Strigolactones interact with nitric oxide in regulating root system architecture of *Arabidopsis thaliana*

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Abstract

Both nitric oxide (NO) and strigolactone (SL) are growth regulating signal components in plants; however, regarding their possible interplay our knowledge is limited. Therefore, this study aims to provide new evidence for the signal interplay between NO and SL in the formation of root system architecture using complementary pharmacological and molecular biological approaches in the model *Arabidopsis thaliana* grown under control conditions. Deficiency of SL synthesis or signalling (*max1* and *max2-1*) resulted in elevated NO and S-nitrosothiol (SNO) levels due to decreased S-nitrosoglutathione (GSNO) reductase (GSNOR) protein abundance and activity indicating that there is a signal interaction between SLs and GSNOR-regulated levels of NO/SNO. This was further supported by the down-regulation of SL biosynthetic genes (*CCD7*, *CCD8* and *MAX1*) in GSNOR-deficient *gsnor1-3*. Based on the more pronounced sensitivity of *gsnor1-3* to exogenous SL (*(rac)*-GR24, 2 μM), we suspected that functional GSNOR is needed to control NO/SNO levels during SL-induced primary root (PR) elongation. Additionally, SLs may be involved in GSNO-regulated PIN1-dependent auxin distribution and PR shortening as suggested by the relative insensitivity of *max1* and *max2* mutants to exogenous GSNO (250 μM). Collectively, our results indicate a connection between SL and GSNOR-regulated NO/SNO signals in roots of *A. thaliana* grown in stress-free environment.

Running title: SL-NO interplay in *Arabidopsis* roots

1. Introduction

Strigolactones (SLs) have been first identified as germination inducers of parasite plants in the 1960s (Cook et al. 1966) and since then, they have been found to be phytohormones due to their multiple roles in regulating growth and developmental processes of higher plants (Umehara et al. 2008, Zwanenburg and Blanco-Ania 2018, Bouwmeester et al. 2019).

SLs as terpenoid lactones can be categorized as canonical SLs containing ABC ring and noncanonical SLs lacking such a ring (Waters et al. 2017). Strigolactones are synthetized from carotenoids in the plastids with the involvement of enzymes such as beta-carotene-isomerase (D27), two carotenoid cleavage dioxygenases (CCD7/MAX3 and CCD8/MAX4), cytochrome P450 (MAX1) and lateral branching oxidoreductase (Alder et al. 2012, Brewer et al. 2016). Following its transport into the cytoplasm, carlactone is converted into 5-deoxystrigol or orobanchol the main precursors of the naturally occurring SLs (Jia et al. 2019). However, our knowledge about the details of SL biosynthesis after carlactone is limited (Bouwmeester et al. 2019). It has been shown that SLs are synthetized in both the root and the shoot and that the strigolactone signal spreads from the root to the shoot system (Foo et al. 2001).

The perception of SLs involves the SL receptor DWARF14 (D14) protein having α/β fold hydrolase activity. Upon SL binding, the strigolactone ligand is hydrolysed and the conformation of D14 changes. Consequently, it can bind the MORE AXILLARY GROWTH2 (MAX2/D3) F-box type protein which assigns DWARF53 and SMXLs repressors for proteasomal degradation resulting in the induction of gene expression (Bouwmeester et al. 2019). The SL-induced gene expression manifests in physiological effects such as the inhibition of shoot branching, shaping of root system architecture, inducing leaf senescence (Pandey et al. 2016, Waters et al. 2017, Marzec and Melzer 2018). Furthermore, SLs have been implicated in plant stress responses to diverse abiotic factors (reviewed by Mostofa et al. 2018) like nutrient deficiency (Bouwmeester and Ruyter-Spira 2011), salinity and drought (Ha et al. 2014, Wang et al. 2019, reviewed by Mostofa et al. 2018) or chilling (Cooper et al. 2018).

