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RESEARCH PAPER

Novel and conserved functions of *S*-nitrosoglutathione reductase in tomato

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Abstract

Nitric oxide (NO) is emerging as a key signalling molecule in plants. The chief mechanism for the transfer of NO bioactivity is thought to be *S*-nitrosylation, the addition of an NO moiety to a protein cysteine thiol to form an *S*-nitrosothiol (SNO). The enzyme *S*-nitrosoglutathione reductase (GSNOR) indirectly controls the total levels of cellular *S*-nitrosylation, by depleting *S*-nitrosoglutathione (GSNO), the major cellular NO donor. Here we show that depletion of GSNOR function impacts tomato (*Solanum lycopersicum*. L) fruit development. Thus, reduction of *GSNOR* expression through RNAi modulated both fruit formation and yield, establishing a novel function for *GSNOR*. Further, depletion of *S. lycopersicum GSNOR* (*SlGSNOR*) additionally impacted a number of other developmental processes, including seed development, which also has not been previously linked with GSNOR activity. In contrast to Arabidopsis, depletion of *GSNOR* function did not influence root development. Further, reduction of *GSNOR* transcript abundance compromised plant immunity. Surprisingly, this was in contrast to previous data in Arabidopsis that reported that reducing *Arabidopsis thaliana GSNOR* (*AtGSNOR*) expression by antisense technology increased disease resistance. We also show that increased *SlGSNOR* expression enhanced pathogen protection, uncovering a potential strategy to enhance disease resistance in crop plants. Collectively, our findings reveal, at the genetic level, that some but not all GSNOR activities are conserved outside the Arabidopsis reference system. Thus, manipulating the extent of *GSNOR* expression may control important agricultural traits in tomato and possibly other crop plants.

Keywords: Climacteric fruit, fruit development, GSNOR, MicroTom, nitric oxide, NO, *S*-nitrosation, *S*-nitrosylation, tomato, tomato fruit.

Introduction

Nitric oxide (NO) underpins a plethora of cellular processes integral to the biology of plants. The chief mechanism for the transfer of NO bioactivity is thought to be *S*-nitrosylation, the addition of an NO moiety to a peptide or protein cysteine thiol to form an *S*-nitrosothiol (SNO) (Spadaro *et al.*, 2010). This redox-based post-translational modification controls a number

of key activities related to growth, development, and environmental interactions, including immune function. Typically, protein SNOs can be denitrosylated by the antioxidant tripeptide, glutathione (GSH), resulting in the reconstitution of the protein Cys thiol and formation of *S*-nitrosogluthionine (GSNO) (Airaki *et al.*, 2011), which functions as a natural NO donor and consequently a reservoir for NO bioactivity (Feechan *et al.*, 2005).

S-Nitrosoglutathione reductase (GSNOR), first identified in bacteria (Liu *et al.*, 2001), is thought to be the major determinant in the control of total cellular SNO levels in Arabidopsis (Feechan *et al.*, 2005; Lee *et al.*, 2008; Leterrier *et al.*, 2011). The enzyme has a high affinity for GSNO (Liu *et al.*, 2001; Achkor *et al.*, 2003). Loss-of-function mutations in *AtGSNOR1* compromise multiple modes of plant disease resistance, while overexpression of this gene conveys increased disease resistance. Further, AtGSNOR1 has been shown to regulate both salicylic acid (SA) biosynthesis and associated signalling (Feechan *et al.*, 2005; Rustérucci *et al.*, 2007; Tada *et al.*, 2008).

In the context of SA signalling (Loake and Grant, 2007; Fu and Dong, 2013), *S*-nitrosylation of the *A. thaliana* SA-binding protein 3 (AtSABP3) at Cys280 suppresses its binding to both the immune activator, SA, and the carbonic anhydrase activity of this protein, negatively regulating disease resistance (Y.J. Wang *et al.*, 2009). Further, NO via GSNO has been shown to protect the TGA1 transcriptional regulator from oxygen-mediated modifications and enhance the DNA binding activity of this protein to its cognate *cis*-element in the presence of the transcriptional co-activator, NPR1. In addition, the translocation of NPR1 into the nucleus may be promoted by NO (Lindermayr *et al.*, 2010). In contrast, GSNO accumulation in *atgsnor1-3* plants has been reported to inhibit the translocation of NPR1 from the cytoplasm to the nucleus, thereby curbing SA signalling and associated plant immunity (Tada *et al.*, 2008; Yun *et al.*, 2016). NO is also proposed to play a central role in signalling activated by the fungal elicitor, cryptogein (Kulik *et al.*, 2015), and is required for disease resistance against *Botrytis cinerea* triggered by oligogalacturonides (Rasul *et al.*, 2012).

Both NO and reactive oxygen intermediates (ROIs) have been implicated in the programmed cell death of pathogen-challenged cells through the hypersensitive response (HR; Delledonne *et al.*, 1998, 2001; Torres *et al.*, 2002). A loss-of-function allele of *atgsnor1* [*paraquat resistance 1-2* (*par1-2*)] conveyed protection against cell death mediated by the herbicide, paraquat (Chen *et al.*, 2009). Further, NO has been shown to regulate the production of ROIs by the *S*-nitrosylation of NADPH oxidase (*AtRBOHD*) at Cys890, reducing ROI production at later stages of the plant defence, curbing development of the HR (Yun *et al.*, 2011). Interestingly, oxidative post-translational modification of GSNOR inhibited the activity of this enzyme, suggesting an additional mechanism of direct crosstalk between ROI and NO signalling (Frungillo *et al.*, 2014; Guerra *et al.*, 2016; Kovacs *et al.*, 2016; Lindermayr, 2018).

*At*GSNOR1 has also been shown to control some key aspects of plant development (Lee *et al.*, 2008; Leterrier *et al.*, 2011; Kwon *et al.*, 2012). For example, *atgsnor1-3* mutants show loss of apical dominance, a subtle change in leaf shape, and increased sensitivity to auxin (Kwon *et al.*, 2012). This line is also reduced in fertility, principally due to very short stamens which do not function as effective self-pollinators (Kwon *et al.*, 2012; Xu *et al.*, 2013). AtGSNOR1 has also been implicated in responses to abiotic stress (Corpas *et al.*, 2011; Fancy *et al.*, 2017; Begara-Morales *et al.*, 2018). Missense alleles of *hot5/atgsnor/par2* cannot acclimate to heat as do dark-grown seedlings, but grow normally and can heat-acclimate in the light. In contrast, null alleles cannot heat-acclimate like light-grown plants (Lee *et al.*, 2008). Thus, *At*GSNOR is required for heat acclimation. In sunflower seedlings exposed to high temperature (38 °C for 4 h), GSNOR activity, protein levels, and transcript abundance have been found to be reduced in hypocotyls, with the simultaneous accumulation of SNOs (Leterrier *et al.*, 2011). The consequence was a rise in protein tyrosine nitration, which is considered a marker of nitrosative stress. Collectively, these findings imply that *At*GSNOR also has an important function in plant development and abiotic stress.

