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RESEARCH PAPER

Haemoglobin modulates NO emission and hyponasty under hypoxia-related stress in *Arabidopsis thaliana*

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Abstract

Nitric oxide (NO) and ethylene are signalling molecules that are synthesized in response to oxygen depletion. Non-symbiotic plant haemoglobins (Hbs) have been demonstrated to act in roots under oxygen depletion to scavenge NO. Using *Arabidopsis thaliana* plants, the online emission of NO or ethylene was directly quantified under normoxia, hypoxia (0.1–1.0% O_2), or full anoxia. The production of both gases was increased with reduced expression of either of the Hb genes *GLB1* or *GLB2*, whereas NO emission decreased in plants overexpressing these genes. NO emission in plants with reduced Hb gene expression represented a major loss of nitrogen equivalent to 0.2 mM nitrate per 24 h under hypoxic conditions. Hb gene expression was greatly enhanced in flooded roots, suggesting induction by reduced oxygen diffusion. The function could be to limit loss of nitrogen under NO emission. NO reacts with thiols to form *S*-nitrosylated compounds, and it is demonstrated that hypoxia substantially increased the content of *S*-nitrosylated compounds. A parallel up-regulation of Hb gene expression in the normoxic shoots of the flooded plants may reflect signal transmission from root to shoot via ethylene and a role for Hb in the shoots. Hb gene expression was correlated with ethylene-induced upward leaf movement (hyponastic growth) but not with hypocotyl growth, which was Hb independent. Taken together the data suggest that Hb can influence flood-induced hyponasty via ethylene-dependent and, possibly, ethylene-independent pathways.

Key words: Ethylene, flooding, haemoglobin, hyponastic growth, hypoxia, nitric oxide (NO).

Introduction

When plant haemoglobin (Hb) was discovered in the early 20th century, it was assumed to have a function in oxygen binding similar to animal Hb (Appleby, 1992). Hb genes have been found in all plant species tested, but, with the exception of symbiotic root nodules, the concentrations of Hb protein are generally too low to make a significant contribution to oxygen transport or facilitation of diffusion (Hebelstrup *et al*., 2007). Three classes of Hb genes can be distinguished. Classes 1 and 2 are similar in structure to animal myoglobins and Hbs (Trevaskis *et al*., 1997), whereas class 3 plant Hbs share the closest structural homology with bacterial truncated Hbs (Watts *et al*., 2001). Each class is represented by a single gene in the genome of *Arabidopsis thaliana* (Trevaskis *et al*., 1997; Watts *et al*., 2001). Plant Hbs may also be classified as either non-symbiotic or symbiotic, depending on whether they are synthesized in high concentration in root nodules with symbiotic nitrogen-fixing bacteria, such as leghaemoglobins, or they are synthesized in other tissues with no relationship to symbiosis (Gupta *et al*., 2011). Most symbiotic Hbs

are of the class 2 type; however, class 1-type symbiotic Hb has also been reported (Heckmann *et al*., 2006).

Hb gene expression is up-regulated in oxygen-depleted organs (Taylor *et al*., 1994; Trevaskis *et al*., 1997). Several studies have demonstrated a role for plant Hbs in catalysing the turnover of nitric oxide (NO) to nitrate (Dordas *et al*., 2003a, 2004; Perazzolli *et al*., 2004; Hebelstrup *et al*., 2006). When Hb is coupled with nitrite reductase activity in hypoxic cells, this forms the Hb/NO cycle, in which excess NAD(P)H is oxidized (Igamberdiev *et al*., 2004). The rate of NO turnover by Hb when operating in this cycle is at least 20 nmol g FW^{-1} h^{-1} (Dordas *et al*., 2004). Accordingly, it was demonstrated that Hb-overexpressing plants have increased survival during hypoxia (Hunt *et al*., 2002; Hebelstrup *et al*., 2006) and have increased ATP levels compared with wild-type and Hb-silenced plants in hypoxic root segments (Sowa *et al*., 1998; Dordas *et al*., 2003a). Plant Hb genes are also expressed in shoots under normoxic conditions (Heckmann *et al*., 2006), with a particularly high activity in shoot meristems and leaf hydathodes (Hebelstrup *et al*., 2006).

