005; Tada et al. 2008), or in sunflower (*Helianthus annuus* L.) resistant to pathogenic oomycete *Plasmopara halstedii* (Chaki et al. 2009). Increased *S*-nitrosothiols in *Arabidopsis* GSNOR mutants facilitate the synthesis of ROS and hypersensitive response in the absence of salicylic acid; however, high *S*-nitrosothiol-mediated *S*-nitrosylation of the NADPH oxidase abolishes its capacity of ROS production and represents a negative feedback loop limiting the hypersensitive response (Yun et al. 2011).

Recent data on the pathophysiology of lettuce downy mildew on *Lactuca* spp. obtained in our laboratory have brought a new complex insights into the role of oxidative and nitrosative processes including the role of NO and ROS and their regulation during plant–pathogen interactions (Sedlářová et al. 2007, 2011). In a previous study, higher GSNOR activities and reduced GSNO levels were observed in non-infected plants of two susceptible genotypes *L. sativa* UCDM2 and *L. serriola* compared to resistant *Lactuca* spp. genotypes (Tichá et al. 2017). To test the hypothesis on the key role of GSNOR in the regulation of *S*-nitrosothiol levels within plant responses to biotrophic mildews, we investigated the changes of GSNOR activity, gene expression and protein level in several *Lactuca* spp. genotypes, differing in their susceptibility to lettuce downy mildew, during 168 h after inoculation (hip) with three biotrophic pathogens *B. lactucae*, *G. cichoraceum* and *P. neolycopersici*. The main goal of present work was to unveil further aspects of nitrosative stress regulation, namely the role of GSNOR in *Lactuca* spp. plants dealing with biotrophic downy and powdery mildew infection, using two distinct host pathogens, *B. lactucae* and *G. cichoracearum* differing in their biology, reproduction and physiology as taxonomically distinct mildews. To compare mechanisms of non-host resistance to biotrophic mildews in *Lactuca* spp., the infection by a non-pathogen, tomato powdery mildew (*Pseudoidium neolycopersici*) (Lebeda et al. 2014b), was also included in our experimental study.

Materials and methods

Plant material and growth conditions

8-week-old plants of *Lactuca* spp. genotypes with different phenotypes of response to lettuce downy and powdery mildews were used: susceptible *L. sativa* L. (cv. UCDM2), *L. serriola* L. (LSE/18) and resistant *L. sativa* L. (cv. Mariska), *L. saligna* L. (CGN 05271) and *L. virosa* L. (NVRS 10.001 602) (Fig. S1). Table 1 provides details on their reactions to all three biotrophic pathogens used in this study (Fig. S2). Seeds were obtained from the collection of Department of Botany, Faculty of Science, Palacký University in Olomouc, Czech Republic. Plants were grown in a mixture of soil/potting medium (1:2 v/v) in a growth chamber at 18/15 °C and 12/12 h (light/dark cycles), illumination of 100 µmol photons m^{-2} s^{-1}.

Table 1 Reaction phenotypes of *Lactuca* spp. genotypes to *Bremia lactucae* (*Bl*), *Golovinomyces cichoracearum* (*Gc*), and *Pseudoidium neolycopersici* (*Pn*)

Host species	Genotype	Plant ID[b]	Reaction phenotype		
			Bl[c]	*Gc*	*Pn*[h]
L. sativa L.	UCDM2	09H5700701	+	+[d]	–[NR]
	Mariska	09H5700699	–	+/–[e]	–[NR]
L. serriola L.	LSE/18	09H5800722	+	+[f]	–[NR]
L. saligna L.	CGN 05271	09H5801010	–	+/–[g]	–[NR]
L. virosa L.	NVRS 10.001 602[a]	09H5800958	–	+/–[g]	–[NR]

Reaction phenotype: +, susceptible; –, resistant; +/–, moderately resistant; – *NR*, non-host resistance

[a]Synonym = LVIR/50

[b]EVIGEZ number; passport data on plant genetic resources maintained in the Czech Republic

[c]Sedlářová et al. (2001)

[d]Lebeda et al. (2013)

[e]Tichá, unpublished

[f]Lebeda et al. (2012)

[g]Mieslerová, unpublished

[h]Mieslerová et al. (2004)

Pathogens, inoculation and incubation

The first pathogen used in our experiments was the causal agent of lettuce downy mildew, *Bremia lactucae* Regel race BL 16 (syn. NL16; avirulent genotype Avr14 + Avr15 + Avr18; IBEB sextet code EU-A63/31/02/00) from UPOC collection of the Department of Botany, Palacký University in Olomouc, included in the Czech National Collection of Microorganisms (collection number UPOC-FUN-013), which was maintained and multiplied according to standard methods on seedlings of *L. sativa* L. (Cobham Green) (Sedlářová et al. 2001, 2007). Whole plants were inoculated by spraying leaves, mainly from abaxial sides, with a suspension of *B. lactucae* conidiosporangia in distilled water (concentration approximately 5×10^5 ml^{-1}). Following inoculation, the cultivation temperature was decreased to 15/10 °C, 12/12 h (light/dark). To establish optimal conditions for pathogen development

the plants were kept in darkness first 24 hpi (Sedlářová et al. 2007).

Lettuce powdery mildew [*Golovinomyces cichoracearum* var. *cichoracearum* (DC.) V.P. Heluta], isolate GC 1/11, from the collection of the Department of Botany, Palacký University in Olomouc, was maintained on susceptible *Lactuca serriola* L. (LSE/57/15) and subcultivated every 2–3 weeks (Lebeda et al. 2012). Leaves, covered by freshly sporulating *G. cichoracearum* mycelium from 80 to 100%, were used to inoculate adaxial side of the fourth true leaf of each *Lactuca* spp. plant by a surface contact (dusting/tapping). An average number of 65 ± 15 conidia were applied per mm^2 of a leaf surface.