Similar to SLs, research over the past 40 years has revealed that the gaseous signal molecule nitric oxide (NO) is a multifunctional growth regulator in plants (Kolbert et al. 2019a). While, the ability of SL synthesis is a unique feature of plants (Walker et al. 2019), any living organism is capable of the synthesis of NO. Algae utilize nitric oxide synthase (NOS)-like enzyme system for producing NO (Foresi et al. 2010, 2015, Weisslocker-Schaetzel et al. 2017) while in higher land plants NOS gene homologue to animal gene has not been found (Jeandroz et al. 2016, Santolini et al. 2017, Hancock and Neill 2019). The ability of NO liberation *via*

NOS-system may be lost during evolution of land plants (Fröhlich and Durner 2011) having nitrate-dependent metabolism. A key process in nitrate-dependent NO synthesis of plants indirectly involves nitrate reductase (NR) activity which transfers electron from NAD(P)H to the NO-forming nitrite reductase (NOFNiR). This enzyme catalyses the reduction of nitrite to NO (Chamizo-Ampudia et al. 2016, 2017). Nitric oxide is synthetized endogenously within the plant body in a wide variety of tissues and NO can also be taken up from the atmosphere or from the soil (Cohen et al. 2009). In biological systems, NO reacts with glutathione to form S-nitrosoglutathione (GSNO) being less reactive and more stable molecule than NO itself. GSNO is able to release NO and can achieve long distance movement of NO signal *via* the xylem (Durner et al. 1999, Díaz et al. 2003, Barroso et al. 2006). Intracellular levels of GSNO are controlled by the activity GSNO reductase (GSNOR) enzyme (Feechan et al. 2005, Lee et al. 2008, Chen et al. 2009) catalysing the conversion of GSNO to GSSG and NH_3 in the presence of NADH (Jahnová et al. 2019).

Unlike SLs, the signal of NO isn't perceived by specific receptor but the transfer of NO bioactivity is achieved by direct modification of target proteins. Cysteine S-nitrosation, tyrosine nitration and metal nitrosylation are three major NO-dependent posttranslational modifications being physiologically relevant (Astier and Lindermayr 2012). Additionally, the link between NO-related signalling and Ca^{2+}-, cGMP-, MAPK-, and PA-dependent signalling has also been revealed in diverse physiological processes (Pagnussat et al. 2004, Lanteri et al. 2008, Astier et al. 2011, Jiao et al. 2018). The physiological effects of NO can be categorized similar to that of SLs. Nitric oxide regulates growth processes at stages of seed development, vegetative and generative development like pollen tube growth, seed germination, root growth, gravitropism, flowering, fruit ripening (reviewed in Kolbert and Feigl 2017). Additionally, NO participates also in responses of plants to abiotic stresses like salinity, drought, heavy metal, low oxygen availability or temperature stresses (Fancy et al. 2017).

Based on the stimulating effect of NO on plant germination, vegetative growth or fruit ripening, NO-releasing substances such as nanoparticles could be effectively applied in agricultural practice (Rodríguez-Ruiz et al. 2019). Similarly, SLs and their agonists and antagonists may have a great potential for agricultural applications. Beyond plant protection, SLs may be used to improve the structure of crops as well (Vurro et al. 2016, Takahasi and Asami 2018).

It is sure that both NO and SL are important growth regulating signals of practical significance in plants, their interplay; however, been poorly examined. The majority of the few articles dealing with SL-NO interplay focuses on the root system of crops like sunflower (Barthi

and Bathla 2015), maize (Manoli et al. 2016) and rice (Sun et al. 2014) grown in the presence of different nutrient supply. To clarify the role of SLs in root development, Marzec and Melzer (2018) recommended to perform experiments with plants grown under stress-free conditions. Because of the above reasons, this study aims to provide new evidence for the signal interplay between NO and SL in the formation of root system architecture using complementary pharmacological and molecular biological approaches in the model *Arabidopsis thaliana* grown under control conditions.

2. Materials and Methods

2.1.Plant material and growth conditions

Seeds of *Arabidopsis thaliana* wild-type (WT, Col-0), and their mutant lines *gsnor1-3* (Chen et al. 2009), *35S:FLAG-GSNOR1* (Frungillo et al. 2014), *max1*, *max2-1* (Stirnberg et al. 2002) were surfaced sterilized with 70% (v/v) ethanol for 1 min, and with 30% sodium hypochlorite solution (1:3) for 15 min then washed five times with sterile distilled water. Seeds (approx. 30 seeds/Petri dish) were then transferred to half strength Murashige and Skoog medium (1% sucrose, 0,8% agar). Petri dishes were kept in a greenhouse at a photon flux density of 150 μmol m^{-2} s^{-1} (12/12 h light and dark cycle) at a relative humidity of 55-60% and 25 ± 2 ºC for 7days.

