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RESEARCH PAPER

Haemoglobin modulates salicylate and jasmonate/ethylene-mediated resistance mechanisms against pathogens

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Abstract

Nitric oxide (NO) plays a role in defence against hemibiotrophic pathogens mediated by salicylate (SA) and also necrotrophic pathogens influenced by jasmonate/ethylene (JA/Et). This study examined how NO-oxidizing haemoglobins (Hb) encoded by *GLB1*, *GLB2*, and *GLB3* in *Arabidopsis* could influence both defence pathways. The impact of Hb on responses to the hemibiotrophic *Pseudomonas syringae* pathovar *tomato* (*Pst*) *AvrRpm1* and the necrotrophic *Botrytis cinerea* were investigated using *glb1*, *glb2*, and *glb3* mutant lines and also CaMV 35S *GLB1* and *GLB2* overexpression lines. In *glb1*, but not *glb2* and *glb3*, increased resistance was observed to both pathogens but was compromised in the 35S-*GLB1*. A quantum cascade laser-based sensor measured elevated NO production in *glb1* infected with *Pst AvrRpm1* and *B. cinerea*, which was reduced in 35S-*GLB1* compared to Col-0. SA accumulation was increased in *glb1* and reduced in 35S-*GLB1* compared to controls following attack by *Pst AvrRpm1.* Similarly, JA and Et levels were increased in *glb1* but decreased in 35S-*GLB1* in response to attack by *B. cinerea*. Quantitative PCR assays indicated reduced *GLB1* expression during challenge with either pathogen, thus this may elevate NO concentration and promote a wide-ranging defence against pathogens.

Key words: *Botrytis cinerea*, haemoglobin, hypersensitive response, nitric oxide, *Pseudomomas syringae*, salicylic acid.

Introduction

Induced resistance to plant pathogens is based on recognition events involving pathogen (microbial)-associated molecular patterns (PAMPs/MAMPs), pathogen-delivered effectors (Thomma *et al.*, 2011), or plant cell-wall fragment defences (Denoux *et al.*, 2008; Galletti *et al.*, 2011). Variation in elicitation events and pathogenic mechanisms leads to two major types of plant defence response. Classification of these responses is, somewhat crudely based into those mediated by salicylic acid (SA) or those dually influenced by jasmonate (JA)/ethylene (Et) (Pieterse and Van Loon, 2004). SA-mediated defences are prominent against (hemi)biotrophic pathogens that undergo a subtle interaction with the host, variously involving aspects such as the production of non-necrotizing toxins or distinct infection structures such as the fungal haustorium (O'Connell and Panstruga, 2006). SA has many roles in plant stress responses including the hypersensitive response (HR), a form of host programmed cell death – which can be triggered following plant recognition via a resistance gene product of avirulence (*Avr*) gene product encoded by the pathogen. In contrast, JA/Et acts in necrotrophic pathogens where rapid tissue maceration or pathogen-regulated host cell death are prominent infection mechanisms, although these do not preclude the exhibition of subtle responses during the interaction (Oliver and Solomon, 2004).

Over the last decade, nitric oxide has emerged as a major signal in plant defence against pathogens. It has established roles in influencing the HR form of cell death against bacterial pathogens

(Delledonne *et al.*, 1998, 2001; Mur *et al.*, 2005, 2006), necrosis induced by oomycete pathogens (Foissner *et al.*, 2000), and responses to the necrotrophic pathogens such as *Botrytis cinerea* (Yoshioka *et al.*, 2009) as well as symbiotic relationships involving legumes and *Sinorhizobium* (Puppo *et al.*, 2002; Uchiumi *et al.*, 2005).

In considering how NO production and distribution could be regulated during plant defence, most effort has concentrated on understanding its means of generation. In this context, cytosolic nitrate reductase (NR; Modolo *et al.*, 2005) has emerged as a major source of NO during plant–pathogen interactions. For efficient NO production, NR requires low concentrations of nitrate and high concentrations of nitrite because NR has a 2-fold higher affinity for nitrite in comparison to nitrate (Rockel *et al.*, 2002). As an alternative, oxidative pathways are based on a nitric oxide synthase (NOS)-like enzyme as well as both hydroxylamine- or polyamine-mediated NO generation (Moreau *et al.*, 2010; Gupta *et al.*, 2011a). In all oxidative NO production pathways, L-arginine acts as substrate or intermediate, thus inhibitors of mammalian NOS that are based on analogues of L-arginine are effective at suppressing NO production in plants (for example, Mur *et al.*, 2005). However, the genetic bases of these biochemically-defined pathways remain to be elucidated and these remain major targets for the plant NO scientist.

At the same time, mechanisms through which NO can be removed from plant cells need to be considered. NO can be eliminated by oxidation to NO_3 following the formation of an oxidized form of Hb (methaemoglobin), from which the reduced form may be regenerated by monodehydroascorbate reductase (Igamberdiev *et al.*, 2011). As such, Hb represents the prime candidate through which plants can modulate patterns of NO production during pathogen interactions (Hebelstrup *et al.*, 2007). Plants contain three classes of Hb. In classes 1 and 2 (Hunt *et al.*, 2001), Hb has protein structures similar to classical vertebrate globins with a 3-on-3 α-helical sandwich embracing a haem-group (Harutyunyan *et al.*, 1995; Hargrove *et al.*, 2000). Class 3 has truncated globins with a 2-on-2 α-helical sandwich (Wittenberg *et al.*, 2002). *Arabidopsis* encodes three Hb genes, *GLB1*, *GLB2*, and *GLB3* respectively in each class (Trevaskis *et al.*, 1997; Watts *et al.*, 2001). Previous studies have shown that *GLB1* and *GLB2* expression is particularly strong in specific cells: leaf hydathodes, meristems, and at lateral root branch points (Hebelstrup *et al.*, 2006). However, expression is also detected at a lower level by quantitative real-time PCR (qRT-PCR) in other cells such as leaf mesophylls. *Arabidopsis* plants with silencing of Hb class 1 (*GLB1*) gene expression develop stunted organs (Hebelstrup *et al.*, 2006) and flowering is delayed (Hebelstrup and Jensen, 2008). These phenotypes are associated with accumulation of NO, suggesting that Hb class 1 plays an essential role in adjustment of NO concentration in plants. These phenotypes are not present in plants with full loss-of-function mutations in class 2 (*glb2*) or 3 (*glb3*) Hb genes, demonstrating a specific role for GLB1 in NO removal (Hebelstrup *et al.*, 2006; Wang *et al.*, 2011).

Roles for Hb have already been suggested in plant defence. Class 1 Hb expression in cotton (*Gossypium hirsutum*) has been shown to be induced by exogenously applied defence hormones SA, JA, Et, H_2O_2, and NO (Seregelyes *et al.*, 2003). Tobacco plants overproducing alfalfa class 1 Hb exhibited reduced leaf necrosis when treated with NO donor and reduced cell death in response to challenge with *Pseudomonas syringae* pv. *phaseolicola* or tobacco necrosis virus (Seregelyes *et al.*, 2003). Some groups have used transgenic *Arabidopsis* lines overexpressing bacterial *Hmp* flavohaemoglobin genes from either *Escherichia coli* or *Erwinia chrysanthemi* (Zeier *et al*., 2004; Bocarra *et al*., 2005). At that time, due to the absence of well-characterized NO generation mutants, *Hmp* transgenics were-used to unequivocally demonstrate that NO influenced the HR (*Zeier et al*., 2004; *Bocarra et al*., 2005). Given these data, it was perhaps surprising that in transgenic plants overexpressing *Arabidopsis* Hb1, the major NO scavenging form, a HR elicited by the bacterial pathogen *P. syringae* pv. tomato DC3000 (*Pst*) *AvrRpm1* was not affected (Perazzolli *et al*., 2004). However, in this study, it should be noted that cell death was only assessed using trypan blue staining and that production was only qualitatively assessed at one early time point (2 h) using the dye 4-amino-5-methylamino-2',7'-difluorescein.

This study presents a more extensive characterization of the effects of different Hb genes during pathogenic challenge in *Arabidopsis*, incorporating on-line, quantitative measurements of NO production. *GLB1* expression is suppressed rapidly in response to *P. syringae* and more slowly following inoculation with *B. cinerea* and in both cases this is likely to potentiate NO effects on both SA- and JA/Et-mediated defences.