Tomato powdery mildew (*Pseudoidium neolycopersici* (L. Kiss) L. Kiss) (isolate C-2) from the UPOC collection (UPOC-FUN-127) was used as non-pathogen (Mieslerová et al. 2004). Maintenance and inoculation was performed from susceptible tomato, *Solanum lycopersicum* cv. Amateur, as described elsewhere (Piterková et al. 2009). Plants inoculated by both powdery mildew species were kept at 20/18 °C, 12/12 h (light/dark).

Inoculation procedures in all experiments were initiated at 8:30 a.m. to keep circadian rhythm of GSNOR and avoid influence of daylong changes in plant metabolism. Fourth to sixth youngest leaves from control and inoculated plants of individual *Lactuca* spp. genotypes were harvested 0, 6, 24, 48, 72 and 168 hpi separately. Plant samples were either fixed and subjected to immunohistochemical staining followed by confocal microscopy, or frozen in liquid nitrogen and stored at − 80 °C until GSNOR activity, gene expression or *S*-nitrosothiol content were measured in the leaf extracts.

Preparation of plant extracts

Leaves were ground using a mortar and pestle in a liquid nitrogen in an extraction buffer [50 mM Tris–HCl pH 7.5, 0.2% (v/v) Triton X-100, 2 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride]. Homogenates were centrifuged at 16,000*g* for 30 min at 4 °C. Supernatants were passed through Sephadex G-25 gel filtration column (NAP-10, GE Healthcare). Columns were equilibrated with cold 10 mM sodium phosphate buffer pH 6.8, and eluted with 50 mM potassium phosphate buffer pH 7.8.

Measurement of *S*-nitrosothiol content by the Saville assay

The *S*-nitrosothiol content was determined by modified Saville method (Gow et al. 2007). Protein extracts (5 µl) were incubated in 96-well microplates for 5 min with 100 µl of 3.5% sulphanilamide in 0.5 M HCl or 100 µl of 3.5% sulphanilamide in 0.5 M HCl containing 1% $HgCl_2$. The formation of the azo dye product was obtained by reaction of the two samples for additional 5 min with equal volumes of 0.1% *N*-(1-naphthyl)-ethylendiamine dihydrochloride in deionized water. Absorbance values were subsequently read at 540 nm with micro-plate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). *S*-Nitrosothiols were quantified as the difference of absorbance between values obtained with and without added $HgCl_2$, comparing the values with a standard curve prepared using GSNO prepared according to (Moore and Mani 2002). The results were calculated per milligram of total protein measured by the Bradford method (Bradford 1976).

GSNOR activity assay

GSNOR activity was assayed in freshly prepared leaf extracts spectrophotometrically by monitoring the absorbance of NADH at 340 nm ($\varepsilon_{340} = 6220\ M^{-1}\ cm^{-1}$) at 25 °C on a micro-plate reader. The plant extracts were incubated in an assay mixture containing 20 mM Tris–HCl pH 8.0 and 200 µM NADH. The reaction was started upon addition of freshly prepared GSNO to the reaction mixture at a final concentration of 400 µM. The activity was expressed as nmol NADH consumed $min^{-1}\ g^{-1}$ of fresh weight.

Quantification of GSNOR by Western blotting

SDS-PAGE was carried out in 12% polyacrylamide gel and using a Mini-Protean electrophoresis cell (Bio-Rad). Separated proteins were electroblotted to 0.45-µm nitrocellulose membrane. The membrane was incubated in a blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS: 20 mM Tris–HCl, 500 mM NaCl, pH 7.5) for 2 h and incubated overnight with anti-SlGSNOR polyclonal rabbit antibody (dilution 1:1000 with blocking buffer). The membrane was washed six times in 0.1% Tween-20 in TBS for 10 min and then incubated in blocking buffer containing goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma-Aldrich, dilution 1:10,000) for 2 h. After washing in TBS containing 0.1% Tween-20 for 1 h, the membrane was incubated with a western blotting luminol reagent (Santa Cruz Biotechnology) for 5 min. The chemiluminiscence was detected with a photographic film (Amersham Hyperfilm ECL, GE Healthcare). The evaluation of signal intensity from gels was performed using ImageJ 1.33 software (National Institute of Health, Bethesda, MD, USA). Data represent means of signal intensities of three replicates for each plant genotypes.

Quantitative PCR

Total RNA from 100 mg of plant tissue was extracted using NucleoSpin Plant RNA kit (Macherey–Nagel). cDNA was synthesized from 1 µg of total RNA using Transcriptor

High-Fidelity Reverse Transcriptase (Roche) at 42 °C for 30 min. qPCR was performed using an Absolute SYBR Green ROX kit (ABgene) and gene-specific primers (Table S1) on CFX96 Touch C1000 Real-Time PCR Detection System (Bio-Rad). The following programme was applied: initial DNA polymerase activation 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. The specificity of PCR amplification was checked with a melting curve programme 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. Relative quantification of *GSNOR* gene expression was normalized to housekeeping genes *actin* and *tubulin* using the Pfaffl method (Pfaffl 2001).

GSNOR and GSNO immunodetection by confocal laser scanning microscopy

Leaves were cut into 5 × 5 mm pieces and fixed in 4% (w/v) formaldehyde in 0.1 M phosphate buffer, pH 7.4, for 3 h at room temperature. Serial sections of 100 µm were sliced on a vibratome (BangCo 1000Plus). After three washes for 10 min with the 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 and 0.1% (v/v) Triton X-100], the free-floating sections were incubated overnight at 4 °C with rabbit polyclonal antibodies against tomato GSNOR (dilution 1:500; Tichá et al. 2017) or rat polyclonal antibodies against GSNO (Agrisera, dilution 1:2000) in the washing buffer. After three washes with the washing buffer, the sections were incubated for 1 h at room temperature with goat anti-rabbit IgG (H + L) DyLight® 488 conjugate or rabbit anti-rat IgG (H + L) DyLight® 488 conjugate (Thermo Fisher Scientific) for tomato GSNOR and GSNO, respectively, diluted 1:1000 in the washing buffer. After incubation and subsequent three washes in the washing buffer, the sections were mounted on glass slides in 10 mM Tris–HCl, pH 7.4 containing 50% (v/v) glycerol. Samples were observed by IX81 microscope attached to the confocal laser scanning unit FV1000 (Olympus). The transmission light images were obtained by a 405-nm excitation using near-ultraviolet diode laser. Fluorescence corresponding to GSNO and GSNOR was visualized by argon laser excitation at 488 nm and signal detection at 505–525 nm. Controls for background fluorescence, usually negligible, were performed without the primary antibody and were observed in the beginning of each experiment.