2.2.Treatments

Stock solution of *(rac)*-GR24 and TIS108 (both purchased from Chiralix B.V., Nijmegen, Netherlands) was prepared in acetone or in DMSO, respectively. Appropriate volumes of stock solutions were added to the medium following sterilization through sterile syringe yielding 2 μM GR24 or 5 μM TIS108 concentrations in the media. These concentrations were chosen in pilot experiments using several doses (1, 2, 5 μM for GR24 and 1, 5, 10 μM for TIS108). Stock solutions of GSNO and 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) were prepared in DMSO and were diluted to the final concentrations (250 μM GSNO and 800 μM cPTIO) with distilled water. Four days after placing the seeds on the media, GSNO and cPTIO solutions were added to the surface of the agar containing the root system. One milliliter of GSNO or cPTIO was added per Petri dish using 2-ml syringe and sterile filter.

2.3.Morphological measurements

Primary root lengths of *Arabidopsis* seedlings were measured and expressed in mm. Lateral roots within the primary root (smaller than stage VII) were considered as lateral root primordia (LR_{prim}), whereas visible laterals which have already grown outside the PR were considered as emerged LRs (LR_{em}, larger than stage VII, Malamy and Benfey 1997, Feigl et al. 2019). Number of LR_{prim} and LR_{em} was determined by using Zeiss Axiovert 200 inverted microscope and 20x objective (Carl Zeiss, Jena, Germany). Lateral root density (pieces mm^{-1}) was calculated by dividing total number of LRs with PR length.

2.4.Detection of NO levels

Levels of NO were detected with the fluorophore, 4-amino-5-methylamino2'-7'-difluorofluorescein diacetate (DAF-FM DA). *Arabidopsis* seedlings were incubated in 10 μM dye solution for 30 min, in darkness, at room temperature and washed two times with TRIS-HCl buffer (10 mM, pH 7,4) according to Kolbert et al. (2012). Stained root samples were observed under Axiovert 200M (Carl Zeiss, Jena, Germany) fluorescent microscope equipped with digital camera (Axiocam HR) and filter set 10 (excitation 450-490 nm, emission 515-565 nm) Fluorescence intensities in the primary roots were measured on digital images using Axiovision Rel. 4.8 software within circles of 38 μm radii.

2.5.Determination of S-nitrosothiol (SNO) contents

The amount of SNO was quantified by Sievers 280i NO analyser (GE Analytical Instruments, Boulder, CO, USA) according to Kolbert et al. (2019b). Briefly, 250 mg of *Arabidopsis* seedlings were mixed with double volume of 1x PBS buffer (containing 10 mM N-ethylmaleimide and 2.5 mM EDTA, pH 7.4) and were grounded using Fast Prep ® Instrument (Savant Instruments Inc., Holbrook, NY). Samples were centrifuged twice for 15 min (20 000 g, 4 ºC). The supernatants were incubated with 20 mM sulphanilamide. 250 μL of the samples were injected into the reaction vessel filled with potassium iodide. SNO concentrations were quantified with the help of NO analysis software (v3.2).

2.6.Western blot analysis of GSNOR protein abundance

Whole *Arabidopsis* seedlings were grounded with extraction buffer (50 mM TRIS-HCl, pH 7.6-7.8) and centrifuged (4 ºC, 9300 g, 20 min). Protein extract was treated with 1% proteinase inhibitor and stored at -80 ºC. Protein concentrations were determined using the Bradford (1976) assay.

Fifteen microliters of denaturated protein extract was subjected to SDS-PAGE on 12 % acrylamid gel. Proteins were transferred to PVDF membranes using the wet blotting procedure (25 mA, 16h). After that, membranes were used for cross-activity assays with rabbit polyclonal antibody against GSNOR (1:2000). Immunodetection was performed by using affinity, isolated goat anti-rabbit IgG-alkaline phosphatase secondary antibody at a dilution of 1:10000, and bands were visualized by using the NBT/BCIP reaction.

2.7.Spectrophotometric measurement of GSNOR activity

The specific activity of GSNOR was measured by monitoring the NADH oxidation in the presence of GSNO at 340 nm (Sakamoto et al. 2002). Plant homogenate was centrifuged (14 000 g, 20 min, 4 ºC) and 100 µg of protein extract was incubated in in 1 ml reaction buffer (20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.2 mM NADH). Data are expressed as nmol NADH min^{-1} mg $protein^{-1}$.