Materials and methods

Plant material

Plants were cultivated in Levington Universal compost in trays with 24 compartment inserts. Plants were maintained in Conviron growth rooms (Controlled Environments, UK) at 24 °C with a light intensity of 110 μmol/m/s and an 8/16 light/dark cycle for 4 weeks. For ease of treatment, plants were transferred to Polysec growth rooms (Polysec Cold Rooms, UK) and maintained under the same conditions. Aerial plant parts were treated at the fully expanded rosette stage at 5 weeks (stage 3.7 as defined by Boyes *et al.* (2001). All of the genotypes were of identical size expected for *glb1* which were slightly smaller (Supplementary Fig. S1, available at *JXB* online) but exhibited no evidence of necrotic flecking which was visible to the naked eye. Plants used for NO and Et measurements were transported to Radboud University, The Netherlands via road and car ferry by the authors. Plants were then kept at Radboud University for 2 days under identical growth conditions as at Aberystwyth to allow plant physiology to normalize prior to gas measurements being attempted.

Pseudomonas syringae *culture and inoculation*

Arabidopsis plants of various genotypes were inoculated with avirulent *P. syringae* pv. *tomato* (*Pst*) strain DC3000 *AvrRpm1* or the disease-forming *Pst* as described previously (Mur *et al.*, 2000) using inocula of 2×10^6 bacterial cells/ml 10 mM $MgCl_2$. Mock-inoculated controls consisted of injecting leaves with 10 mM $MgCl_2$. Bacterial inoculations for NO or Et measurements involved vacuum infiltration of six 5-week-old short-day (8 h light period) *Arabidopsis* rosettes (excised just above the soil line) in plants treated with 2×10^6 bacterial cells/ml and 10 mM $MgCl_2$ or 10 mM $MgCl_2$ alone (mock inoculation). Rosettes were immersed in a bell vacuum infiltrator and a vacuum applied for 5 min. The vacuum was carefully released during which the intracellular spaces of the leaves were observed to become filled with bacterial suspension or 10 mM $MgCl_2$ alone as appropriate. The rosettes were then quickly dried with a paper towel and then enclosed within a cuvette so

that measurement could commence. The mass of plant material used was in the range of 4–6 g per cuvette.

B. cinerea *culture and inoculation*

This study used the grape *B. cinerea* isolate IMI169558 (Thomma *et al.*, 1997). *B. cinerea* was cultured and harvested as stated in Johnson *et al.* (2007) and diluted to a concentration of 1×10^5 spores/ml in potato dextrose broth (PDB, Formedium, UK). For assessments of infection phenotypes, single leaves (leaf stage 7 or 8 as defined by Boyes *et al.*, 2001) were inoculated with 5 μl of spore suspension, pipetted onto the adaxial surface of the leaf. Controls were inoculated with PDB. Plants remained under Stewart Micropropagators to sustain a relative humidity of 50–80% and lightly watered every 24 h. For the NO and Et measurements, 1×10^5 spores/ml suspension or PDB controls were sprayed onto a whole plant to run off.

Estimations of electrolyte leakage and in planta *bacterial populations*

Cell death was estimated by electrolyte leakage in 1-cm-diameter cores as described in Mur *et al.* (2000).

Scoring B. cinerea *lesion phenotypes*

A weighted scoring method was used to categorize *B. cinerea* lesion phenotypes (Lloyd *et al.*, 2011). Susceptible symptoms (water-soaking, chlorosis, and spreading necrosis) were conferred a range of negative scores and the resistant symptoms (necrosis limited to inoculation site) were given positive scores. A weighted score could be produced arithmetically from the lesion scores of replicates.

Salicylic acid and jasmonic acid measurements

SA and JA concentrations in samples were determined by liquid chromatography–mass spectrometer (LC-MS) using the Micromass LCT–Time of Flight, as described in Clarke *et al.* (2004) and Allwood *et al.* (2006), respectively. Absolute SA and JA concentrations were derived by comparison with deuterated standards (d_6-SA, C/D/N Isotopes, Quebec, Canada; d_6-JA standard were kindly provided by Claus Wasternack Leibniz Institute of Plant Biochemistry, Halle, Germany), which were added to the samples at first extraction.

Ethylene measurements using photoacoustic laser spectroscopy

Ethylene production was monitored in real time using a gas flow-through in-line system fitted with a photoacoustic laser-based ethylene detector (ETD-300, Sensor Sense), which is able to detect on-line 300 parts per trillion volume of ethylene within 5 s (Cristescu *et al.*, 2008). Gas was regulated by an automated valve control box (VC-6, Sensor Sense). Ethylene emanation from a single inoculated rosette within a glass cuvette (254 ml volume) was alternately monitored for 15 min (5 s per acquisition point), at a controlled continuous flow rate of 1.5 l h^{-1} by flushing with air and preventing accumulation-induced effects. KOH and $CaCl_2$ scrubbers were incorporated into the system to remove CO_2 and H_2O respectively. As assays of Et production from *Arabidopsis* plants inoculated with *B. cinerea* involved measuring both the plant and the 18-cm^3 module with Levington Universal compost, a reference cuvette was set up containing only a module of compost. The negligible level of ethylene produced from this compost was subtracted from experimental cuvettes. Each experiment was repeated to give similar results and the outcomes of one representative experiment are shown.

Nitric oxide measurements using a quantum cascade laser-based sensor

The configuration of the quantum cascade laser (QCL)-based sensor for NO detection is shown in Supplementary Fig. S2. A detailed description is given elsewhere (Cristescu *et al.*, 2008). Briefly, the QCL emitting around 1900 cm^{-1} passes through an absorption multi-pass cell in which enters the airline transporting the NO released by a single inoculated rosette within a glass cuvette (~500 ml). The NO production is directly detected by measuring the attenuation of the laser intensity due to the NO absorption in the cell.

Infected materials were placed in glass cuvette and flushed with air at a controlled continuous flow rate of 1 l h^{-1}. Multiple cuvettes could be monitored in sequence, each being measured for ~13 min. Each experiment was repeated to give similar results and the outcomes of one representative experiment are shown.

Gene expression analysis and Western blotting

The expression of *GLB1* and *GLB2* was assessed in rosette leaves from *Arabidopsis* (Col-0) using qRT-PCR. The method has previously been described in (Hebelstrup *et al.*, 2010). Total RNA was purified using a FastRNA Pro Green kit (MP Biomedicals, France). Total RNA (3 μg) was used to generate first-strand cDNA by using Superscript Reverse Transcriptase II (Invitrogen) and random hexamer oligonucleotide primers in a total volume of 20 μl, as described in the manufacturer's guidelines. The finished product was diluted 10 times to be ready for qRT-PCR. qRT-PCR was performed in an ABI Prism 7900HT Sequence Detection System. Power SYBR Green PCR Master Mix (Applied Biosystems) was used as the basis for the reaction in a total volume of 10 μl (5 μl PCR Master Mix, 1 μl primer mix containing 5 μm each primer, 3 μl H_2O, 1 μl cDNA). Expression levels were normalized using the housekeeping gene *ACT2* by the method described by Pfaffl (2001). The following primer pairs were used to amplify the genes of interest: *GLB1*: 5'-CTCTTCATCAAGATCTTTGAGATTGC-3' and 5'-GACAAAAACAGACATTGCGTGAGG-3'; *GLB2*: 5'-TCACTTCTTCTCACAGATACTGGA-3' and 5'-CTTGAAGACTTTAACAGCATGAGC-3'; *ACT2*: 5'-AGCGCTGAGGCTGATGATATTCAAC-3' and 5'-TCTAGAAACATTTTCTGTGAACGATTC-3'. For Western blotting, plants were grown on 1 × Murashige-Skoog medium with 0.8% agar under 18/6 light/dark cycle with fluorescent lighting at intensities between 50 and 100 μmol/m^2/s. Polyclonal rabbit antiserum against purified recombinant GLB1 and GLB2 proteins (Trevaskis *et al.*, 1997) were used for protein detection. The proteins were extracted by grinding plant material in 0.01 M $NaPO_4$ buffer (pH 7.0) and 1mM EDTA, centrifuging (10 000 *g* for 5 min), and collecting the supernatants. Total protein (50 μg, as measured using the BioRad protein detection reagent) was used per lane for SDS PAGE separation (15% acrylamide) and Western blots were prepared and probed as described previously (Trevaskis *et al.*, 1997).