Statistical analysis

Statistically significant differences of measured variables among studied genotypes were assessed by one-way analysis of variance (ANOVA) and further analyzed using Bonferroni Multiple-Comparison Test in NCSS 11.0.9 Statistical Software. Bars show the mean ± SD of a representative experiment performed in triplicate. For pairwise comparison, Student's *t* test was used. The level of significance is indicated in each figure.

Results

Increased *GSNOR* gene expression and GSNOR protein abundance triggered by pathogen inoculation

In all *Lactuca* spp. genotypes, the *GSNOR* expression was induced after inoculation by all three pathogens, namely at 6 and 72 hpi (Fig. 1). Induction of *GSNOR* expression was triggered by inoculation with *B. lactucae,* although less pronounced when compared to plants inoculated with *G. cichoracearum*. Among the studied genotypes, a high increase in *GSNOR* expression was recorded in general in *L. sativa* cv. UCDM2 interaction with all three pathogens. Among plants inoculated with *B. lactucae* or *P. neolycopersici* the highest levels of GSNOR expression were found at 6 or 72 hpi in *L. sativa* UCDM2, *L. virosa* and *L. saligna* at 6 or 72 hpi, respectively. In plants inoculated with *G. cichoracearum*, the highest increase was observed in *L. sativa* UCDM2 in both time intervals, followed by *L. sativa* cv. Mariska at 6 hpi and *L. saligna* at 72 hpi (Fig. 1). On the other hand, except for increase registered 6 hpi with *B. lactucae*, *L. serriola* (LSE/18) showed minor modulations of *GSNOR* expression, namely following inoculation *P. neolycopersici*.

The highest changes in relative abundance of GSNOR protein among all three studied pathosystems were observed in plants inoculated with *P. neolycopersici,* whereas similarly to gene expression the lowest changes were induced by *B. lactucae* (Fig. 2). Among the five studied genotypes inoculated by *B. lactucae*, the GSNOR protein content increased most strongly in highly resistant *L. virosa* 6–72 hpi (Fig. 2a). Following inoculation with powdery mildews the increase of GSNOR level was found at 6 and 72 hpi, mainly in *L. sativa* cv. UCDM2 and *L. virosa* (Fig. 2b, c).

Modulation of GSNOR activity in inoculated plants

Slight fluctuations in GSNOR activities over 168 h were recorded in non-inoculated control plants (Suppl. Fig. S3). In accordance with observed changes in *GSNOR* gene expression and protein abundance, a general pattern of GSNOR activity changes in response to biotrophic mildews infection involves a two-phase increase, i.e. peaks at 6 and 72 hpi (Fig. 3). Compared to interactions with both

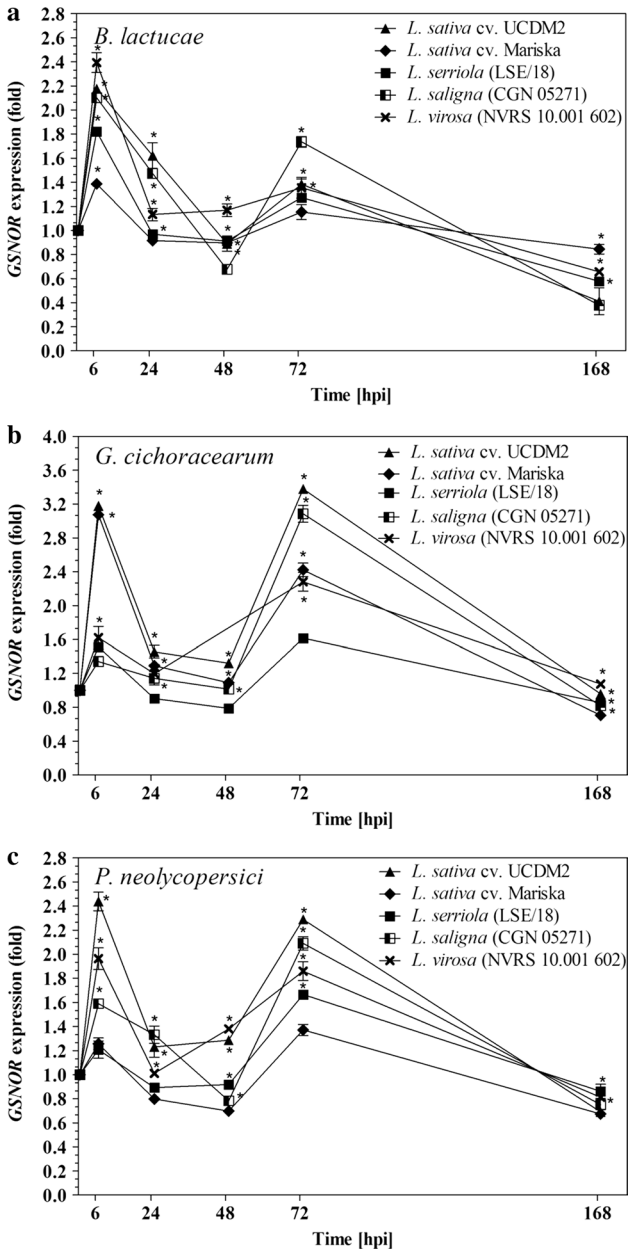

Fig. 1 Quantification of *GSNOR* gene expression during 168 h of *B. lactucae* (**a**), *G. cichoracearum* (**b**) and *P. neolycopersici* (**c**) pathogenesis on *Lactuca* spp. genotypes, determined by quantitative real-time PCR. Inoculated leaves were collected 0, 6, 24, 48, 72 and 168 h post inoculation (hpi). *GSNOR* gene expression was normalized to actin and tubulin mRNA levels. Data represent mean ± SD ($n \geq 3$). Significant differences among the genotypes (one-way ANOVA at $P < 0.05$ are denoted by asterisks (*)