2.8.Qantitative real time PCR analysis

The expression rates of *Arabidopsis* genes *(NIA1, NIA2, GLB1, GLB2, GSNOR1, CCD7, CCD8, D14, MAX1, MAX2)* were determined by quantitative real-time reverse transcription PCR (RT-qPCR). RNA was purified from 90 mg of 7-days-old seedlings by using a NucleoSpin RNA Plant mini spin kit (Macherey-Nagel) according to the manufacturer's instruction. Furthermore, an additional DNAase digestion and purifying step was applied (ZYMO Research) and cDNA was synthetized using RevertAid reverse transcriptase. Primer3 software was used for designing primers. The primers used for RT-qPCR analyses are listed in Table S1. The expression rates of the NO- an SL associated genes were detected by quantitative real time PCR machine (qTOWER 2.0, Jena Instruments) using SYBR Green PCR Master Mix (Thermo Mix) (Gallé et al. 2019). Data were analysed by using qPCRsoft3.2 software (Jena Instruments). Data were normalized to the transcript levels of the control samples, *ACTIN2* (At3918780) and *GAPDH2* (At1913440) were used as internal controls (Papdi et al. 2008). Each reaction was carried out in three replicates using cDNA synthesized from independently extracted RNAs.

2.9.Measurement of NO liberation capacity of GSNO

Nitric oxide-sensitive electrode (ISO-NOP 2 mm, World Precision Instrument) was calibrated using a method of Zhang (2004). Donor solution (1 ml 250 µM GSNO in distilled water) was prepared and placed under illumination (150 µmol m^{-2} s^{-1}) in the greenhouse in order to stimulate conditions similar to treatment conditions. To ensure constant mixing of the solution magnetic stirrer was applied during the measurement. NO concentration (nM) was calculated from a standard curve. The standard curve and the results are presented in Fig S1.

2.10. Statistical analysis

All results are expressed as mean ± SE. Graphs were prepared in Microsoft Excel 2010 and in SigmaPlot 12. For statistical analysis, Duncan's multiple range test (one-way ANOVA, $P \leq 0.05$) was used in SigmaPlot 12. For the assumptions of ANOVA, we used Hartley's F_{max} test for homogeneity and the Shapiro-Wilk normality test.

3. Results and Discussion

3.1. Root system of GSNOR- and SL mutant *Arabidopsis* seedlings

Compared to the wild-type (*Col-0*), the primary root of *gsnor1-3* mutant was 57% shorter, its root system contained very few lateral roots, and consequently its LR density was low indicating that GSNOR activity is necessary for normal root development (Lee et al. 2008, Holzmeister et al. 2011, Kwon et al. 2012). Similarly, *35S:FLAG-GSNOR1* seedlings had shortened primary roots and reduced numbers of laterals resulting in WT-like LR density, and the LR primordia to emerged LR ratio was similar to that of Col-0. This means that not only the reduced GSNOR activity but also the overexpression of GSNOR enzyme negatively affect root elongation and lateral root development. As for the *max1* mutant, WT-like PR length was accompanied by increased number of emerged lateral roots and by consequently enhanced LR density compared to Col-0. The primary root of *max2-1* mutant proved to be slightly (by 14%) shorter than in Col-0 and the LR number was significantly increased. The branched root systems of *max1* and *max2-1* suggest that MAX1-dependent SL biosynthesis and MAX2-associated SL-signalling inhibits LR development as was published previously by others (Kapulnik et al. 2011, Ruyter-Spira et al. 2011, Villaécija-Aguilar et al. 2019). The LR_{prim} : LR_{em} ratio was similar in Col-0 and the mutants suggesting that SLs similarly influence both the initiation and the emergence of LRs.

3.2. Levels of NO and SNO in GSNOR- and SL mutant *Arabidopsis* seedlings

As shown in Fig 2, the level of NO and SNO in *gsnor1-3* was higher than in Col-0, while in *35S:FLAG-GSNOR1* plants, the increased endogenous NO level was accompanied by lower SNO levels than in the WT (Kolbert et al. 2019b). Additionally, in *max1* and *max2-1* significantly increased NO level and SNO content was detected compared to Col-0 (Fig 2).

Expressions of genes involved in NO metabolism (*NIA1*, *NIA2*, *GLB1*, *GLB2*) in *max1* mutants were similar to Col-0 but all examined genes were slightly down-regulated in *max2-1* (Fig 3). However, the changes were small and were not detectable in both *max* mutants, suggesting that these genes may not play a significant role in the regulation of NO in the absence of SLs.