While the genetics of tomato (*Solanum lycopersicum*) *GSNOR* (*SlGSNOR)* have largely been unexplored, the crystal structure for the corresponding enzyme has been solved to 1.9 Å resolution (Kubienová *et al.*, 2013), being the first plant GSNOR to be structurally determined. Here, taking a genetic approach, we uncover key roles for *SlGSNOR* in plant development, fruit formation, and immunity in tomato. Collectively, these data imply that the function of GSNOR is conserved across plant species. Further, manipulating levels of *GSNOR* expression may provide novel mechanisms for the incorporation of disease resistance and advantageous developmental traits into crop plants.

Materials and methods

DNA constructs

Tomato *GSNOR* (Solyc09g064370, http://solgenomics.net/locus/34669/view) was amplified using *SlGSNOR-Pst*I-F and *SlGSNOR-Not*I-R primers (Supplementary Table S1 at *JXB* online) and cloned behind the *CaMV2x35S* promoter in the pGreenI0029 binary expression vector to make the *GSNOR* overexpression (OE) DNA construct. For the *SlGSNOR*-RNAi DNA construct, sense and antisense DNA fragments of 369 bp were amplified using *SlGSNORS-Xho*I-F and *SlGSNORS-Kpn*I-R for the sense fragment, and *SlGSNORA-Cla*I-F and *SlGSNORA-Xba*I-R for the antisense fragment (Table 1), and cloned in the pHANNIBAL intermediate vector separated by a pDK intron to make the *CaMV35S*:sense:intron:antisense:terminator RNAi cassette. The cassette was then transferred to the pGreenI0029 binary vector. RNA extraction was performed using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. A total of 1 µg of RNA was reverse transcribed to synthesize cDNA using the Omniscript RT kit (Qiagen) according to the manufacturer's instructions. A 1 µl aliquot of this cDNA was subsequently used in a semi-quantitative PCR for the amplification of DNA fragments. All DNA fragments were amplified in a 40 µl reaction using Phusion® High Fidelity DNA polymerase (New England Biolabs). Both the OE and RNAi *SlGSNOR* DNA constructs were confirmed by colony PCR and sequenced before transformation in tomato cultivar MicroTom.

Tomato transformation

The *SlGSNOR*-OE and *SlGSNOR*-RNAi DNA constructs were transformed into tomato cv. MicroTom. Briefly, seeds of tomato variety

MicroTom were surface sterilized in 70% ethanol for 40 s, rinsed with sterile distilled water, kept in 40% sodium hypochlorite (NaOCl)+3 drops/100 ml Tween-20, for 15 min, and rinsed with sterile distilled water five times. The seeds were germinated on germination medium [1/2 MS (Murashige and Skoog, 1962) salts and vitamins, 3% sucrose, 0.8% agar] in the dark at 25±2 °C for 7 d. Single *Agrobacterium tumefaciens* colonies carrying *SlGSNOR*-OE and *SlGSNOR*-RNAi constructs were grown at 28 °C with shaking at 250 rpm for 24 h. The cultures were centrifuged at 12 000 rpm for 10 min and bacteria were washed and re-suspended in liquid MS medium to OD_{600}=0.7. MicroTom explants were prepared by cutting both ends of the cotyledons and dipped in *A. tumefaciens SlGSNOR*-OE and *SlGSNOR*-RNAi suspension cultures for 5 min. The explants were then incubated on shoot induction medium [SIM: 1/2 MS salts and vitamins, 3% sucrose, 0.8% agar, 2 mg l^{-1} 6-benzylaminopurine (BA), 0.01 mg l^{-1} indole-3-butyric acid (IBA)] for 2 d and rinsed with sterile distilled water. The explants were then screened on SIM plates containing 25 mg l^{-1} kanamycin+500 mg l^{-1} cefotaxime (transgenic shoot selection medium: TSM). Transgenic shoots were transferred to fresh TSM every 4 weeks to ensure stringent selection. Transgenic shoots (~0.5 cm) were then transferred to transgenic shoot elongation medium (TEM: MS salts and vitamins, 3% sucrose, 0.8% agar, 30 mg l^{-1} kanamycin+500 mg l^{-1} cefotaxime) at 25±2 °C in the light. Plants were transferred to fresh TEM every 3–4 weeks and then to sterile soil in pots after rooting. Homozygous transgenic plants were obtained as described previously (Harrison *et al.*, 2006).

Growth of tomato plants

Seeds from wild-type (WT) and transgenic plants were germinated either on 1/2 MS medium (1.1 g of MS salt and 5 g of sucrose dissolved in 300 ml of water at pH 5.7–5.9 and volume adjusted to 500 ml after adding 4 g of agar) or a special peat-based UC (University of California) compost [100 litres of medium grade peat (Sinclair Horticulture), 25 litres of horticultural sand, 375 g of garden limestone (J. Arthur Bowers), 150 g of Osmocote Exact 3–4 months (Scotts) and 3 g of Intercept-70WG (Scotts)]. Seedlings were transplanted to new pots 1 week after germination and grown at 21 °C under long days (16 h light/8 h dark) at 800 µmol m^{-2} s^{-1} PAR (photosynthetically active radiation) light intensity.

Growth and inoculation of PstDC3000

Pseudomonas syringae pv. *tomato* DC3000 (*Pst*DC3000) was grown in LB liquid medium [tryptone 10 g l^{-1}, yeast extract (Oxoid) 5 g l^{-1}, NaCl (VWR, UK) 10 g l^{-1}], with 50 µg ml^{-1} rifampicin at 28 °C overnight. Cells were pelleted by centrifugation before re-suspension in 10 mM $MgCl_2$. Plants were inoculated as described by Mudgett and Staskawicz (1999) with some modification. Three-week-old plants were sprayed with a *Pst*DC3000 virulent suspension with cell density adjusted to 2×10^8 cfu ml^{-1} at OD_{600}. Plants were kept covered inside plastic bags under high humidity for 24 h to allow the opening of stomata for successful bacterial entrance/inoculation.