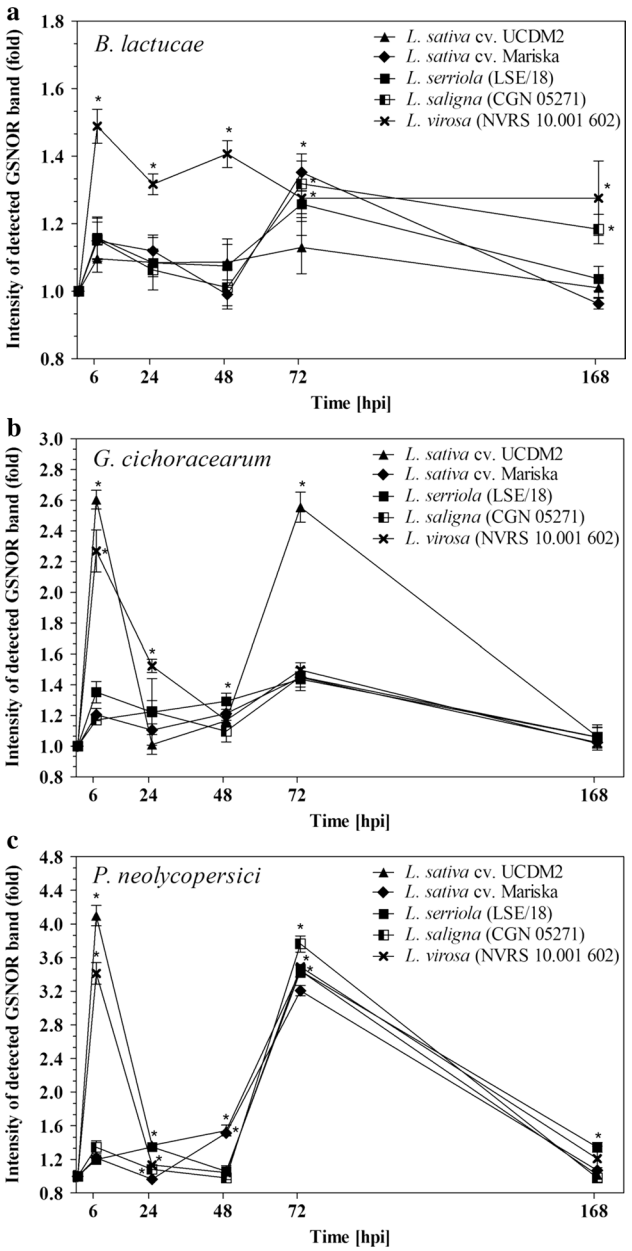

Fig. 2 Immunodetection of GSNOR protein during 168 h of *B. lactucae* (**a**), *G. cichoracearum* (**b**) and *P. neolycopersici* (**c**) pathogenesis on *Lactuca* spp. genotypes. Inoculated leaves were collected 0, 6, 24, 48, 72 and 168 hpi. Samples were subjected to 12% SDS-PAGE gel and western blot analysis using a nitrocellulose membrane probed with anti-*Sl*GSNOR polyclonal rabbit antibody (1:1000) and goat anti-rabbit IgG peroxidase conjugate (1:10,000). Detected band intensities were quantified by ImageJ 1.33 software using a non-infected control plant of each genotype as a reference value (= 1). Data represent mean ± SD ($n \geq 3$). Significant differences among the genotypes (one-way ANOVA at $P < 0.05$ are denoted by asterisks (*)

powdery mildews, only minor changes in GSNOR activity can be detected following inoculation by *B. lactucae* (Fig. 3a). The early increase of GSNOR activity (at 6 hpi) seems to be linked with resistance to *Bremia lactucae* as it occurred in all resistant genotypes but not in susceptible ones. However, no such general trend in GSNOR activity could be found in results obtained with powdery mildews (Fig. 3b, c). In susceptible *L. sativa* cv. UCDM2, the activity of GSNOR was slightly induced 72 hpi with *B. lactucae,* i.e. by approximately 60%. Conversely, inoculation by