Higher NO levels of the *max* mutants may be associated with higher SNO levels. GSNOR is a key regulator of SNO metabolism (Lindermayr 2018), thus we assumed that *max*

mutants show differences in association with GSNOR enzyme. Although, there were no relevant differences in the rates of *GSNOR1* expression in the plant lines (Fig 4A), the GSNOR protein abundance was significantly lower in *max* mutants compared to Col-0 (Fig 4 BC) and also the activity of the enzyme was decreased in *max1* and *max2-1* mutant seedlings (Fig 4D) which may provide explanation for the elevated SNO and NO levels (Fig 3). These results indicate that SL deficiency posttranscriptionally influence GSNOR enzyme, therefore we examined the responses of GSNOR deficient and -overexpressing *Arabidopsis* lines to exogenous application of SL analogue GR24 and SL synthesis inhibitor TIS108.

3.3. The effect of SL analogue and inhibitor on root system and NO-associated genes in *Arabidopsis*

Similar to previously published results, GR24 treatment induced PR elongation in Col-0 *Arabidopsis* plants (Ruyter-Spira et al. 2011, Sun et al. 2014, Marzec 2016), while TIS108 caused 50% inhibition of it (Fig 5A). In case of *gsnor1-3*, SL analogue did not trigger PR elongation and TIS108 reduced PR length by 67% compared to the control. These suggest that the root system of *gsnor1-3* is more sensitive to modifications of SL levels meaning that functional GSNOR enzyme is needed to control NO/SNO levels and to the positive effect of GR24 on PR elongation. Presumably, in case of GSNOR deficiency, NO/SNO levels are not properly regulated and high NO/SNO levels may cause PR shortening instead of elongation (Fernández-Marcos et al. 2011). The root elongation response of *35S:FLAG-GSNOR1* to SL analogue or inhibitor did not differ from that of Col-0 indicating that overexpressing GSNOR enzyme has no effect on SL-induced elongation (Fig 5A). Treatment with GR24 resulted in reduced LR_{em} number and unchanged LR_{prim} number (Fig 5B) suggesting that SLs influence LR emergence but not LR initiation. Jiang and co-workers (2016) published contrasting results in rice where GR24 treatment reduced only the number of LR primordia. It is conceivable that the effect of GR24 on LR development depends, *inter alia*, on the plant species. In GSNOR overexpressing line, GR24-induced inhibition of LR emergence proved to be more pronounced than in Col-0. Additionally, in the stunted root system of *gsnor1-3*, the number of LR primordia was completely reduced by GR24. These results regarding the inhibitory effect of SL analogue GR24 support previously published results (Kapulnik et al. 2011, Ruyter-Spira et al. 2011, Arite et al. 2012, Marzec 2016, De Cuyper et al. 2015). In Col-0 roots, TIS108 decreased the number of both staged-lateral roots, but in *35S:FLAG-GSNOR1* it increased the number of LR primordia. Based on these we can assume that in case of normal GSNOR level reduced SL level inhibits LR initiation, while in the presence of increased GSNOR activity SL inhibition leads

to the induction of LR initiation. These signal interactions may be complex and the knowledge of other contributing factors would be necessary to fully explain the observed effects. It can be a concern that the effect of the analogue and the inhibitor is not always the opposite. At the same time, it is conceivable that an optimal SL level is needed for normal root growth. Increasing (by the addition of GR24) or lowering (by the addition of TIS108) the optimal SL level may result in similarly inhibited growth processes.

Treatment with GR24 resulted in significantly increased NO content in *Arabidopsis* roots (Kolbert 2019c). As for NO-associated genes, the expressions of *NIA1* and *NIA2* as well as *GSNOR1* didn't show any relevant modification in the presence of GR24 (Fig 6). In contrast, nitrogen regulatory protein P-II homolog (*GLB1*) and non-symbiotic hemoglobin 2 (*GLB2*) genes were upregulated by GR24. The *GLB* genes encode plant hemoglobins which may act as NO scavengers (Hebelstrup and Jensen 2008, Hebelstrup et al. 2012, Mira et al. 2015). In this experimental system; however, *GLB1* and *GLB2* upregulation induced by GR24 did not lead to NO scavenging, but instead GR24 induced NO production (Kolbert 2019c). This seems to be an interesting contradiction that needs further research.