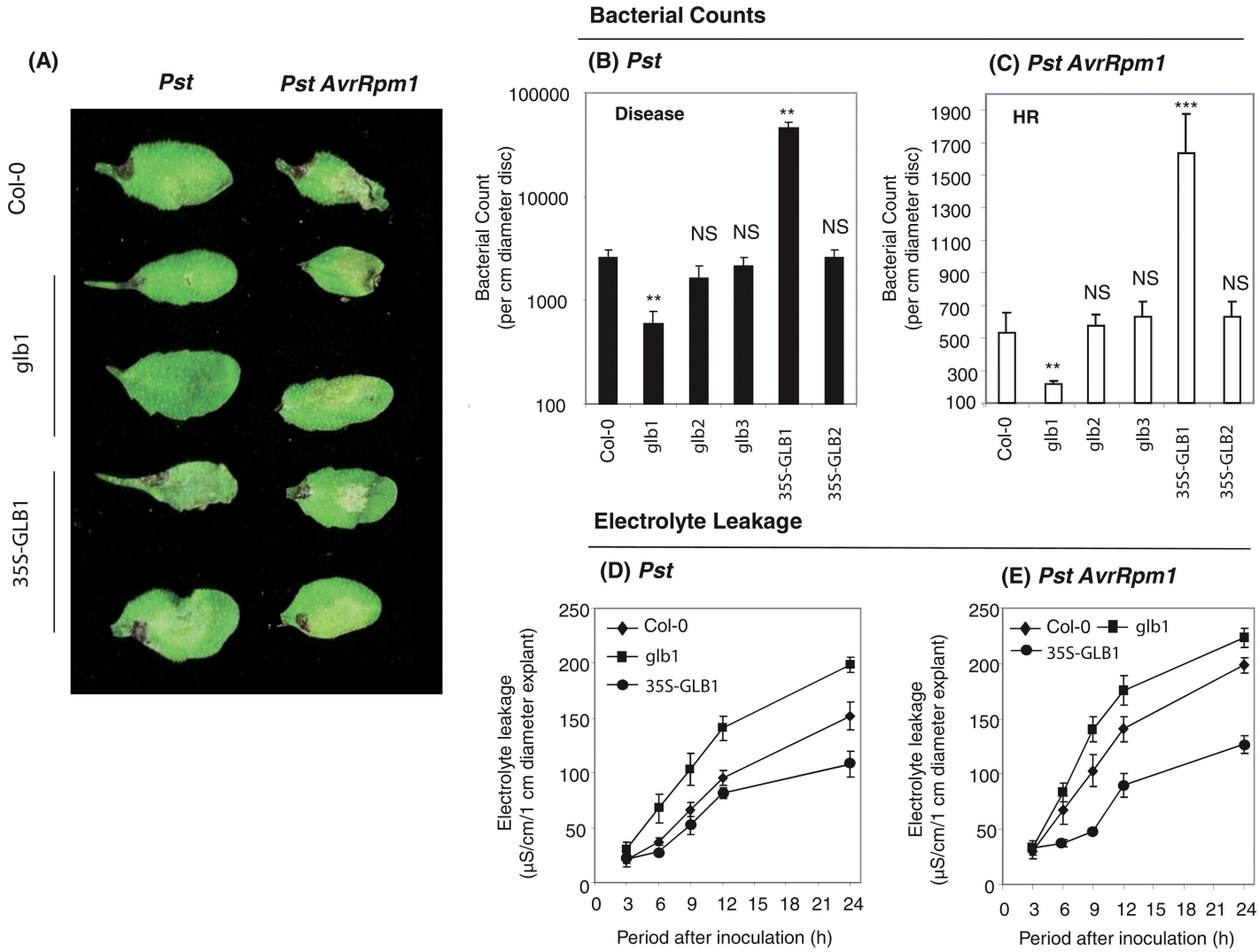
Statistical analysis

Data were subjected to analysis of variance using MiniTab version 14, after which residual plots were inspected to confirm data conformed to normality.

Results

Resistance to P. syringae *is influenced by* GLB1

These investigations into the role of Hb during pathogen challenge in *Arabidopsis* were based on the RNAi-suppressed line *glb1*, the *glb2 Spm* mutant (Hebelstrup *et al.*, 2006), a *glb3* T-DNA tagged line (Wang *et al.*, 2011), and also CaMV 35S-*GLB1* and 35S-*GLB2* overexpression lines (Hebelstrup and Jensen, 2008; Supplementary Fig. S3). Where described collectively, these will be referred to as Glb lines. Each Glb line and Col-0 controls were challenged with the virulent *P. syringae* pv. *tomato* DC3000 (*Pst*) and the HR-eliciting strain *P. syringae* pv. *tomato* DC3000 *AvrRpm1* (*Pst AvrRpm1*). With inoculation



AQ2 **Fig. 1.** Haemoglobin effects on *Pseudomonas syringae* pv. *tomato* interactions with *Arabidopsis.* (A) Lesion phenotypes at 48 h post inoculation (hpi) with the virulent disease-forming strain *P. syringae* pv. *tomato* DC3000 (*Pst*) and the avirulent, hypersensitive response (HR)-eliciting strain *Pst AvrRpm1* in Col-0, the haemoglobin (Hb) *GLB1* RNAi-suppressed *Arabidopsis* line *glb1*, and the *GLB1* overexpression line 35S-*GLB1.* Single representative examples are given for *Pst AvrRpm1* and *Pst*-elicited lesions forming in Col-0, but two examples are given for lesions forming in *glb1* and 35S-*GLB1* to represent the range of phenotypes that were obtained. (B and C) Bacterial populations at 48 hpi of *Pst* (B, filled bars) and *Pst AvrRpm1* (C, open bars) with from 1-cm-diameter cores sampled from inoculated tissue of Col-0, the *glb1* RNAi-suppressed line, the Hb *glb2* and *glb3* mutants, and the 35S-*GLB1* and -*GLB2* overexpression lines. Results are mean ± SE (n = 6). Levels of significant difference to results obtained for Col-0 are indicated: NS, no significant difference; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (D and E) Electrolyte leakage from 1-cm-diameter discs sampled from leaves of Col-0 (filled diamonds), *glb1* (filled squares), or 35S-*GLB1* (filled triangles) immediately following inoculation with *Pst* (D) or *Pst AvrRpm1* (E). Results are mean ± SE (n = 6).

of *Pst*, typical chlorotic symptoms were observed at 48 h post inoculation (hpi) in Col-0 but were noticeably reduced in *glb1* and accentuated in 35S-*GLB1* where greater cell death was observed (Fig. 1A). With inoculation of *Pst AvrRpm1* at 48 hpi, cellular collapse was observed with no observable differences between each *Arabidopsis* line (Fig. 1A) which were in agreement with the observations of Perazzolli *et al.* (2004). Following inoculation with *Pst AvrRpm1*, no observable change in HR forming in *glb1* and 35S-*GLB1* compared to that in the wild-type line. Following careful demarcation of the area of inoculation, this study found no evidence of altered lesion spread in the Glb lines (data not shown). Phenotypes in *glb2*, *glb3*, and 35S-*GLB2*, following inoculation with either *Pst* strain, were identical to those in Col-0 (data not shown).

Bacterial population sizes with the inoculated leaves were assessed at 48 hpi (Fig. 1B and C). The numbers of *Pst AvrRpm1* and *Pst* in *glb1* were significantly reduced and increased in 35S-*GLB1* significantly. With all other genotypes, bacterial numbers were not significantly different to those in Col-0. The kinetics of cell death were quantified using electrolyte leakage (Fig. 1D and E). When inoculating with either *Pst AvrRpm1* or *Pst*, cell death was increased in *glb1* and decreased