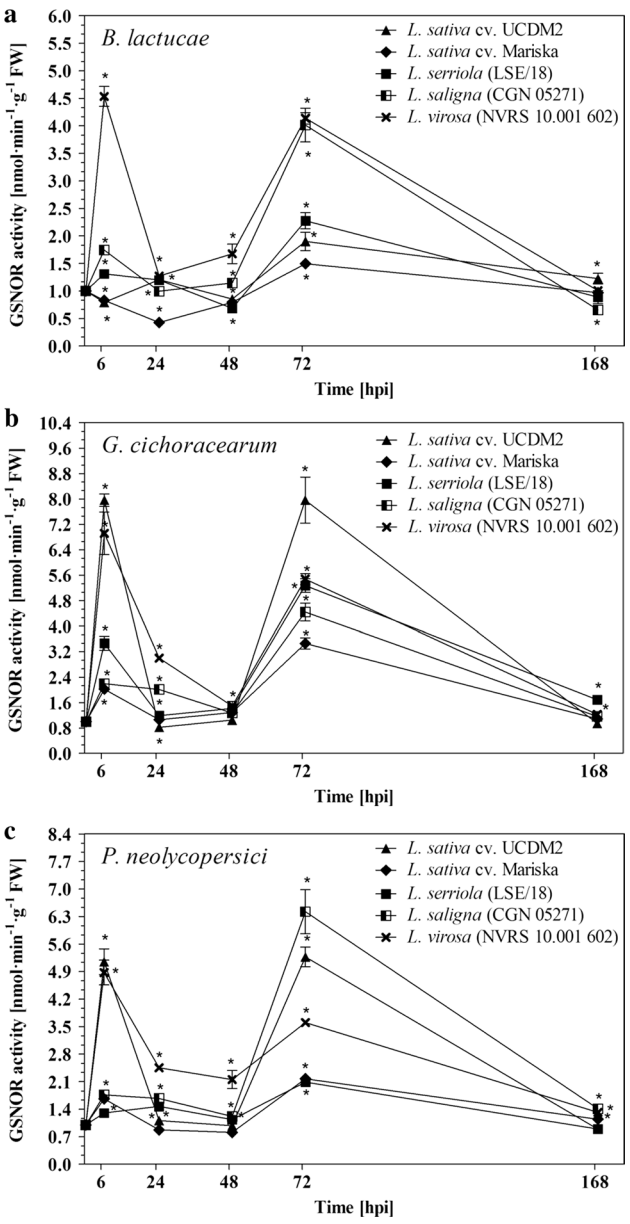

Fig. 3 GSNOR activity during 168 h of *B. lactucae* (**a**), *G. cichoracearum* (**b**) and *P. neolycopersici* (**c**) pathogenesis on *Lactuca* spp. genotypes. Inoculated leaves were collected 0, 6, 24, 48, 72 and 168 hpi. GSNOR enzyme activity in leaf extracts was evaluated spectrophotometrically at 30 °C by monitoring the absorbance changes at $\lambda = 340$ nm. Data represent mean ± SD ($n \geq 3$). Significant differences among the genotypes (one-way ANOVA at $P < 0.05$) are denoted by asterisks (*)

powdery mildew caused a strong GSNOR activity enhancement at 6 and 72 hpi, approximately eight-times in reaction to *G. cichoracearum* and six-times to non-pathogen *P. neolycopersici*. In moderately resistant *L. sativa* cv. Mariska (Table 1), the GSNOR activity was decreased 6 and 24 hpi but later increased 72 hpi with *B. lactucae* by approximately 20% (Fig. 3a). In contrast, inoculation by *L. sativa* cv. Mariska both powdery mildews led to a high increase of GSNOR activity by approximately 100 and 250% at 6 and 72 hpi, respectively (Fig. 3b, c). In both lettuce cultivars GSNOR activities at 24, 48 and 168 hpi equalled to those in controls. In susceptible *L. serriola* (LSE/18) exposed to *B. lactucae* the activity of GSNOR increased only at 72 hpi by approximately 120%. Similar minor changes were recorded also after *P. neolycopersici* inoculation. Oppositely, *G. cichoracearum* infection resulted in GSNOR activity increased approx. 3.5 times at 6 hpi and 6.5 times at 72 hpi (Fig. 3).

Within resistant response of *L. saligna* (CGN 05271) to *B. lactucae* GSNOR activity was found increased by approximately 50% at 6 hpi whereas by 250% at 72 hpi. The pattern of moderately resistant reaction of this genotype to *G. cichoracearum* infection included an enhancement of GSNOR activity by 120% at 6 hpi, 100% at 24 hpi and 300% at 72 hpi. *P. neolycopersici* inoculation elevated GSNOR activity by ca 50% at 6 hpi, 40% at 24 hpi and 450% at 72 hpi (Fig. 3). In the only *L. virosa* (NVRS 10.001 602), a genotype with the highest degree of resistance to *B. lactucae*, the enhanced GSNOR activity was recorded in reaction to all three pathogens at all studied time intervals. Following an initial increase at 6 hpi, the GSNOR activity dropped to values of control 24 hpi by *B. lactucae* and 48 hpi by *G. cichoracearum* with second peak at 72 hpi. Interactions with *P. neolycopersici* were characterized by GSNOR activities continuously elevated 6–72 hpi (Fig. 3).

Increased GSNOR and decreased GSNO levels in *L. virosa* leaves confirmed by immunohistochemical detection

The differences found on the level of GSNOR protein as assessed by quantitative western blot analysis of plant leaf extracts pointed us to investigate the localization of GSNOR in situ by immunohistochemical approach. We exploited rabbit polyclonal antibodies raised to tomato GSNOR, which were confirmed previously to show cross-reactivity to other plant GSNOR including LsGSNOR (Tichá et al. 2017). We selected the time point of 72 hpi for immunohistochemical analysis, as we consider it more relevant in relation to the timing of different phases of plant–pathogen interaction, namely with the pathogen penetration and development of pathogenic structure in penetrated leaf tissue, and increase production of NO and ROS as described previously (Sedlářová et al. 2007, 2011). In parallel, we also assessed levels of GSNO as GSNOR substrate using available commercial antibodies by immunostaining experiments, which were performed at 72 hpi when highest changes in GSNOR protein level were observed. Obtained results revealed GSNOR localization in both mesophyll and vascular bundles within the cross-sections of all studied *Lactuca* spp.

leaves (data not shown). Observed intensities of GSNOR immunostaining compared among pathosystems at 72 hpi followed the pattern of GSNOR immunodetection in leaf extracts by western blotting; therefore, only microphotographs of GSNOR localization in *L. virosa* are presented as representative examples (Fig. 4a). Increased signal for GSNOR immunostaining in *L. virosa* during *B. lactucae* and *P. neolycopersici* pathogenesis was accompanied by decreased fluorescence signals of its substrate GSNO compared to leaves of control plants (Fig. 4b).

Decreased total *S*-nitrosothiols content in response to pathogen inoculation

GSNOR is considered the key enzyme controlling intracellular levels of GSNO and hence to play important role in the regulation of protein S-nitrosylation (Salgado et al. 2013). Although GSNO represents the major low-molecular *S*-nitrosothiol, the major pool of total intracellular *S*-nitrosothiols is supposed to be composed by S-nitrosylated protein fraction. We, therefore, tested if observed modulations of GSNOR induced *Lactuca* spp. leaves by pathogen infection resulted in changes of total *S*-nitrosothiol content. Interestingly, in control non-inoculated plants the highest levels of *S*-nitrosothiols were found in resistant *L. virosa* and *L. saligna* but also in susceptible *L. sativa* cv. UCDM2 (Fig. 5). In all studied genotypes, the *S*-nitrosothiol content was reduced after inoculation with all three pathogens both in early and late time intervals after pathogen inoculation, though with different intensity. The strongest decrease of total *S*-nitrosothiols was detected in *L. virosa–P. neolycopersici* interaction at 6 and 72 hpi.