Arabidopsis plants in which class 1 Hb gene expression is silenced through RNA interference (RNAi) show a number of shoot and leaf phenotypes: Flowering is delayed, apical meristems tend to reverse from bolting stage to rosette stage (Hebelstrup and Jensen, 2008), and leaves are stunted with enlarged hydathodes (Hebelstrup *et al*., 2006). These phenotypes coincide with NO accumulation in the affected organs, which hints at a role for plant Hb in modulation of NO signalling in development and hormone responses (Hebelstrup *et al*., 2007). Indeed, NO formation and/or perception are part of signalling pathways of several hormones, and a number of observations in different studies suggest that Hb can interfere with the action of several hormones by modulating NO levels (Hill, 2012). NO is, for instance, a central component in abscisic acid (ABA)-induced stomatal closure (Neill *et al*., 2008), and NO generation has been shown to interfere with various auxin-dependent responses such as root development (Pagnussat *et al*., 2003; Correa-Aragunde *et al*., 2004) and auxin-mediated gravitropism (Hu *et al*., 2005). Moreover, NO is involved in the elicitation of programmed cell death (PCD; Delledonne *et al*., 1998) by controlling the biphasic ethylene formation during the hypersensitive response in plants subjected to pathogens (Mur *et al*., 2012). Recently, it was shown how Hb, most probably acting via modulation of NO production, influenced the generation of the defence hormones salicylic acid, ethylene, and jasmonic acid (Mur *et al*., 2012).

The ethylene precursor ACC (1-aminoacyl cyclopropane-1-carboxylic acid) is constitutively generated in root tips. ACC can accumulate in response to oxygen depletion and subsequently it can be transported to the shoots via xylem vessels, where the oxygen concentration is high enough to allow conversion to ethylene by ACC oxidase (ACO; Jackson, 2002). Ethylene can accumulate to high levels by entrapment in flooded organs coinciding with low oxygen levels (Visser and Voesenek, 2005) and trigger various adaptive responses in flooding-tolerant species (Bailey-Serres and Voesenek, 2008). For example, some species escape submergence by ethylene-induced upward leaf movement, called hyponastic growth, followed by petiole elongation to reach above the water surface and restore (e.g. oxygen) gas exchange. Ethylene also induces a marked hyponastic growth response in *A. thaliana* plants (Millenaar *et al*., 2005, 2009). This system has been exploited to gain an understanding of the molecular, hormonal, and physiological mechanisms controlling leaf movement (Van Zanten *et al*., 2010). Ethylene, NO, and non-symbiotic Hbs are all associated with hypoxia linked to flooding. *Electron paramagnetic resonance* (*EPR*) was used to measure NO production from alfalfa root cultures which could be modulated through the overexpression or antisense suppression of Hb in transgenic lines (Dordas *et al*., 2003a). Notably, lines with reduced Hb expression exhibited cellular disruption and reduced ATP levels, thus implicating Hb in the maintenance of cellular viability during hypoxia. As ethylene production was elevated in Hb knockdown maize cell lines under conditions of hypoxia (Manac'h-Little *et al*., 2005), it can be hypothesized that altered Hb would augment ethylene production during hyponastic growth.

This study provides online quantitative measurements of NO and ethylene emission from *Arabidopsis* plants by a method that allows the direct comparison of the effects of various degrees of hypoxia or full anoxia. This allowed the determination of at what oxygen concentration NO production is triggered and the estimatation of how much nitrogen is lost through NO emission. This release is modulated through manipulation of *GLB1* and possibly *GLB2*. Investigating responses to flooding indicated that only *GLB1* expression was increased, suggesting that this was the major Hb which was limiting NO and ethylene emission during flooding stress and thus preventing the initiation of PCD and promoting the exhibition of the flooding tolerance mechanism. The functional relevance of *GLB1* expression during hyponastic growth was at least partly independent of ethylene and NO action. The observed increase in the cellular content of nitrosylated thiol (*S*-nitrosothiols) suggested that this is a mechanism through which responses to flooding are effected.

Materials and methods

Plant materials and growth conditions

Transgenic 35S:Glb1 and 35S:Glb2 overexpression *A. thaliana* (L.) Heynh. (Col-0) lines are described in Hebelstrup *et al*. (2007), and the Hb silencing line Hg:*Glb1* and knock-out mutant *glb2* are described in Hebelstrup *et al*. (2006). The gene expression level is limited to 2–3% of that of wild-type plants in the Hg:*Glb1* line (Hebelstrup *et al*., 2006). Increased content of Glb1 protein in the 35S:Glb1 line and increased content of Glb2 protein in the 35S:Glb2 line was confirmed by western blotting (Supplementary Fig. S1 available at *JXB* online). The class 3 Hb (*Glb3*) knock-out mutant (*glb3*) is described in Wang *et al*. (2011). The *ein2-1* mutant (Col-0) is described in Guzman and Ecker (1990). The overexpressing lines (35S:Glb1 and 35S:Glb2) and the mutant lines *glb2* and *glb3* have no visible phenotype, while the silencing line Hg:*Glb1* show a number of phenotypes as listed in the Introduction.