PstDC3000 colony counts

*Pst*DC3000 was inoculated to the tomato WT and transgenic plants as described above. The plants were examined for disease symptoms at regular intervals. Leaf samples were collected at 0, 2, and 4 days post-infection (DPI). Using 12 plants per line, three leaf discs (1 cm^2) were collected per plant. Each leaf disc was ground in a microfuge tube in 500 µl of 10 mM sterile $MgCl_2$ using a tissue lyser (Qiagen/Retsch) for 2 min at 30 shakes per second. A 200 µl aliquot of the bacterial suspension was transferred to a new microfuge tube, and serial dilutions were made to 10^{-2}. After the serial dilution, 10 µl of each dilution was plated on NYG plates [Bacto peptone 5 g l^{-1}, yeast extract (Oxoid) 3 g l^{-1}, glycerol (Fisher Scientific) 20 ml l^{-1}, Bacto agar 15 g l^{-1}] containing 50 µg ml^{-1} rifampicin. The plates were incubated for 2 d at 28 °C and the number of bacterial colonies for each sample counted and recorded in the best countable dilution. The experiment was repeated three times.

PR1 gene expression

Leaf samples collected for colony counts, from *Pst*DC3000-infected WT and transgenic plants at 0, 2, and 4 DPI, were used to cut 1 cm^2 leaf discs for colony count assay. The rest of the leaf samples were used to extract RNA for *PR* gene expression analysis. RNA was isolated from plant samples as described earlier, and quantification of reverse transcription–PCR (RT–PCR) was carried out to check *PR1* expression in response to infection. Tomato actin was used as a reference gene. Primers for tomato *PR1* and *actin* genes are given in Supplementary Table S1.

Salicylic acid measurement

Free and conjugated endogenous SA levels were determined using HPLC, as described by Aboul-Soud *et al.* (2004) with minor modifications. A 200 mg aliquot of leaf tissue per sample was collected and promptly frozen in liquid nitrogen. Samples were then ground in liquid nitrogen using a mortar and pestle, and transferred to a 2 ml microfuge tube, followed by the addition of 1 ml of 90% methanol (Fisher Scientific), and vortexed for 1 min, before the sample thawed. The sample was then centrifuged at 15 000 *g* for 5 min and the supernatant transferred to a new tube. The pellet was re-extracted in 1 ml of 100% methanol, centrifuged, and the two supernatants pooled together and dried in a speed vacuum centrifuge (SpeedVac DNA110, Savant) at medium temperature. The residue resulting from drying the supernatant was then re-suspended in 1 ml of 5% trichloroacetic acid, followed by the addition of 1 ml of ethyl acetate:cyclopentane (Fluka):isopropanol (Fisher Scientific) (50:50:1) and vortexed for 1 min. The organic phase was transferred to a new tube. The aqueous phase was re-extracted with another 1 ml of the organic 50:50:1 mix, and the two supernatants pooled together and evaporated under heat in the vacuum centrifuge. The aqueous phase was then acidified to pH 1 by addition of 50 µl of absolute HCl, boiled for half an hour to release SA from any acid-labile conjugated forms, and extracted with the organic mix twice. The two supernatants were pooled together and dried in the vacuum centrifuge. The residues were dissolved in 100 µl of 100% methanol before 100 µl of H_2O was added to give a final 50% (v/v) methanol concentration. The samples were filtered through a 0.25 µM filter (Millex-GP, Millipore Corporation, Billerica, MA, USA) and subjected to HPLC analysis. Samples were taken at 4 DPI. SA samples of 1 mM and 10 mM were used as standard.

Results

SlGSNOR *depletion negatively affects seed development and germination but not root development*

After the transformation of *SlGSNOR*-OE and *SlGSNOR*-RNAi constructs in tomato (cv. MicroTom), *SlGSNOR*-RNAi and *SlGSNOR*-OE lines were generated. RT–PCR results showed a significant reduction in *Sl*GSNOR expression in the RNAi lines, whereas a significant increase in *Sl*GSNOR expression was observed in the OE lines (Fig. 1A). A significant impact of *SlGSNOR* knockdown on the germination percentage was observed in the RNAi lines, with >80% reduction in germination (Fig. 1B). This shows that the accumulation of *SlGSNOR1* transcripts is tightly regulated in tomato MicroTom and a significant reduction in its expression leads to lethality, as *SlGSNOR*-RNAi lines with greater than ~60% reduction in *SlGSNOR1* expression were not viable (Fig. 1C). Both *SlGSNOR*-RNAi and *SlGSNOR*-OE lines conveyed significant effects on the overall development of tomato plants, ranging from seed germination to fruiting and net yield per plant. Reduced GSNOR expression in *SlGSNOR*-RNAi lines drastically affected seed development and reduced the number of seeds produced in the fruits of the resulting transgenic plants. In representative RNAi lines that could not be maintained, the seeds were small,