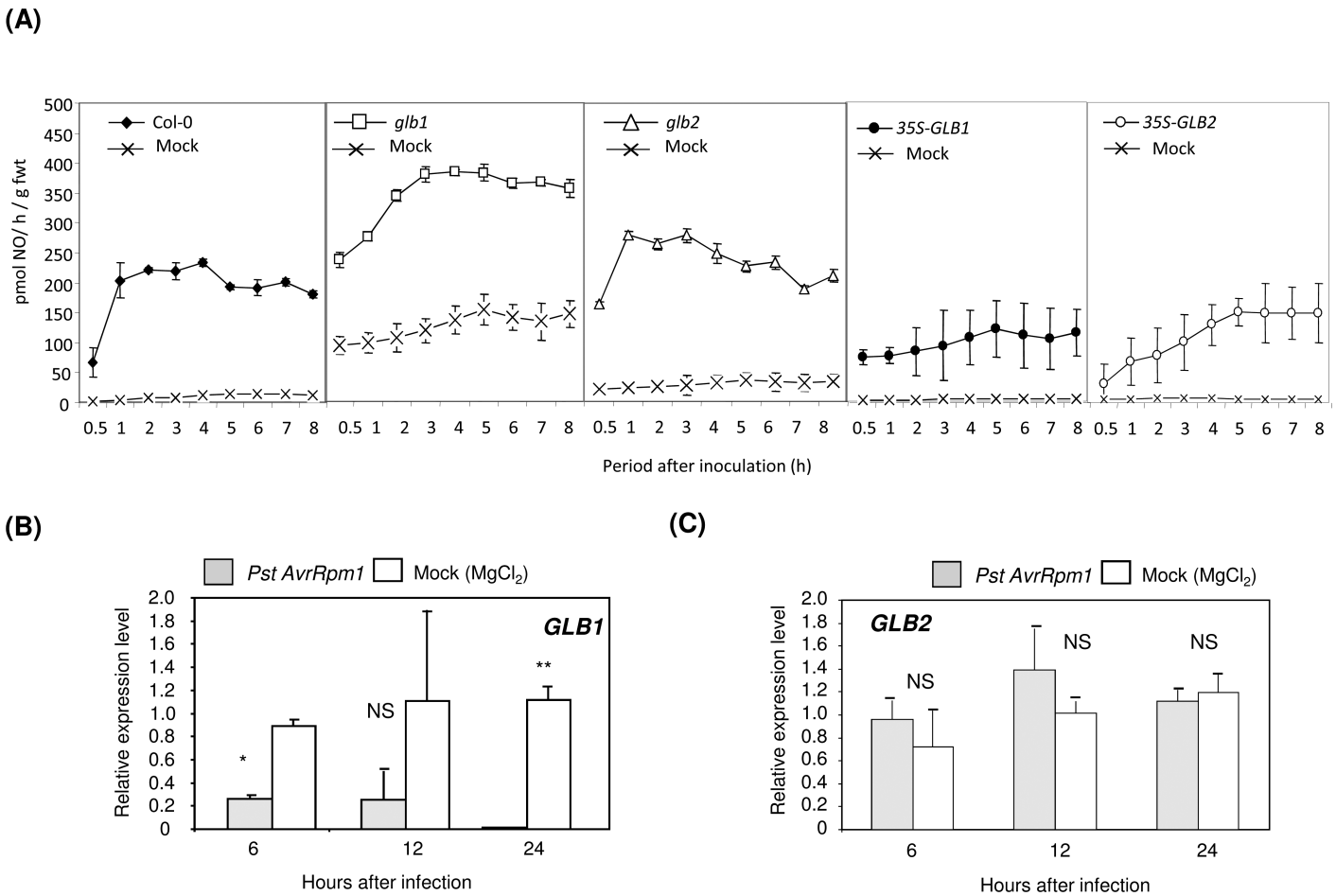


Fig. 2. Nitric oxide production from *Arabidopsis* lines with modified haemoglobin expression on inoculation with strains of *Pseudomonas syringae* pv. *tomato*. (A) NO production was determined from *Arabidopsis* Col-0 and the haemoglobin (Hb) *GLB1* RNAi-suppressed *Arabidopsis* line *glb1*, the Hb mutant *glb2*, and the 35S-*GLB1* and -*GLB2* overexpression lines, following inoculation with the hypersensitive response (HR)-eliciting *P. syringae* pv. *tomato* DC3000 (*Pst*) *AvrRpm1* strain or mock inoculation with 10 mM $MgCl_2$. NO was determined using a quantum cascade laser system. ANOVA of NO production from *Pst avrRpm1*-challenged *glb1* and 35S-*GLB1* suggested significant differences (in both cases $P < 0.01$) to that from Col-0 plants. (B and C) Quantitative real-time PCR was used to assess gene expression 24 h post inoculation in three biological replicates with either the HR-eliciting *Pst AvrRpm1* (grey blocks) or mock inoculation ($MgCl_2$; white blocks) of *GLB1* (B) and *GLB2* (C). Results are mean ± SE of three replicates of three different plants. Levels of significant difference to mock-inoculated controls at the same time point are indicated: NS, no significant difference; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

in 35S-*GLB1*. Electrolyte leakage did not significantly differ from Col-0 in *Pst* and *Pst AvrRpm1* inoculations of *glb2*, *glb3*, and 35S-*GLB2* (data not shown). These data were consistent with *GLB1* expression influencing both HR-associated resistance and basal defences deployed during disease development.

Modification of GLB expression can influence plant-derived NO production

Since Hb has a well-established NO dioxygenase activity (Gupta *et al.*, 2011b), and to focus on HR-linked defence, this study sought to related observed responses to *Pst AvrRpm1* in *glb1* and 35S-*GLB1* to NO production. NO production was determined using a QCL, the underlying principles of which and its use to measure plant-derived NO have been recently described (Yordanova *et al.*, 2010; Mur *et al.*, 2011). As noted previously (Mur *et al.*, 2006) in Col-0, NO generation was rapidly initiated and achieved maximal rates of production at 1–2 hpi (Fig. 2A). NO production from mock-inoculated uninfected *glb1*, but not *glb2* (or *glb3* plants, data not shown) was significantly increased compared with Col-0 and, in the case of *glb1*, was produced at similar rates as during the HR in Col-0. Following challenge of *glb1* with *Pst AvrRpm1*, NO production was approximately double that seen with Col-0, but in *glb2*, the patterns of NO production did not differ from Col-0. Measuring NO in *Pst AvrRpm1*-inoculated 35S-*GLB1* and 35S-*GLB2* lines (Fig. 2A) indicated that in both instances, production was reduced compared to Col-0. NO production was also assessed from *Pst*-challenged *Arabidopsis* (Supplementary Fig. S4A). Increased NO production was not observed until after 6 hpi with Col-0 and was markedly reduced in 35S-*GLB1* but increased in *glb1*. This correlated with the patterns of symptom development (Fig. 1A).

The expression of *GLB1* (Fig. 2B) and *GLB2* (Fig. 2C) was assessed in Col-0 at 24 h after inoculation with *Pst AvrRpm1* by qRT-PCR. Inoculation with *Pst AvrRpm1* significantly reduced *GLB1* compared to controls within 6 hpi and was barely detectable by 24 hpi (Fig. 2B). *GLB2* expression did not significantly differ from controls throughout the first 24 hpi with *Pst AvrRpm1* (Fig. 2C).

NO contributes to salicylic acid-mediated defence potentiation in HR

Increased Hb expression has been shown to suppress pathogen-initiated SA accumulation (Seregelyes *et al.*, 2003). Thus, this study sought to relate *GLB1*-associated effects to SA accumulation in response to *Pst AvrRpm1* at 48 hpi (Fig. 1B). SA concentrations in *Pst AvrRpm1*-challenged *glb1* were significantly elevated and reduced in 35S-*GLB1*. SA accumulation in mock-inoculated *glb1* was also increased compared to Col-0 (Fig 3A).

Both NO and SA are required to enhance Et biosynthesis during a *P. s.* pv. *phaseolicola*-elicited HR in tobacco (Mur *et al.*, 2008) so Et production was measured in *Pst AvrRpm1*-challenged *Arabidopsis* Col-0, *glb1*, and 35S-*GLB1* using laser photoacoustic detection. Challenge of Col-0 led to the biphasic rise in Et production (Mur *et al.*, 2009) and both peaks were increased in *glb1* (Fig. 3B) but decreased in 35S-*GLB1* (Fig. 3C). Hence, one effect of increased NO accumulation in *glb1* may be increase SA-influenced Et production. To substantiate this observation, this study examined SA and Et levels in *Pst*-challenged plants (Supplementary Fig. S4B and C). In *glb1*, *Pst*-elicited SA accumulation (Supplementary Fig. S4B) and ethylene production (Supplementary Fig. S4C) were increased, which correlated with the increased resistance to this virulent strain observed on this plant genotype (Fig. 1).

Resistance to the necrotrophic fungus B. cinerea *is influenced by GLB1*

Et plays a minor role in resistance to *Pst AvrRpm1* (Mur *et al.*, 2009), unlike the situation following challenge with *B. cinerea* where it is a major defence determinant (Thomma *et al.*, 1997). Inoculations with *B. cinerea* are carried out by drop inoculation on to adaxial surfaces so that the degree of host resistance is indicated by the size of lesion and the speed with which the pathogen penetrates through to the abaxial surface (Fig. 4A). At 72 hpi, lesion development in *glb1* was markedly delayed compared to controls, with little penetration through to the abaxial surface. In 35S-*GLB1* plants, abaxial penetration was rapid and whole leaf collapse was evident (Fig. 4A). A semi-quantitative scoring scheme has recently been developed to describe *B. cinerea* lesion development (Lloyd *et al.*, 2011), which facilitates comparisons between responses to infection in different genotypes (Fig. 4B). This comparison included the NR Col-0 *nia1* mutant, which is most compromised in NO generation (Neill *et al.*, 2008; Miao *et al.*, 2010; Lu *et al.*, 2011). Also included was the L *er nia2* mutant which is mutated in the gene encoding the most active form of NR but apparently is not a major source of NO (Neill *et al.*, 2008). Lesion scores at 24, 48, and 72 hpi were identical in Col-0 and *glb3*, further suggesting that *GLB3* does not play an observable role in plant defence. In contrast, the *glb1* line was the most resistant phenotype and 35S-*GLB1* the most susceptible line, whilst responses in *nia1* and *glb2* were very similar. In line with the negligible effect of NIA2 in NO-mediated events, lesions in *nia2* and L *er* were identical.