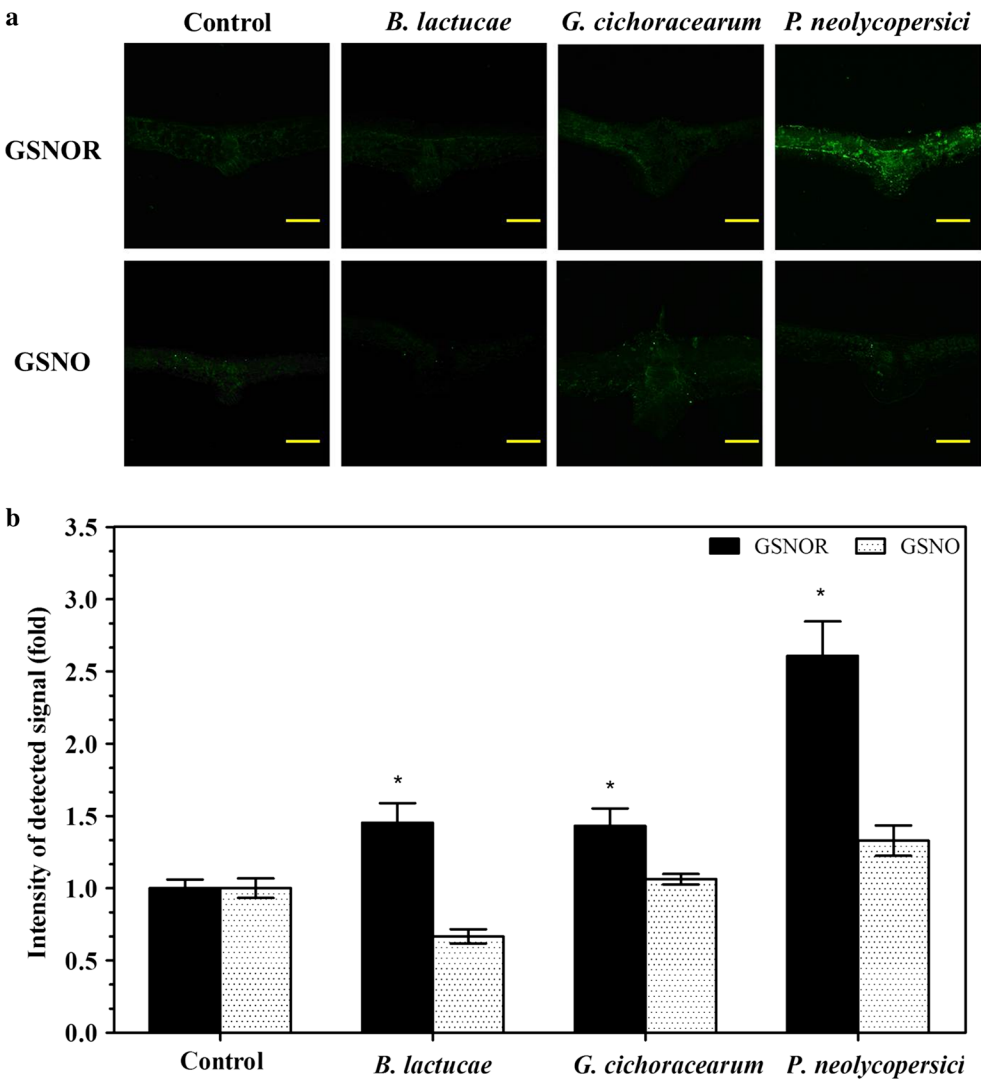

Fig. 4 Immunohistochemical localizations of GSNOR protein and its substrate GSNO (**a**) in cross-sections of control and inoculated *L. virosa* leaves 72 hpi. The bar represents 200 µm. Quantification of GSNOR and GSNO fluorescence signals detected by CLSM (**b**) using ImageJ software. Data represent mean ± SD ($n \geq 3$). Significant differences between control and inoculated samples (*t* test at $P < 0.05$) are denoted by asterisks (*)