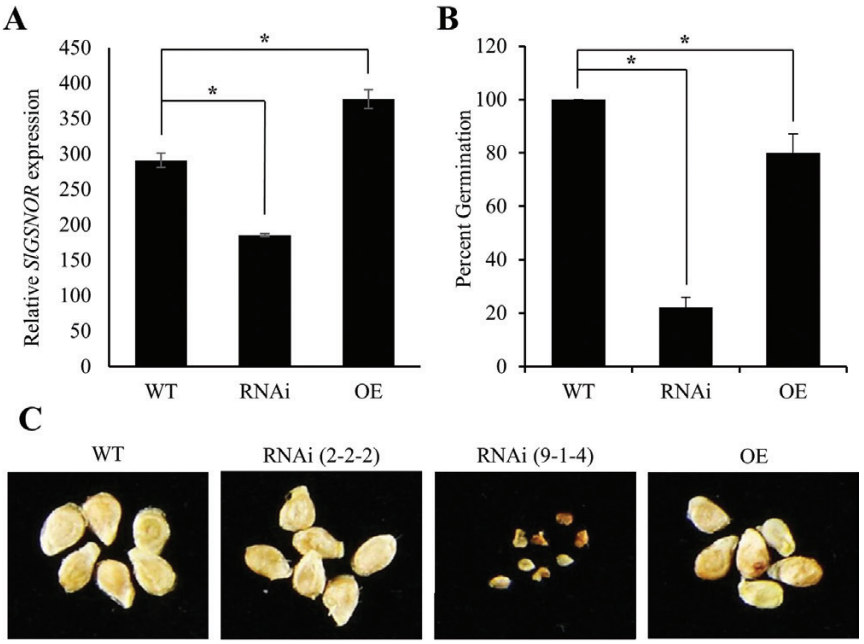

Fig. 1. *SlGSNOR* suppression negatively affects seed development and germination. (A) Quantification of RT–PCR results showing *SlGSNOR* transcript levels in representative tomato WT, RNAi, and OE plants. (B) Percentage germination of tomato WT, RNAi, and OE seeds. A highly significant reduction in germination frequency was recorded for the seeds produced by RNAi plants as compared with WT plants. (C) Phenotype of the WT, RNAi, and OE seeds. *SlGSNOR*-RNAi plants produced misshapen, small, and deformed seeds as compared with WT and OE plants. Reduction of *SlGSNOR* expression by up to ~60% resulted in lethality and the seeds could not germinate, However, the OE plants produced seeds with a normal phenotype. Statistical analyses were performed through one-way ANOVA test at a 95% level of confidence. Statistically significant differences are shown by an asterisk (*). Error bars represent the SD. (This figure is available in colour at *JXB* online.)

misshapen, and lacked endosperm. Consequently, these seeds failed to germinate. In a representative fertile *SlGSNOR*-RNAi line (2-2-2), seed germination was reduced by 80% relative to the WT. The *SlGSNOR*-OE plants, however, also showed an average reduction of 20% in germination frequency (Fig. 1B), although OE plants produced normal healthy seeds (Fig. 1C). Counterintuitively, the *SlGSNOR*-RNAi plants also showed faster germination as compared with WT and *SlGSNOR*-OE plants on either MS medium or soil, and showed the appearance of fresh green tissues at least 1 d before the WT and OE plants.

Suppression of *SlGSNOR* in tomato did not seem to have a major impact on the root system of plants under optimal environmental conditions. The root length of 1- and 5-week-old plants was analysed (Fig. 2A, B). Root length in *SlGSNOR*-RNAi plants was not significantly different from that of WT plants. In a similar fashion, root length in *SlGSNOR*-OE plants was also not visibly different from that of WT plants (Fig. 2A, B). Quantitative analysis and associated statistical testing confirmed that the root length of *SlGSNOR*-RNAi plants and *SlGSNOR*-OE plants is not statistically different from that of the WT (Fig. 2C). Collectively, our data imply that reducing *SlGSNOR* gene expression impacts tomato seed development and germination, but not root development. In contrast, increasing *SlGSNOR* expression does not impact either seed development, seed germination, or root development.

SlGSNOR *is required for leaf development*

Multiple developmental phenotypes were found to be different in the transgenic plants as compared with WT plants. Therefore, the average leaf area for WT, *SlGSNOR*-RNAi, and *SlGSNOR*-OE tomato plants was calculated using 5-week-old plants. Leaf area was measured as an average of multiple leaves of different sizes, ranging from the smallest to the largest and from the bottom to the top of the plants. The smallest average leaf area of 468.49 mm^2 was recorded for *SlGSNOR*-RNAi plants, compared with 734.35 mm^2 for the WT. Tomato transgenic plants overexpressing *SlGSNOR* had the largest leaves, with an area of 783.10 mm^2 (Fig. 3A, B) as compared with leaves of WT plants, although the difference was not statistically significant.

SlGSNOR *regulates fruit production and flower development*

WT and transgenic *SlGSNOR*-RNAi and *SlGSNOR*-OE tomato plants were analysed for their respective yields. Both the *SlGSNOR*-RNAi and *SlGSNOR*-OE plants showed reduced yield per plant as compared with the WT. However, *SlGSNOR*-RNAi plants produced an average of 73.56 g of fruit per plant as compared with 63.96 g for *SlGSNOR*-OE and 179.10 g for WT plants (Fig. 4A, B). However, fruits produced by the *SlGSNOR*-OE plants were significantly larger in size as compared with those of WT plants, whereas those produced by the *SlGSNOR*-RNAi plants were smaller in size (Fig. 4B). Consistent with the *atgsnor1* Arabidopsis mutant, the tomato *SlGSNOR*-RNAi plants produced misshapen flowers with carpels beyond the reach of stamens, which presumably negatively impacted self-fertilization (Fig. 4C–H). In contrast, *SlGSNOR* overexpression had no effect on the floral phenotype; these lines resembled WT plants with respect to these traits (Fig. 4C–H).