To further quantify the differences in *B. cinerea* lesion development amongst the *Glb* lines, cell death was estimated by electrolyte leakage in explants (Fig. 4C). Compared to Col-0 controls, *glb1*, but not *glb2*, *glb3* or 35S-*GLB2*, electrolyte leakage was significantly increased at both 24 and 48 hpi. Electrolyte leakage was also significantly increased in 35S-*GLB1* but this may reflect the rapid fungal penetration in this line (Fig. 4A). The equivocal nature of such results limited the value of the electrolyte leakage assay with *B. cinerea* inoculations of Glb lines.

NO potentiates ethylene- and jasmonic acid-mediated plant defence against B. cinerea

As above (Fig. 2), the observed changes in lesion development were related to patterns of NO generation. Using QCL, NO production was determined from Col-0, *glb1* (Fig. 5A), and 35S-*GLB1* (Fig. 5B) challenged with *B. cinerea* compared to uninfected controls. Spraying plants with *B. cinerea* led to rapid production of NO in Col-0, achieving similar levels to those seen with control *glb1* plants. NO production in *B. cinerea*-infected *glb1* was significantly higher and in 35S-*GLB1* significantly lower than in infected wild-type controls. *GLB1* (Fig. C) and *GLB2* (Fig. 5D) expression in response to *B. cinerea* was investigated using qRT-PCR. At 48 h, *GLB1* expression was reduced in *B. cinerea*-inoculated samples but *GLB2* was increased compared to mock-inoculated controls.

Et synthesis was determined in Col-0, *glb1*, and 35S-*GLB1* following inoculation with *B. cinerea* using laser photoacoustic detection (Fig. 6A). In all genotypes, a minor, transient rise in Et biosynthesis was observed 3–6 hpi only to increase for a second time at ~5–7 hpi. In Col-0 and *glb1*, this rise in Et production persisted for at least 12h before subsiding. In 35S-*GLB1*, the second increase in Et generation greatly reduced. As Et acts with JA in conferring resistance to *B. cinerea* (Thomma *et al.*, 1997), the concentration of JA was measured in Col-0, *glb1*, and 35S-*GLB1* at 48 hpi (Fig. 6B). JA accumulation in mock-inoculated and inoculated 35S-*GLB1* did not appear to significantly differ from equivalent treatments in Col-0. However, in *B. cinerea*-inoculated *glb1* JA accumulation was significantly increased over infected Col-0.

Discussion

GLB1 negatively affects plant responses to pathogens

Over the last decade, NO has emerged as a major signal that mediates plant defence against pathogens. Previously, plant lines overexpressing bacterial flavohaemoglobin genes have been used as a means of demonstrating the importance of NO to the system under investigation, whether defence (Zeier *et al.*, 2004; Bocarra *et al.*, 2005), senescence (Mishina *et al.*, 2007),

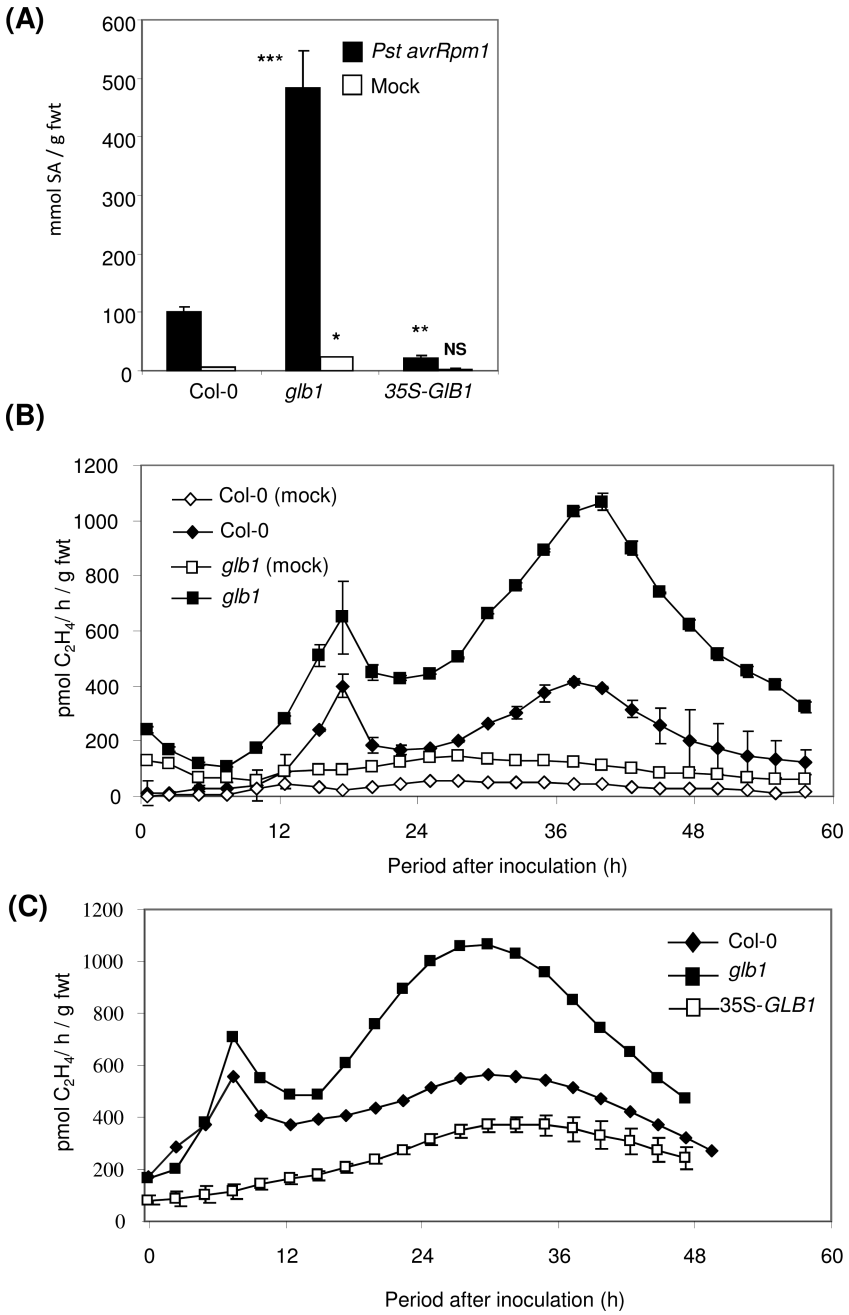


Fig. 3. Haemoglobin effects on salicylic acid (SA) accumulation and ethylene production elicited by *Pseudomonas syringae* pv. *tomato*. (A) SA accumulation at 48 h post inoculation of *Arabidopsis* Col-0, the haemoglobin (Hb) *GLB1* RNAi-suppressed *Arabidopsis* line *glb1*, and the *GLB1* overexpression line 35S-*GLB1*, inoculated with *P. syringae* pv. *tomato* DC3000 *avrRpm1* (*Pst avrRpm1*) or mock inoculated with 10 mM $MgCl_2$. Results are mean ± SE (n = 6). Statistical comparisons were made between inoculated Col-0 and *glb1* or 35S-*GLB1* plants and also between mock-inoculated and *glb1* or 35S-*GLB1* plants. Levels of significant difference are indicated: NS = no significant difference; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. (B and C) Ethylene production was determined using laser photoacoustic detection with *Arabidopsis* Col-0 and either *glb1* (B) or 35S-*GLB1* (C) following inoculation with *Pst avrRpm1* or mock inoculation with 10 mM $MgCl_2$.

or UV-B protection (Tossi *et al.*, 2011). In contrast, when Perazzolli *et al.* (2004) overexpressed endogenous Hb in *Arabidopsis*, no effect on a HR elicited by *Pst AvrRpm1* was observed. However, in Perazzolli's study, both cell death and NO production were qualitatively estimated at a single time point. Thus, the present study embarked on a fuller, quantitative assessment of the potential roles of Hb in plant–pathogen interactions, focusing on defences employed against a hemibiotrophic bacterial pathogen *Pst AvrRpm1* and the necrotrophic fungus *B. cinerea*.