3.4. The effect of NO donor and scavenger on SL-associated genes and root system of *Arabidopsis*

We were interested also in reverse interplay, i.e., whether under- or overproduction of GSNOR enzyme affects the expression of SL-associated genes (Fig 7). The examined genes *(CCD7, CCD8, MAX1)* involved in the synthesis of SLs showed down-regulation in GSNOR-deficient *Arabidopsis* compared to Col-0. This indicates that in case of low GSNOR activity, SL biosynthesis is inhibited. This further supports the interaction between GSNO metabolism and SL production in *Arabidopsis*. In addition, *CCD7* was down-regulated also in GSNOR overproducing *35S:FLAG-GSNOR1* seedlings. In contrast, the expressions of SL signalling genes *(D14* and *MAX2)* were not altered by GSNOR deficiency or overproduction. However, this was not supported by pharmacological treatments (GSNO or cPTIO), because we didn't observe relevant up- or downregulation of SL-associated genes *(CCD7, CCD8, MAX1, MAX2, D14*) in the presence of NO donor (GSNO) or scavenger (cPTIO) treatments (Fig 8). From the applied 250 μM GSNO solution approx. 220 nM NO liberated over 15 min during the same circumstances as the plant treatments took place (Fig S1).

To further investigate this interaction, GSNO and cPTIO treatments were applied and responses of *max* mutants were examined (Fig 9). Exogenous GSNO treatment resulted in 50%

297 root shortening in Col-0, whereas this effect was absent in *max* mutants suggesting that the
298 examined SL mutants are GSNO-insensitive and that SLs are needed for GSNO-induced root
299 shortening. According to Fernández-Marcos et al. (2011) GSNO inhibits root meristem activity
300 through the reduction of PIN1-dependent auxin transport. Since SLs were proved to negatively
301 regulate PIN proteins in *Arabidopsis* roots (Ruyter-Spira et al. 2011), we can assume that GSNO
302 may exert its effect on PINs *via* inducing SL synthesis and/or signalling. The NO scavenger
303 cPTIO shortened primary roots to a similar extent in all three plant lines *(Col-0, max1, max2-*
304 *1)*. Moreover, GSNO inhibited LR initiation and slightly increased LR emergence of Col-0,
305 while cPTIO supplementation decreased the number of both types of LR. In *max1* and *max2-1*
306 seedlings, LR emergence seemed to be insensitive to NO donor or scavenger. However, GSNO
307 treatment caused reduction in the number of LR primordia of the *max1* mutant, and cPTIO
308 treatment decreased LR initiation in both *max* mutants. Just like the matching effects of SL
309 analogue and inhibitor, the effects of NO donor and scavenger proved also to be often similar
310 to each other, indicating the necessity of an optimal NO level for optimal root development.

4. Conclusion

This study combines molecular biological and pharmacological approaches in order to reveal interactions between NO and SLs as growth regulating signals in the model plant *Arabidopsis thaliana*. We observed that SL-deficiency resulted in elevated NO and SNO levels due to decreased GSNOR protein abundance and activity indicating that there is a signal interaction between SLs and GSNOR-regulated levels of NO/SNO. This was further supported by the down-regulation of SL biosynthetic genes (*CCD7, CCD8* and *MAX1*) in *gsnor1-3* containing elevated NO/SNO levels. Based on the more pronounced sensitivity of *gsnor1-3* to exogenous SL (GR24), we suspected that functional GSNOR is needed to control NO/SNO levels during SL-induced PR elongation. Furthermore, SLs may be involved in GSNO-regulated PIN1-dependent auxin distribution and PR shortening as suggested by the relative insensitivity of *max1* and *max*2 mutants to exogenous GSNO. Collectively, our results indicate a connection between SL and NO/SNO signals in *Arabidopsis thaliana* roots and the details of this interaction should be examined in the future.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contribution

D. O. performing the experiments, writing the manuscript draft; G. F. performing experiments, reviewing the manuscript; Á. M. performing experiments; A. Ö. performing experiments, reviewing the manuscript; Zs. K. conceptualizing the research, designing and directing the project, reviewing manuscript draft and wrote the final manuscript.