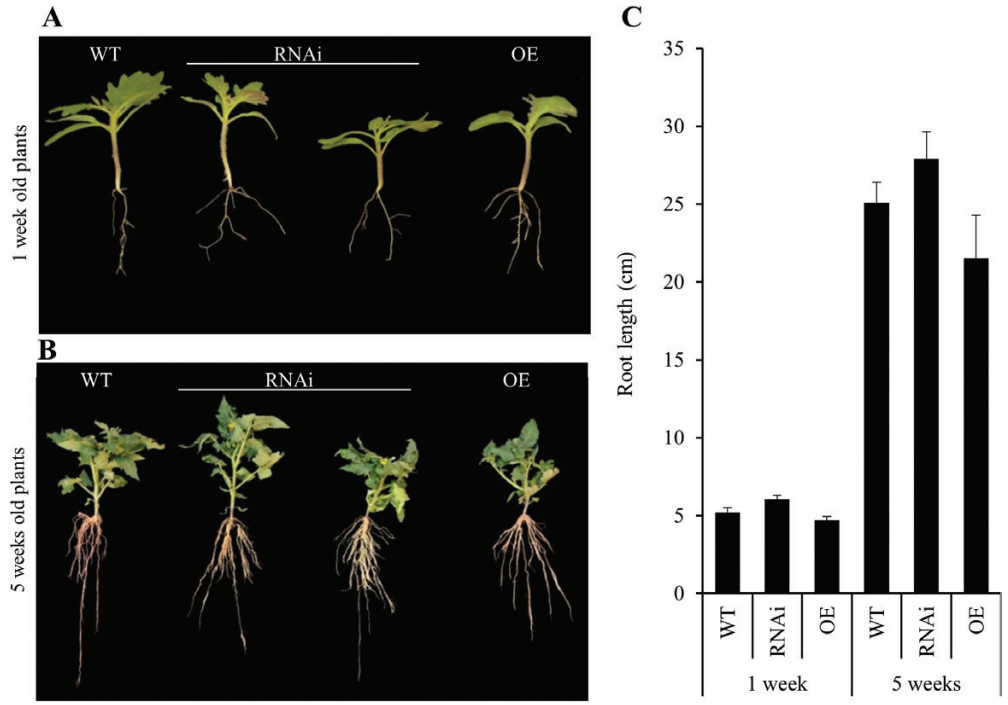

Fig. 2. *SlGSNOR* suppression does not negatively impact root development. No significant differences were found between the root length of WT, RNAi, and OE plants after 1 week (A) or 5 weeks of growth (B). Root length measurements of WT, RNAi, and OE plants were statistically analysed using a two-way ANOVA test at a 95% level of significance. Error bars represent the SD. (This figure is available in colour at *JXB* online.)

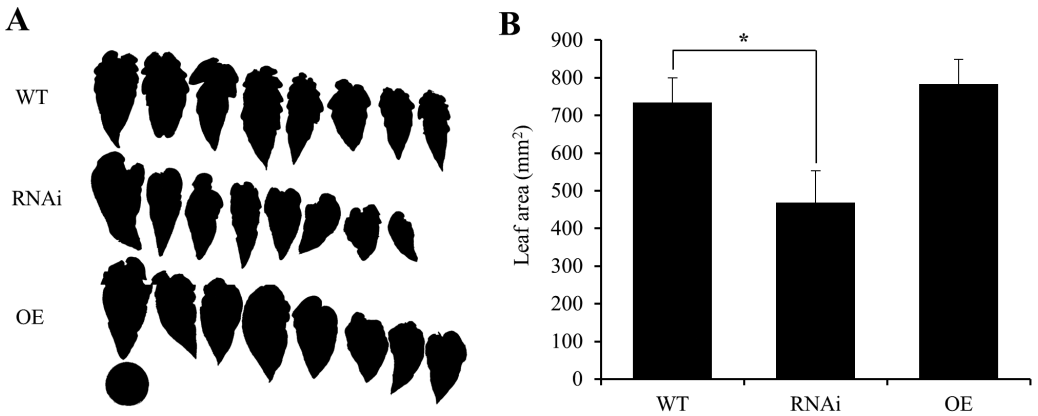

Fig. 3. *SlGSNOR* suppression reduces average leaf area in tomato. (A) Scanned image showing the arrangement of WT, *SlGSNOR*-RNAi, and *SlGSNOR*-OE plant leaves along with a UK 10 pence coin as a scale marker. (B) Significantly reduced average leaf area was recorded for the RNAi plants compared with WT and OE plants. However, the increase in the leaf area for OE plants compared with the WT was not significant. Statistical analysis was performed using one-way ANOVA with 95% confidence Statistical analyses were performed through one-way ANOVA test at a 95% level of confidence. Statistically significant differences are shown by an asterisk (*). Error bars represent the SD.

Overexpression of SlGSNOR *promotes resistance to bacterial pathogens*

The tomato cv. MicroTom is not well characterized with respect to microbial pathogens. The well-characterized leaf pathogen, *Pst*DC3000, is thought to be an opportunistic pathogen of this tomato cultivar, with little increase in growth over time (Takahashi *et al.*, 2005). Three-week-old WT, *SlGSNOR*-RNAi, and *SlGSNOR*-OE plants were spray inoculated with a *Pst*DC3000 suspension of 2×10^8 cfu ml^{-1} in 10 mM $MgCl_2$ and 0.02% Silwet L77. This ensured even application of inoculum and avoided potential injury. Development of disease symptoms was monitored daily. Disease symptoms appeared in *SlGSNOR*-RNAi plants after 7 d. Chlorosis, the appearance of typical dark brown lesions surrounded by chlorotic areas, was clearly visible, especially near leaf margins, on the leaves of *SlGSNOR*-RNAi plants. These symptoms are typical of *Pst*DC3000 infection in susceptible tomato plants. In contrast, WT and *SlGSNOR*-OE plants showed delayed and reduced symptom development (Fig. 5A). Leaf samples were collected from the inoculated plants at 2 and 4 DPI for the determination of bacterial titre. *SlGSNOR*-RNAi plants supported an increased bacterial titre relative to WT plants at both time