This study used a variety of different *Arabidopsis* lines with silencing, overexpression, or mutation of the three Hb genes GLB1, GLB2, and GLB3. No GLB1 loss-of-function mutant has been identified in any mutation-tagging studies, suggesting that such a mutation is lethal. Instead a previously described silencing

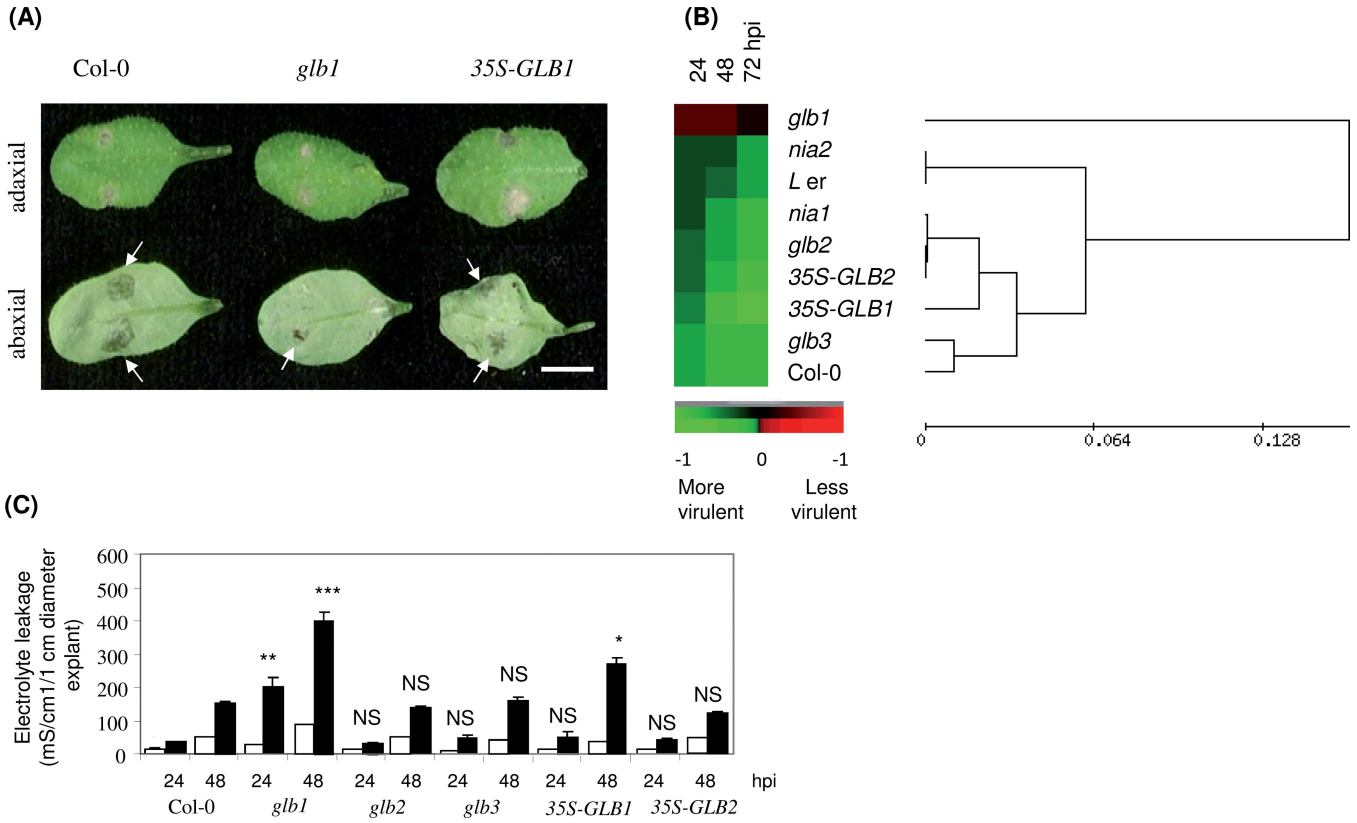


Fig. 4. Haemoglobin effects on *Botrytis cinerea* interactions with *Arabidopsis*. (A) Lesion development in *Arabidopsis* Col-0, the haemoglobin (Hb) *GLB1* RNAi-suppressed line *glb1*, and the *GLB1* overexpression line 35S-*GLB1* at 72 h post-inoculation (hpi) with *B. cinerea* strain IMI169558. Attached leaves were inoculated on the adaxial side with 5 μl of 1 × 10^5 spores ml^{-1} in potato dextrose broth. Leaves were detached only for photography and representative lesion phenotypes on the adaxial and abaxial sides are shown. (B) *B. cinerea* lesion phenotypes occurring in Col-0, the *glb1* RNAi-suppressed line, the Hb *glb2* and *glb3* mutants ,and the 35S-*GLB1* and -*GLB2* overexpression lines and also the nitrate reductase mutants *nia1* and *nia2* (*Landsberg erecta* [L *er*] is included because the *nia2* mutant is derived from this ecotype), scored at 24, 48, and 72 hpi according to Lloyd *et al.* (2011): see Materials and Methods. A heat map and dendogram was derived to show the relationships between patterns of lesion development. (C) Electrolyte leakage from 1-cm-diameter discs sampled from leaves of Col-0, *glb1*, *glb2*, *glb3*, 35S-*GLB1*, or 35S-*GLB2* following inoculation with *B. cinerea* (filled bars) or mock inoculated (open bars) with potato dextrose broth. Results are mean ± SE (n = 6). Levels of significant differences to results equivalent treatments of Col-0 are indicated: NS, no significant difference; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

line, where gene expression level is limited to 2–3% of that of the wild-type line, was used (Hebelstrup *et al.*, 2006). A number of developmental phenotypes, which may interfere indirectly with pathogen susceptibility, have been described for this line (Hebelstrup *et al.*, 2006; Hebelstrup and Jensen, 2008). However, most of these phenotypes disappear when the plants are grown under short-day conditions, as done in this study, where the only difference is a small decrease in size of the glb1 line (Supplementary Fig. S3). Interestingly, the mock-inoculated *glb1*-suppressed line emitted a constant level of NO which was elevated compared to controls and which also featured to a lesser extent with the *glb2* line. This latter observation is in line with the previous observation that GLB2 is less important for NO removal compared to GLB1 and that basal production of NO requires removal for efficient plant growth (Hebelstrup *et al.*, 2006; Hebelstrup and Jensen, 2008).

To ascertain the relative roles of each individual Hb gene on NO removal, the corresponding suppressed lines were challenged with virulent *Pst* stains and HR-eliciting *Pst AvrRpm1* (Fig. 1). As suggested by Perazzolli *et al.* (2004), the HR phenotype elicited by *Pst AvrRpm1* did not appear to differ between controls and Glb modulated lines. However, quantitatve estimates of cell death using electrolyte leakage as well as of bacterial numbers suggested that, in *GLB1*-suppressed lines, resistance and cell death in response to both strains were significantly enhanced. Phenotypes obtained following inoculation of the Glb lines with *Pst AvrRpm1* correlated with the observed rates of NO production. Thus, for example, overproduction of *GLB1* and to a lesser extent *GLB2* could suppress NO production (Fig. 2A) and this was associated with reduced host cell death and increased bacterial numbers, reaffirming the role of NO in resistance against *Pst* strains.