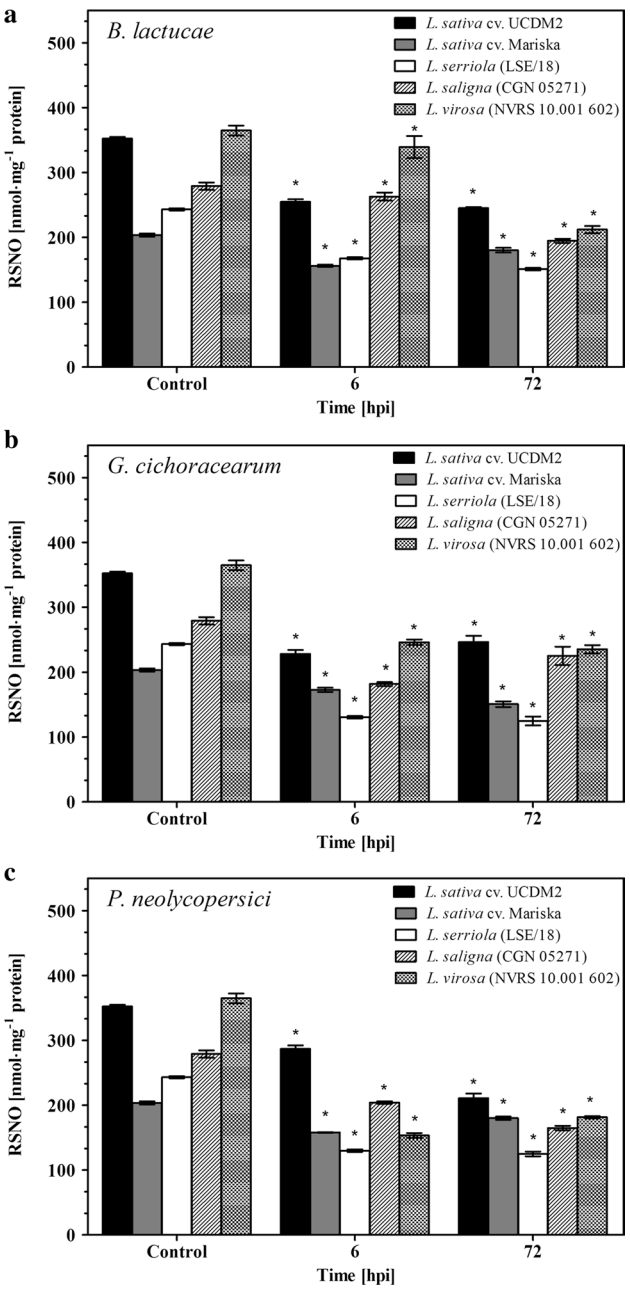

Fig. 5 Total *S*-nitrosothiol profiles during pathogenesis of *B. lactucae* (**a**), *G. cichoracearum* (**b**) and *P. neolycopersici* (**c**) on *Lactuca* spp. genotypes. *S*-Nitrosothiol content was determined by modified Saville method at $\lambda = 540$ nm. Data represent mean ± SD ($n \geq 3$). Significant differences between control and inoculated samples (*t* test at $P < 0.05$) are denoted by asterisks (*)

Discussion

Multiple studies on plant interactions with biotrophic pathogens uncovered pronounced modulations of ROS and RNS levels in infected plant tissues associated with key time points of the pathogenesis: pathogen recognition, penetration of germination tubes and establishment of pathogenic structures inside plant cells (Tománková et al. 2006; Piterková et al. 2009, 2011). Resistance mechanisms to *B. lactucae* in lettuce (*L. sativa*) and related wild species (*L. serriola, L. saligna, L. virosa*) studied herein have been previously linked with H_2O_2 accumulation 6–12 hpi followed by an increased activity of peroxidase and catalase at 48–96 hpi (Sedlářová et al. 2007). Major changes in ROS metabolism and local NO concentrations were observed in resistant *L. virosa*, which response to *B. lactucae* infection involves extensive hypersensitive response, leading to a successful cessation of pathogen growth (Sedlářová et al. 2007, 2011; Lebeda et al. 2008). Accordingly, the present results revealed the greatest changes in *GSNOR* gene expression, protein content and activity occurring in resistant *L. virosa* namely 6 hpi with *B. lactucae*. This early point of plant responses to *B. lactucae* pathogenesis seems to be crucial as the strong increase of GSNOR activity at 6 hpi was recorded only in the resistant but not in moderately resistant or susceptible genotypes. GSNOR irreversibly catabolizes GSNO, an essential reservoir of NO bioactivity, and changes in GSNO and NO levels represent critical aspects of plant-resistance mechanisms (Malik et al. 2011). Data presented herein support the evidence that GSNOR plays a key role in ameliorating the effects of nitrosative stress which would be manifested by increased GSNO levels triggered by high increase in NO and ROS production in the early phase of plant–pathogen recognition and interactions, similarly to GSNOR role in plant responses to abiotic stress conditions (Lee et al. 2008). The suggested role of increased *GSNOR* expression and activity in the control of nitrosative stress in resistant genotypes occurs both in early and later phase of pathogenesis at 6 and 72 hpi, respectively, similarly to reported biphasic increase in NO production in these time intervals in resistant genotypes (Sedlářová et al. 2011). As depicted in Fig. 2, we did not observe any significant decrease of the GSNOR level at the time interval of 168 hpi, where the GSNO level returned more or less to the initial level at 0 h. However, as shown in Fig. 1, the GSNOR gene expression at 168 hpi was slightly but significantly decreased compared to 0 hpi in all *Lactuca* spp. genotypes infected with *B. lactucae* and *P. neolycopersici*, and in all genotypes except *L. serriola* infected with *G. cichoraceum*. We consider as an important fact that on the level of measured GSNOR enzyme activity there was no substantial decrease detected at 168 hpi compared to the initial measurement at 0 hpi. In another recent study, we have also observed a similar trend in the pathosystem of three *Solanum* spp. genotypes infected with *P. neolycopersici*, where GSNOR expression increased at 24 hpi but decreased at 72 hpi compared to 0 hpi namely in the resistant genotype *S. habrochaites* (Činčalová, personal communication).

Activities of nitrate reductase and GSNOR, key enzymes involved in NO and RNS homeostasis, and of several

antioxidant enzymes were shown to be reduced by NO or RNS through a feedback mechanism involving *S*-nitrosylation (Begara-Morales et al. 2016).

Unlike the previously studied role of ROS and RNS role in plant responses to infection by *B. lactucae*, until now no data have been available for biochemical aspects of *Lactuca* spp. interactions with *G. cichoracearum*, where histological and molecular inputs of defence mechanisms have not been examined in great detail so far (Lebeda and Mieslerová 2011). According to recent results, it is evident that the interactions between *L. sativa*, *L. serriola* and *L. saligna* and *G. cichoracearum* are race-specific, i.e. gene-for-gene interactions are expected (Lebeda et al. 2012). Another limited study observed necrotic spots as signs of a hypersensitive response (HR) at sites of pathogen penetration in some *Lactuca* spp. accessions; however, pathogen development was not completely restricted by the necrosis (Lebeda and Mieslerová 2011). In the present study, we report a rather surprising finding on a higher intensity of GSNOR induction on gene expression, protein and activity levels in susceptible genotypes *L. sativa* cv. UCDM2 and *L. serriola* at both 6 and 72 hpi. In *A. thaliana* plants challenged with the oomycete *Hyaloperonospora arabidopsidis*, avirulent isolate Emwa1, high *S*-nitrosothiols were suggested to contribute to increased ROS production and cell death within the hypersensitive response, but also to inhibition of ROS-producing activity of NADPH oxidase by reversible S-nitrosylation (Yun et al. 2011). Whether such mechanisms operate on molecular level in *Lactuca* spp.—downy/powdery mildew pathosystems requires further investigation.