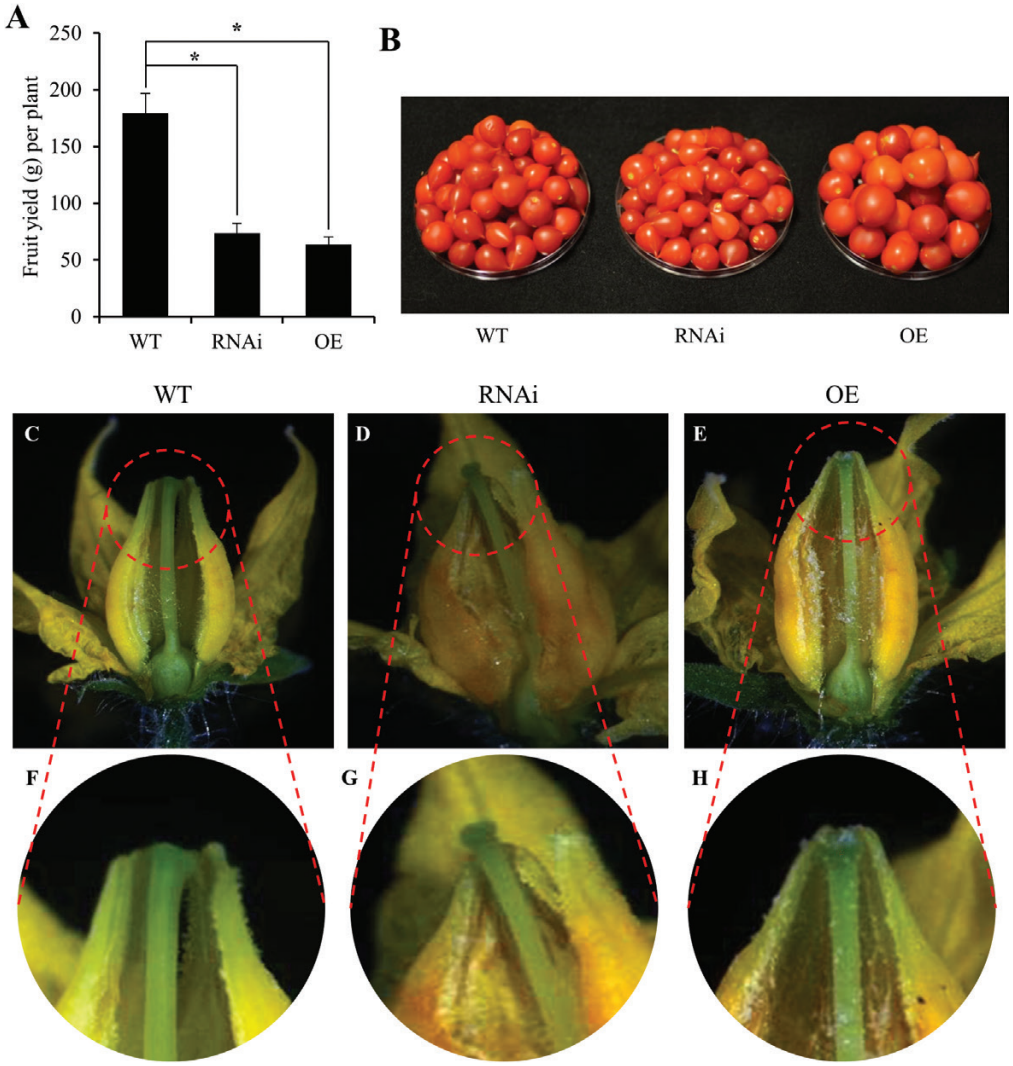

Fig. 4. Manipulation of *SlGSNOR* levels impacts fruit production and flower development. (A) Average yield (g per plant) of WT, *SlGSNOR*-RNAi, and *SlGSNOR*-OE tomato plants. Both *SlGSNOR*-RNAi and *SlGSNOR*-OE transgenic lines showed a highly significant reduction in yield. (B) OE plants produced large, healthy fruits with a good number of healthy viable seeds, while RNAi plants produced small fruits typically <2.5 cm in diameter with few and mostly non-viable seeds. (C–H) *SlGSNOR*-RNAi plants produced misshapen flowers with long carpels extending beyond the reach of the stamens (D, G) compared with the WT (C, F) and OE plants (E, H). Statistical analyses were performed through one-way ANOVA test at a 95% confidence level. Statistically significant differences are shown by an asterisk (*). Error bars represent the SD. (This figure is available in colour at *JXB* online.)

points. Conversely, the number of bacteria in *SlGSNOR*-OE plants was significantly reduced relative to the WT (Fig. 5B). Thus, reduction of *SlGSNOR* expression promotes enhanced disease susceptibility. In contrast, increased *SlGSNOR* expression enhances disease resistance against *Pst*DC3000. Thus, modulation of *SlGSNOR* transcript abundance impacts the level of basal disease resistance.

In Arabidopsis, *GSNOR* has been shown to be a positive regulator of both SA synthesis and associated signalling (Feechan *et al.*, 2005; Tada *et al.*, 2008). Therefore, to explore the molecular mechanism underpinning the regulation of basal disease resistance by *SlGSNOR*, we first determined the levels of SA in WT, *SlGSNOR*-RNAi, and *SlGSNOR*-OE plants. The basal and pathogen-induced levels of SA in *SlGSNOR*-RNAi plants were 55% and 57.74%, respectively, of those present in WT plants. The concentrations of SA found in the *GSNOR*-overexpressing plants were 360% and 132.8% higher than those in the WT before and after infection, respectively (Fig. 5C).

Presumably, these changes in the levels of SA impact the expression of SA-dependent genes, including the well-established SA marker gene, *PR1* (Uknes *et al.*, 1992). We therefore determined the level of *PR1* gene expression in WT, *SlGSNOR*-RNAi, and *SlGSNOR*-OE plants in response to pathogen challenge. Reduced *PR1* transcript accumulation was observed in *SlGSNOR*-RNAi lines 2 and 4 DPI with respect to the WT. Conversely, *PR1* gene expression was significantly increased in *SlGSNOR*-OE plants at 2 and 4 DPI relative to the WT (Fig. 5D).

Discussion

Our data show that depletion of *SlGSNOR* levels impacts growth and development of tomato cv. MicroTom. Reduction of *Sl*GSNOR function results in the loss of apical dominance, changes in leaf shape, perturbations in seed development and germination, and, significantly, a reduction in the yield of