Whilst the regulation of NO is undoubtedly important to defence, the qRT-PCR data suggested that, with *Pst AvrRpm1*-inoculated *Arabidopsis* at least, modulation of Hb expression can also influence NO levels (Fig. 3A and 3D). Within 6 hpi with *Pst AvrRpm1*, *GLB1* expression was significantly reduced in Col-0 and nearly undetectable by 24 hpi, suggesting that in a natural

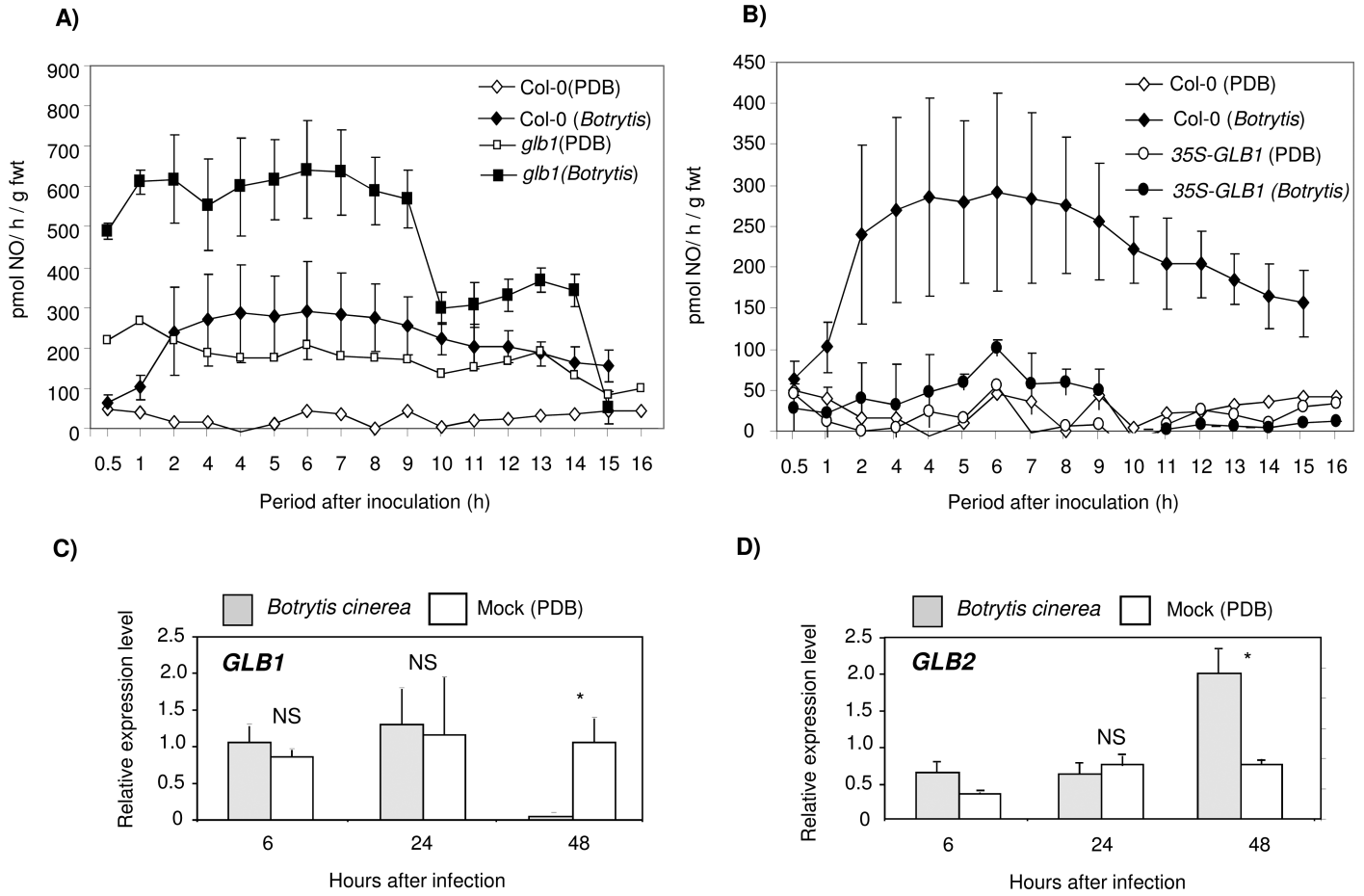


Fig. 5. Nitric oxide production from *Arabidopsis* lines with modified haemoglobin expression on inoculation with *Botrytis cinerea*. (A and B) NO production was determined from *Arabidopsis* Col-0 and the haemoglobin (Hb) *GLB1* RNAi-suppressed *Arabidopsis* line *glb1* or the *GLB1* overexpression lines 35S-*GLB1* following inoculation with *B. cinerea* strain IMI169558 or mock inoculated with potato dextrose broth (PDB). NO was determined using a quantum cascade laser system. Results in Fig. 5A are derived from two experiments conducted within a 48-h period and in Fig. 5B represent the pooling of two similarly contemporaneous experiments undertaken 1 week after those represented in Fig. 5A, albeit with equivalently aged plants. Results are mean ± SE (n = 3) except for controls, where only the result from a single replicate is depicted. ANOVA of NO production from *B. cinerea*-infected *glb1* and 35S-*GLB1* suggested significant differences ($P < 0.05$ and $P < 0.01$, respectively) to measurements from wild-type plants. (C and D) Quantitative real-time PCR was used to assess gene expression 48 h post inoculation in three biological replicates with either *B. cinerea* (filled bars) or mock inoculation (PDB; open bars) of *GLB1* (C) and *GLB2* (D). Results are mean ± SE of three PCR replicates. Levels of significant difference to mock-inoculated controls at the same time point are indicated: NS, no significant difference; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

avirulent *Pst–Arabidopsis* Col-0 interaction *GLB1* expression is suppressed in a manner analogous to the *glb1* mutant with, presumably, comparable effects on NO accumulation. As *GLB2* expression was not significantly affected throughout the time course, it was unlikely that the observed reduction of *GLB1* expression was due either to plant cell collapse during the HR or to global changes in gene expression.

The data for *GLB1* expression following inoculation with *B. cinerea* indicated that suppression was not observed until 48 hpi, when necrosis at the infection site is first evident (Lloyd *et al.*, 2011). This suppression clearly did not match the observed patterns of NO production (Fig. 5A) and it may be that it reflected different kinetics when spotting *Botrytis* on *Arabidopsis* as opposed to spraying. Unfortunately, it did not prove possible to detect NO production from spot *Botrytis*-inoculated *Arabidopsis* plants (data not shown). As infections of 35S-*GLB1* with *Botrytis* were markedly affected (Fig. 4A), this later *GLB1* suppression may act to increase NO production to aid in confining the pathogen to the site of inoculation. Indeed, with the 35S-*GLB1* line, *Botrytis* was less well confined to the point of inoculation (Fig. 4A).

Some indirect evidence could suggest that NO plays no role in resistance to *B. cinerea*. Thus, Benito *et al.* (2010) demonstrated that a *Botrytis* Hb mutant was not affected in its ability to infect its host. Further, *B. cinerea* also transiently produces NO early in its infection process (Benito *et al.*, 2010). Indeed, it may be that the fungus responds to external, *in planta*-generated NO to promote pathogenesis of host tissue (Turrion-Gomez and Benito, 2011). This offers an apparently contradictory scenario where NO is being used by the host for defence and by the pathogen to promote virulence. Rationalization of these disparate data may require careful spatiotemporal measurement of