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Figure legends

Fig 1 Primary root length (mm, A), lateral root number (pieces root^{-1}, B) and lateral root density (pieces mm^{-1}) in 7-days-old Col-0, GSNOR- and SL mutant *Arabidopsis* lines grown under control conditions. Different letters indicate significant differences according to Duncan's test (n=20, P≤0.05). (D) Representative photographs taken from 7-days-old *Arabidopsis* seedlings of different mutant lines grown on ½ MS medium under control conditions. Bars=1 cm.

Fig 2 Nitric oxide levels (pixel intensity, A) and SNO levels (pmol mg protein^{-1}, C) in Col-0, GSNOR- and SL mutant *Arabidopsis* seedlings grown under control conditions for 7 days. Different letters indicate significant differences according to Duncan's test (n=10 or 5, P≤0.05). (B) Representative microscopic images showing DAF-FM DA-stained root tips of examined *Arabidopsis* lines. Bar=100 μm.

Fig 3 Relative transcript level of selected NO-associated genes (*NIA1, NIA2, GLB1, GLB2*) in control Col-0, *max1* and *max2-1 Arabidopsis* seedlings. Different letters indicate significant differences according to Duncan's test (n=3, P≤0.05). Data were normalized using the *A. thaliana ACTIN2* and *GAPDH2* genes as internal controls. The relative transcript level in Col-0 control samples was arbitrarily considered to be 1 for each gene.

Fig 4 Relative transcript level (A) of *GSNOR1* in Col-0, *max1* and *max2* seedlings. (B-C) Protein abundance of GSNOR in *max* mutants and *35S:FLAG-GSNOR1* (as a positive control). Anti-actin was used as a loading control. (E) GSNOR activity (nmol NADH min^{-1} mg protein^{-1}) in Col-0, *max1* and *max2* seedlings. Different letters indicate significant differences according to Duncan's test (n=3 or 5, P≤0.05).

Fig 5 Primary root length (mm, A), lateral root number (pieces root^{-1}, B) and lateral root density (pieces mm^{-1}, C) in Col-0, *gsnor1-3* and *35S:FLAG-GSNOR1 Arabidopsis* seedlings grown in the absence (-GR24/-TIS108) or in the presence of GR24 (1 μM) or TIS108 (5 μM). Different letters indicate significant differences according to Duncan's test (n=20, P≤0.05).

Fig 6 Relative transcript level of selected NO-associated genes (*NIA1, NIA2, GSNOR1, GLB1, GLB2*) in Col-0 *Arabidopsis* grown under control conditions (-GR24/-TIS108) or in the presence of GR24 (1 μM) or TIS108 (5 μM). Different letters indicate significant differences according to Duncan's test (n=3, P≤0.05). Data were normalized using the *A. thaliana ACTIN2* and *GAPDH2* genes as internal controls. The relative transcript level in Col-0 control samples was arbitrarily considered to be 1 for each gene.

Fig 7 Relative transcript level of selected SL-associated genes in Col-0, *gsnor1-3* and *35S:FLAG-GSNOR1 Arabidopsis* seedlings grown under control conditions. Different letters indicate significant differences according to Duncan's test (n=3, P≤0.05). Data were normalized using the *A. thaliana ACTIN2* and *GAPDH2* genes as internal controls. The relative transcript level in Col-0 control samples was arbitrarily considered to be 1 for each gene.

Fig 8 Relative transcript level of selected SL-associated genes *(CCD7, CCD8, MAX1, MAX2, D14)* in Col-0 *Arabidopsis* grown under control conditions or supplemented with GSNO (250 μM) or cPTIO (800 μM). Data were normalized using the *A. thaliana ACTIN2* and *GAPDH2* genes as internal controls. The relative transcript level in Col-0 control samples was arbitrarily considered to be 1 for each gene.

Fig 9 Primary root length (mm, A), lateral root number (pieces $root^{-1}$, B) and lateral root density (number mm^{-1}, C) in Col-0, *max1, max2-1 Arabidopsis* seedlings grown in the absence (-GSNO/-cPTIO) or in the presence of GSNO (250 μM) or cPTIO (800 μM) for 3 days. Different letters indicate significant differences according to Duncan's test (n=20, P≤0.05).

Fig S1 Concentration (nM) of liberated NO by 250 μM GSNO solution following different duration of illumination (0, 15, 30, 45, 60, 90, 120, 180, 360 min). Insert: Calibration curve of ISO-NOP electrode. Calibration was carried out using different concentrations of SNAP according to Zhang (2004).

Table S1 Primers used in this study. (*Papdi et al. 2008)

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