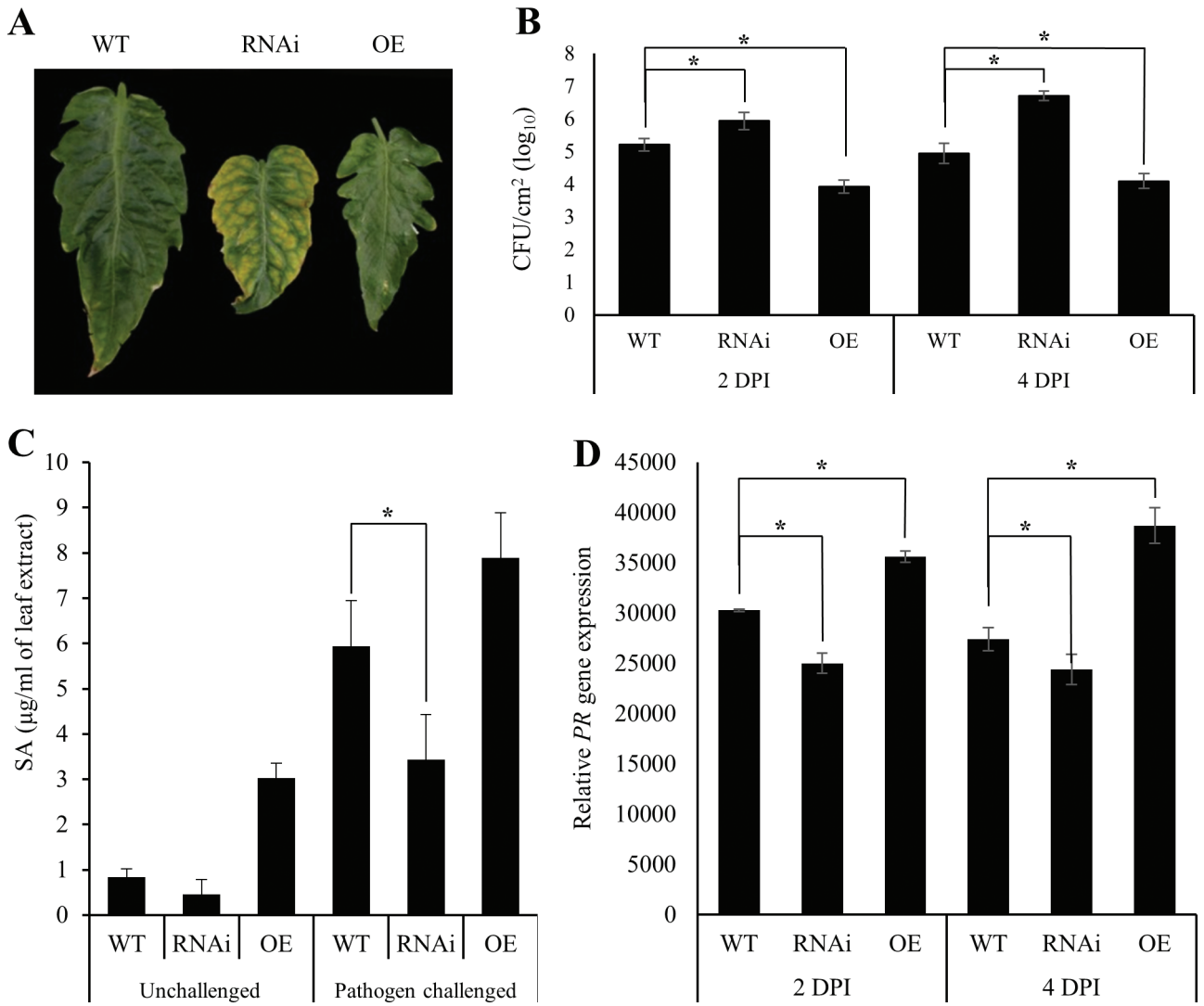

Fig. 5. Overexpression of *SlGSNOR* promotes disease resistance. (A) Development of disease symptoms in the stated lines after 1 week of infection with *Pst*DC3000. Yellow chlorotic areas surrounding dark brown lesions can be seen on the leaves of RNAi plants. (B) Graph showing bacterial growth after 2 d and 4 d of infection. Significantly higher bacterial growth was observed in RNAi plants, whereas the OE plants supported significantly lower bacterial growth after 2 d and 4 d of infection. (C) Total salicylic acid (SA) levels were measured in unchallenged leaves, in addition to plants infected with *Pst*DC3000 after 4 d of infection. *SlGSNOR*-OE plants showed a significantly higher level of basal and induced SA compared with WT plants. On the other hand, the RNAi plants produced lower quantities of SA both before and after infection. (D) Expression of the SA marker gene, *PR1*, was found to be significantly higher in the OE plants after 2 d and 4 d of infection by *Pst*DC3000 as compared with that in WT plants. RNAi plants showed significantly lower *PR1* expression compared with WT plants. Statistical analyses were performed using two-way ANOVA test at a 95% level of confidence. Statistically significant differences are shown by an asterisk (*). Error bars represent the SD. (This figure is available in colour at *JXB* online.)

tomato fruit. In contrast, overexpression of *Sl*GSNOR has no impact on the growth of tomato cv. MicroTom. Our findings also highlight a key role for *Sl*GSNOR in disease resistance. Thus, depletion of *SlGSNOR* levels resulted in enhanced disease susceptibility to the bacterial pathogen, *Pst*DC3000. Conversely, overexpression of *SlGSNOR* promoted disease resistance due to increased SA accumulation and associated expression of SA-dependent genes.

While *At*GSNOR has been extensively studied in Arabidopsis, there been no previous information on the genetics of GSNOR in crop plants. In contrast, the effect of NO on seed germination, root architecture, and fruit ripening has been studied employing NO donors in crop plants (Zandonadi *et al.*, 2010; Semchuk *et al.*, 2011). Analysis of GSNO in the main organs of pepper plants established that this metabolite was most abundant in roots, followed by leaves and stems. These findings directly correlated with the content of NO in each organ and inversely correlated with GSNOR activity (Airaki *et al.*, 2011). Subcellular localization of GSNO in pea leaves established the presence of GSNO in the cytosol, chloroplasts, mitochondria, and peroxisomes (Barroso *et al.*, 2013). While these studies have provided excellent and compelling circumstantial evidence for a key role for GSNO and, by extension, GSNOR in plant developmental processes in crop plants, direct genetic evidence has not been established. Our data show that depletion of *SlGSNOR* does indeed impact a number of development processes outside the model plant, Arabidopsis. Thus, reduction of *SlGSNOR* transcript accumulation decreases individual leaf size and consequently total leaf area. Seed size is also reduced and seed

from *SlGSNOR*-RNAi plants exhibit a lower germination frequency. Significantly, fruit size and total fruit yield are also reduced.

NO bioactivity has been strongly linked to plant reproductive biology (Bright *et al.*, 2009; Zafra *et al.*, 2010). Thus, NO can act as a negative regulator of pollen tube growth in plants such as *Lilium longiflorum*, Arabidopsis, and *Paulownia tomentosa* (Prado *et al.*, 2004, 2008; He *et al.*, 2007). Conversely, NO has been reported as a positive stimulus of pollen tube growth in *Pinus bangeana*, functioning in a dose-dependent manner (Y. Wang *et al.*, 2009). In *SlGSNOR*-RNAi plants, our data show that the structure of the reproductive organs was impacted; these lines developed long carpels, resulting in the stigma being spatially removed from the surrounding anthers, decreasing pollen transfer and, by extension, self-fertility. These phenotypes parallel those observed in Arabidopsis plants possessing null mutations in *AtGSNOR* (Kwon *et al.*, 2012), implicating conservation of GSNOR function from Arabidopsis to tomato across a number of developmental processes with visible outcomes. Interestingly, null mutations in Arabidopsis *AtGSNOR* also perturb root development, resulting in shorter roots. However, in contrast, depletion of *SlGSNOR* transcripts did not visibly impact root development. Perhaps sufficient *Sl*GSNOR activity was still present in the relevant root cells of these plants to enable the completion of key growth and/or developmental processes in this organ. Alternatively, a role for GSNOR function in root development may not be conserved between Arabidopsis and tomato. GSNOR is a single-copy gene in both tomato and Arabidopsis. Our findings suggest that strong reduction of *SlGSNOR* expression resulted in the formation of non-viable seeds. Thus, null mutants of *SlGSNOR* might not be maintained in tomato cv. MicroTom and perhaps other tomato cultivars.