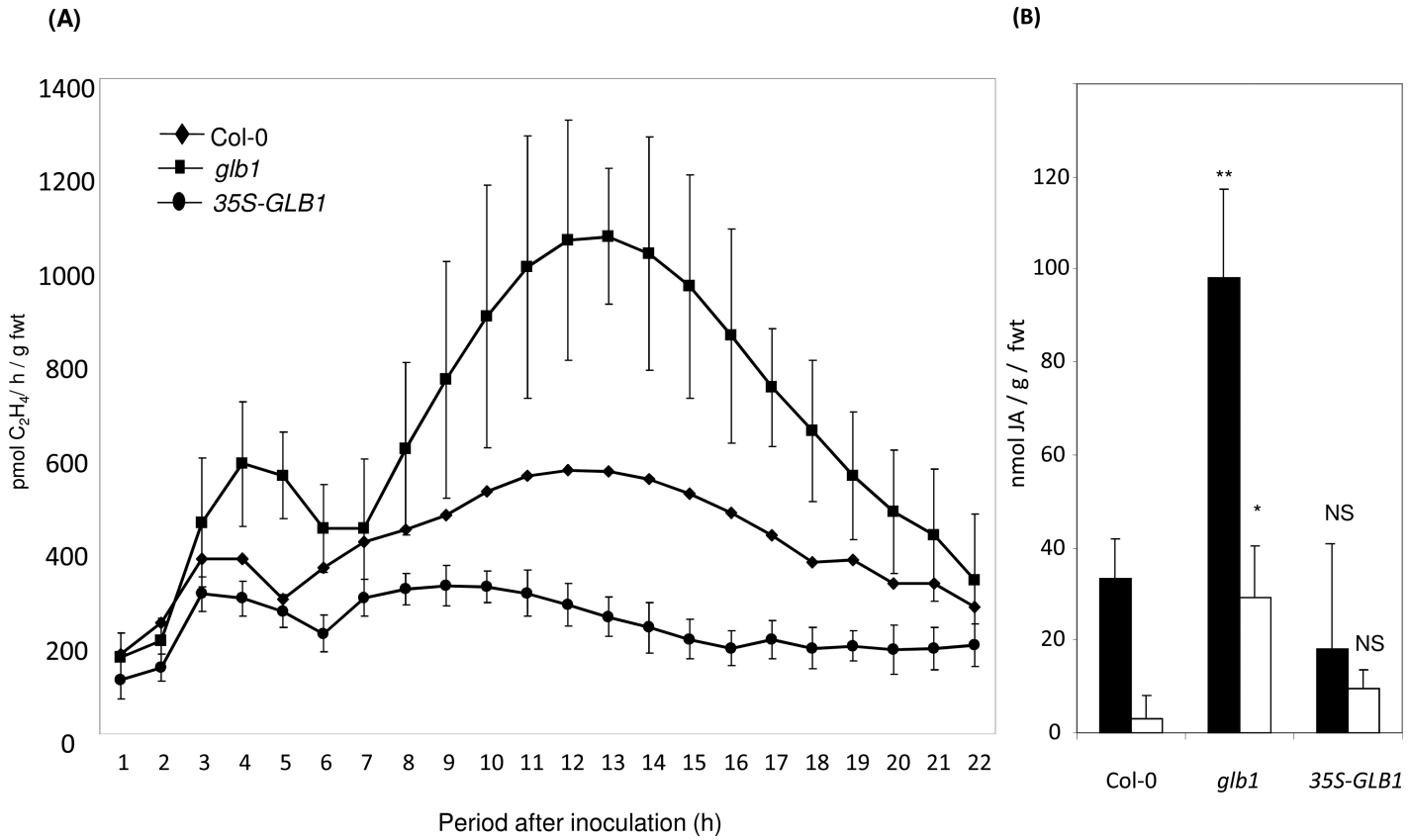


Fig. 6. Haemoglobin effects on ethylene production and jasmonic acid (JA) accumulation elicited by *Botrytis cinerea*. (A) Ethylene production was determined using laser photoacoustic detection (LPAD) from *Arabidopsis* Col-0, the haemoglobin (Hb) *GLB1* RNAi-suppressed *Arabidopsis* line *glb1*, and the *GLB1* overexpression line 35S-*GLB1* following inoculation with *B. cinerea* strain IMI169558 or mock inoculated with potato dextrose broth. Results are mean ± SE (n = 3). (B) JA accumulation at 48 h post inoculation of *Arabidopsis* with *B. cinerea* (filled bars) or mock inoculated with potato dextrose broth (open bars). Results are mean ± SE (n = 6). Statistical comparisons were made between *B. cinerea*-infected Col-0 and *glb1* or 35S-*GLB1* plants and also between controls in Col-0 and *glb1* or 35S-*GLB1* plants. Levels of significant difference are indicated: NS, no significant difference; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Results in Part A are derived from directly contemporaneous experiments as two LPAD were used in parallel.

NO concentrations, as the relative concentration of NO could play a vital role in governing its action (Lamattina and Beligni, 2001; Turrion-Gomez and Benito, 2011). It may be that altered host *GLB1* expression at specific points in the infection process could play a role in favour of defence over promotion of pathogen invasiveness.

NO is a potentiator of both SA- and Et/JA-associated defences

A central event in the potentiation of defence and cell death against bacterial pathogens is the initiation of SA biosynthesis (Mur *et al.*, 2000). The initiation of SA biosynthesis by NO is a well-established event (Klessig *et al.*, 1998; Zottini *et al.*, 2007), so unsurprisingly, SA levels were increased in *glb1* and decreased in 35S-*GLB1* following inoculation with *Pst AvrRpm1* (Fig. 3A). Such SA production has previously been linked with a potentiated oxidative burst (Mur *et al.*, 2000), but establishing the *in planta* kinetics of the oxidative burst in *Arabidopsis* is technically demanding. The use of the H_2O_2-detecting stain 3,3-diaminobenzidine did not suggest any changes in *Pst AvrRpm1*-challenged *glb1* or 35S-*GLB1* compared to Col-0 (data not shown). However, the kinetics of Et production shares many features of the oxidative burst (Mur *et al.*, 2008, 2009). The first transient phase of Et is PAMPs-elicited whilst the second persistent rise was seen only with HR-eliciting bacteria Further, Et production was potentiated by SA (Mur *et al.*, 2008, 2009). The current study observed increased Et production in *glb1* but this was greatly compromised in 35S-*GLB1* (Fig. 3C). The mammalian NOS inhibitor L-NAME was previously used to demonstrate a link between bacterially elicited NO and Et production (Mur *et al.*, 2008). However, those experiments failed to suppress the first transient rise in Et production leading to the hypothesis that this could be influenced by other signals, particularly reactive oxygen species (ROS). However, in *glb1*, both the transient and persistent rise in Et production was augmented and, crucially, both were compromised in 35S-*GLB1*, which demonstrates the limitations of using L-NAME in plant studies. Somewhat surprisingly, Qu *et al.* (2006) noted that overexpression of a cotton Hb gene in *Arabidopsis* led to enhanced disease resistance to pathogenic challenge and the activation of defence genes *PR1* and *PDF1.2*. This contrasts with the well-established role of NO

in the initiation of SA biosynthesis for which *PR1* is a well-established gene marker (Klessig *et al.*, 1998), because Hb overexpression leads to scavenging of NO, as demonstrated in this work and several previous studies (Hebelstrup *et al.*, 2007) This could indicate that cotton Hb can have distinctive effects than scavenging of NO when expressed in *Arabidopsis*. Equally, it should be noted that no NO measurements were done by Qu *et al.* (2006).

Et is much more prominent in conferring resistance to *B. cinerea* than to *Pst AvrRpm1* (Thomma *et al.*, 1997). The current measurements established that the degree of resistance correlated with the NO and Et rates of production (Fig 4A, Fig. 5A and 5B). Interestingly, Et production was more rapid in these experiments than in Lloyd *et al.*, 2011). As suggested above for NO production, this may indicate that applying fungal spore suspensions resulted in more rapid responses than simply spotting the pathogen on to the leaf surface as in Lloyd *et al.* (2011). JA accumulation in *glb1* and 35S-*GLB1* suggested that, following challenge with *B. cinerea*, JA accumulation correlated with the rate of NO production (Fig. 6). Thus, NO seems likely to contribute to both Et and JA defences against necrotrophs. This situation appears different to that in *Nicotinia attenuata* grazed by tobacco hornworm (*Meduca sexta*). Silencing of *NOA1*, a gene linked with NO generation (Guo and Crawford, 2005) in *N. attenuata* increased JA biosynthesis during *M. sexta* feeding (Wunsche *et al.*, 2011a,b) Similarly, silencing S-nitrosoglutathione reductase (GSNOR), which removes NO-derived S-nitrosylaton adducts, so that the effects of NO would be amplified, less JA biosynthesis was observed in *N. attenuata* on feeding with *M. sexta* (Wunsche *et al.*, 2011a). Although these observations appear at odds with the current results obtained using *B. cinerea* and *Arabidopsis* (Fig. 6B), it may be that specific pest/pathogen and host species interactions influence the outcome of NO–JA interactions.

The role of ROS during NO–Et/JA interactions following attack with *Botrytis* also requires consideration. Asai and Yoskioka (2009) demonstrated how NO acting with SA is important in suppressing *B. cinerea* disease development in *Nicotiana benthamiana*. In contrast, ROS production actively aided disease progression. Similarly, Govrin and Levine (2000) showed that host ROS generation is an important determinant of *B. cinerea* virulence. Thus, NO could reduce *B. cinerea* aggressiveness by reducing H_2O_2 production. Indeed, Malolepsza and Rozalska (2005) observed that NO reduced H_2O_2 concentration in tomato infected with *B. cinerea.* When the current study attempted to assess ROS levels in Col-0, *glb1*, and 35S-*GLB1* lines with *B. cinerea* using 3,3-diaminobenzidine staining, no differences could be detected (data not shown) but this could reflect the insensitivity of this assay for ROS.

Taking all of these observations together suggests that NO acts as a broadly acting defence initiator which acts to potentiate multiple signalling pathways. Further, NO production is regulated at least in part through modulated host Hb expression.

Supplementary material

Supplementary material is available at *JXB* online.

Supplementary Fig. S1. *Arabidopsis rosettes used to assess trace gas emissions following pathogen attack.*

Supplementary Fig. S2. *The quantum cascade laser-based sensor adapted for nitric oxide detection.*

Supplementary Fig. S3. *GLB1 and GLB2 expression in the overexpression lines.*

Supplementary Fig. S4. *Nitric oxide, salicylic acid, and ethylene production and from Arabidopsis lines with modified haemoglobin expression on inoculation with Pseudomonas syringae pv. tomato.*

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