Interestingly, in leaves of *Cucumis sativus* cv. Stela susceptible to *Golovinomyces orontii* (syn. *G. cichoracearum*) significantly higher GSNOR activity was found under normal conditions in non-inoculated plants (Kubienova et al. 2014). In *A. thaliana*, the response to infection with the adapted powdery mildew (*Golovinomyces orontii*) characterized by compatible interaction were compared to incompatible interaction with the non-adapted powdery mildew pathogen, *Erysiphe pisi* (Schlicht and Kombrink 2013). The amount and timing of rapid and transient NO accumulation at infection sites was correlated to the resistance phenotype. Increased NO levels, induced by chemicals or expression of a NO-producing enzyme, resulted in enhanced resistance, but only sustained NO production prevented fungal leaf colonization, which was not achieved by a short NO burst which reduced only the initial penetration success.

We have previously investigated the role of NO and RNS in the resistance mechanisms of *Solanum* spp. to *Pseudoidium neolycopersici* (Piterková et al. 2011, 2013; Lebeda et al. 2014b). In this pathosystem, a pathogen-induced down-regulation of GSNOR activity was recorded, except for the highly resistant genotype *S. habrochaites* early after inoculation (Činčalová et al., unpublished results). Sampling revealed variation in GSNOR parameters as a local response in inoculated leaf in comparison with systemic responses in leaves above or below inoculation site, which supported previous assumption of NO involvement in both local and systemic responses of tomato to *P. neolycopersici* (Piterková et al. 2009). Whereas *P. neolycopersici* downregulated GSNOR in tomato host plants, as reported herein it upregulated GSNOR in non-host plants of lettuce and wild *Lactuca* spp. at 6 and 72 hpi, both recognized as crucial time points of pathogen development.

Immunohistochemical staining showed rather well-correlated decreases of GSNO signal in samples concomitant with GSNOR signal increase; however, no significant translocation of GSNOR or GSNO signals within *Lactuca* spp. leaf cross-sections was observed following pathogen inoculation. Redistribution of GSNO from hypocotyl cortex to epidermis was reported as a feature of sunflower resistance to downy mildew infection (Chaki et al. 2009). Representing the major cache for NO bioavailability, GSNO has been suggested as phloem mobile constituent for the long distance transport of NO as an important redox signalling molecule (Durner and Klessig 1999). In present study, GSNO signal was found in both constituents of vascular bundles, i.e. phloem and xylem, as well as in mesophyll cells of both spongy and palisade parenchyma, while similar localization was observed for lettuce GSNOR. We assume that such more general distribution might be affected also by a plant's growth habit, i.e. lettuce rosette, in contrast with *Arabidopsis* (Rusterucci et al. 2007) or tomato (Kubienová et al. 2013). Intracellular localization of GSNOR in plant cells has been previously attributed mainly to the cytoplasmic compartment and organelles with redox changes, such as peroxisomes (Barroso et al. 2013; Xu et al. 2013).

Several reports confirmed the importance of plant GSNOR in the regulation of bacterial and fungal infections. However, a relatively low number of studies published so far and conflicting results for diverse plant–pathogen interactions hinder generalization of GSNOR function in plant resistance. Decreased GSNOR activity concomitant with increased level of *S*-nitrosothiols resulted in decreased basal and non-host resistance of *A. thaliana* to bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Feechan et al. 2005). In contrast, increased resistance to *Hyaloperonospora parasitica* in transgenic *A. thaliana,* with GSNOR downregulated using an antisense strategy, was found linked with increased *S*-nitrosothiol levels and constitutive activation of *PR-1* gene (Rusterucci et al. 2007). GSNOR activity increased in hypocotyls of resistant but not susceptible sunflower genotype infected with sunflower downy mildew (*Plasmopara halstedii*) while GSNO accumulated in cortical cells was redistributed into epidermis (Chaki et al. 2009). In addition, also pathogens with different life strategies have been involved as found in published studies. For example,

necrotrophic fungus *Botrytis cinerea* causes an increase of GSNOR activity and reduction of the *S*-nitrosothiol content in tomato cell cultures which authors related with the susceptibility to pathogen (Pietrowska et al. 2015). GSNOR activity was induced also by hemibiotrophic oomycete *Phytophthora infestans* in susceptible potato. Study of systemic acquired resistance in potato plants which were primed with inducers (β-aminobutyric acid, 2,6-dichloroisonicotinic acid and laminarin) and later inoculated with *P. infestans* suggested that the amplification of signal for systemic acquired resistance promoted potato resistance, manifested by the upregulation of GSNOR activity tuned with the *S*-nitrosothiol pool depletion (Janus et al. 2013). In *A. thaliana,* GSNOR was also suggested as a key regulator of systemic defence responses though in vice versa action; GSNO accumulation was required to activate the jasmonic acid-dependent wound responses, and also to activate systemic acquired resistance in which GSNO was proposed to act synergistically with salicylic acid (Espunya et al. 2012).

In conclusion, we showed that the regulation of the GSNOR enzyme and *S*-nitrosothiols, together with previously reported ROS and NO signalling, plays an important role in the plant responses to pathogen attack. Apart from common trends for all five studied *Lactuca* spp. genotypes, e.g. GSNOR upregulation at 6 and 72 hpi and decreased *S*-nitrosothiol content under mildew infestation, differentially modulated changes in *GSNOR* expression and activity were uncovered. Specifically, the resistance to the causal agent of lettuce downy mildew *Bremia lactucae* was found to be accompanied with the increase of GSNOR activity at 6 hpi. Findings of induced GSNOR and decreased *S*-nitrosothiol levels also in susceptible *Lactuca* spp. genotypes suggest that a highly concerted action of ROS, NO in the modulation of molecular components of plant immunity requires a time- and site-specific regulation to achieve efficient responses within both the host- and non-host plant resistance to biotrophic pathogens. Apparently, the modulation of GSNOR play an important role in mildew pathogenesis on *Lactuca* spp. similarly to previously described interactions with pathogens in a model plant *A. thaliana* and crop species such as tomato or sunflower. Further experiments are necessary to identify the exact components of plant signalling and defence pathways which might be targets of *S*-nitrosylation modulated with responses of *Lactuca* spp. genotypes to infection.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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