Ripening of both climacteric (e.g. tomato) and non-climacteric (e.g. pepper) fruits is another area where NO function and associated *S*-nitrosylation have been explored (Corpas *et al.*, 2018). Climacteric fruits continue ripening after being picked, a process accelerated by ethylene. Non-climacteric fruits can ripen only when still attached to their respective plant. These fruits have a short shelf-life if harvested when ripe. The application of NO gas or NO donors to a number of different climatic fruits has been shown to delay fruit ripening. In this context, NO can repress both ethylene metabolism and signalling, while simultaneously inducing antioxidative enzymes, which are thought to prevent oxidative damage. Intriguingly, NO gas has also been shown to delay fruit ripening in non-climacteric fruits and, in addition, increase the amount of ascorbate (vitamin C) (Rodríguez-Ruiz *et al.*, 2017; Corpas *et al.*, 2018). Thus, NO treatment of non-climatic fruits may convey dual advantages: extending both fruit shelf-life and quality. This exciting research has clearly uncovered a potential biotechnological application of NO (Manjunatha *et al.*, 2010; Corpas *et al.*, 2018). Our findings suggest that continuous depletion of *SlGSNOR* function and, by extension, increasing GSNO and associated global *S*-nitrosylation, both decreases the size of individual fruits and reduces the overall fruit yield. It will now be interesting for future studies to explore the biochemical composition and shelf-life of these fruits. Therefore, to maximize the potential utility of NO to augment both fruit shelf-life and quality, insights into the molecular mechanisms whereby NO and cognate *S*-nitrosylation control these processes will be important, because our data suggest that too much NO/GSNO can have adverse effects on both individual fruit size and total yield.

The role of GSNO and NO during immunity in crop plants remains relatively unclear, because a genetic analysis has not complemented the biochemical studies to date. Two sunflower (*Helianthus annuus* L.) cultivars either resistant or susceptible to infection by the downy mildew pathogen, *Plasmopara halstedii*, were employed to investigate the role of GSNO and related reactive nitrogen intermediates (RNIs) in the immune response of this plant. In the susceptible cultivar, an increase in both protein tyrosine nitration and SNOs was detected, independent of NO generation, suggesting that microbial pathogens induce nitrosative stress in susceptible sunflower cultivars. Conversely, in the resistant cultivar, there was no increase in either protein tyrosine nitration or SNOs, implying an absence of nitrosative stress. Therefore, protein tyrosine nitration might mark nitrosative stress in plants during microbial infection (Chaki *et al.*, 2009). In potato, after challenge with an avirulent *Phytophthora infestans* isolate, relatively high levels of GSNO and SNOs concentrated in the main vein of potato leaves, implying a possible mobile function of these compounds in the transfer of NO bioactivity. In contrast, during a virulent *P. infestans* infection, low-level production of NO and ROIs occurred; it was proposed that this might result in the delayed up-regulation of *PR* genes and the subsequent compromised resistance towards this pathogen (Arasimowicz-Jelonek *et al.*, 2016). Moreover, in lettuce (*Lactuca sativa*), a GSNOR-mediated decrease of SNOs was found to be a general feature of lettuce responses to both downy and powdery mildew infection, while resistance to *Bremia lactucae*, the causal agent of lettuce downy mildew, was found to parallel an increase of GSNOR activity. Thus, modulation of GSNOR activity appears to play a key role in lettuce–mildew interactions (Tichá *et al.*, 2018).

In Arabidopsis, loss-of-function mutations in *AtGSNOR* result in an increase in total *S*-nitrosylation and a reduction in SA biosynthesis and associated signalling, compromising disease resistance (Feechan *et al.*, 2005; Tada *et al.*, 2008; Yun *et al.*, 2016). In complete contrast, depletion of *AtGSNOR* transcripts has been reported to result in disease resistance (Rustérucci *et al.*, 2007). This may reflect the complex role of (S)NO in plant immunity. Thus, depleting *AtGSNOR* levels may increase SNO levels less relative to a null mutation in *AtGSNOR* and this difference in relative SNO concentrations may result in different immune ouputs (i.e. resistance versus susceptibility, respectively). If this posit is correct, our depletion of *SlGSNOR* transcripts to a similar extent in tomato might be predicted to lead to increased disease resistance. Our findings show that depletion of *GSNOR* function in tomato results in decreased SA biosynthesis and signalling, leading to compromised basal resistance. This surprisingly contrasts with previous data showing that depletion of *AtGSNOR* transcripts results in disease resistance (Rustérucci *et al.*, 2007); however, it is similar to other data proposing that null mutations in *AtGSNOR* compromise plant immunity (Feechan *et al.*, 2005;

Tada *et al.*, 2008; Yun *et al.*, 2011). Therefore, it appears unlikely that these previous findings (Rustérucci *et al.*, 2007) can be explained by differences in relative SNO concentrations between *AtGSNOR*-depleted lines and those possessing null mutations in *AtGSNOR* (Feechan *et al.*, 2005; Tada *et al.*, 2008; Yun *et al.*, 2011).

Importantly, our findings show that the function of *GSNOR* in disease resistance appears to be conserved from Arabidopsis to tomato. Significantly, the overexpression of *AtGSNOR* in Arabidopsis conveyed broad-spectrum disease resistance, without constitutive SA accumulation or associated signalling (Feechan *et al.*, 2005). Rather, *AtGSNOR* overexpression supported a potentiation of SA-dependent gene expression following attempted pathogen infection. Moreover, this resistance was not associated with a negative impact on growth or a yield penalty under laboratory conditions (Feechan *et al.*, 2005), suggesting that manipulation of GSNOR activity might provide a novel mechanism to convey broad-spectrum disease resistance in crop plants. Our data suggest that the overexpression of *SlGSNOR* also does not negatively impact growth, but it does decrease total fruit yield. However, on the positive side, overexpression of *SlGSNOR* significantly increased the size of individual tomato fruits, which might be attractive for some markets. Therefore, manipulating *SlGSNOR* expression via traditional crop breeding or gene editing approaches might provide novel strategies to convey disease resistance and perhaps also modulate the properties of tomato fruits.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. List of primers used in RT–PCR.

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