Plants used for hyponastic growth experiments were grown on a fertilized mixture of pot-soil and perlite (1:2; v/v) as described previously by Millenaar *et al*. (2005), under the following conditions: 20 °C, 70% (v/v) relative humidity, 200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), and short-day photoperiod. Plants were automatically watered to saturation each day, at the start of the photoperiod. For waterlogging experiments,17-day-old seedlings were used. The seedlings were first grown on commercial modified Murashige–Skoog medium (Duchefa, Prod. No M0245.0010) with 0.8% agar for 12 d and then transferred to soil pots in growth chambers with a 16/8 h daylength regime at 20 °C and a light intensity of 175 μmol m^{-2} s^{-1}. For measurements of NO emission and *S*-nitrosothiol accumulation, 35-day-old and 40-day-old

Arabidopsis rosettes were used, respectively. These plants were sown in soil pots, then stratified in the dark at 4 °C for 4 d and grown in growth chambers under the same conditions as above, with the exception that plants used for NO emission experiments were grown at 24 °C with a light intensity of 110 μmol m^{-2} s^{-1} and an 8 h photoperiod.

Analysis of haemoglobin expression by western blotting

Plants were grown on 1× MS medium (as above) containing 0.8% agar under an 18 h day with fluorescent lighting at intensities between 50 μmol m^{-2} s^{-1} and 100 μmol m^{-2} s^{-1}. The proteins were extracted by grinding plant material in 10 mM sodium phosphate buffer, pH 7.0 containing 1 mM EDTA, centrifuging at 10 000 *g* for 5 min, and collecting the supernatants. A 50 μg aliquot of total protein (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). Polyclonal rabbit antiserum against purified recombinant Glb1 and Glb2 proteins (Trevaskis *et al*., 1997) were used for Hb protein detection.

S-*Nitrosothiol analysis*

Arabidopsis rosettes were incubated for 24 h in the dark in either a normoxic (atmospheric air) or a hypoxic atmosphere (0.1% O_2, 99.9% N_2). Leaves were snap-frozen and ground in liquid nitrogen. An equal amount of HEN buffer (25 mM HEPES, pH 7.7, 1 mM EDTA, and 0.1 mM neocuproine—which inhibits denitrosylation) was added. *S*-Nitrosothiol content was determined by the Saville method as described by Lindermayr *et al*. (2005). A standard curve (0–100 μM) for *S*-nitrosothiol was made using *S*-nitrosogluthatione (GSNO) in water.

NO and ethylene emission measurements

NO and ethylene emissions were measured online using laser-based spectroscopic detectors (Sensor Sense B.V. and Trace Gas Facility, Nijmegen, The Netherlands) as described previously (Cristescu *et al*., 2008; Clarke *et al*., 2009). Plants measured in parallel experiments were placed in sealed containers and flushed with gas mixtures at 2 l h^{-1}. Normoxia was obtained by flushing with atmospheric air. Hypoxia was obtained by flushing with mixtures of N_2 and O_2 (0, 0.1, 0.3, 0.43, and 1.0% O_2). Internal ethylene concentrations were measured using the method described in Millenaar *et al*. (2005); Supplementary Fig. S2 at *JXB* online.

qRT-PCR

Five days after seedling transfer to soil, the pots were flooded, so that the roots of the wild-type plants of two different *Arabidopsis* accessions (Col-0 and C24) were submerged. The water was supplied with a complex nutrient medium for plant growth (Hornum complete fertilizer; P. Brøste A/S, Lyngby, Denmark). This waterlogging treatment was maintained for 22 d. Roots or shoots (including rosette leaves) from three different plants from each treatment (Col-0+C24: flooded and non-flooded) were harvested, and expression of *Glb1* and *Glb2* was determined by qRT-PCR as described previously (Hebelstrup *et al*., 2006).

Triple response assay

Sterilized seeds were placed in Petri dishes containing 5 ml of half-strength MS–agar [4 g l^{-1} plant-agar (Duchefa), 0.22 g l^{-1} Murashig–Skoog (Duchefa)], containing different concentrations of ACC (Duchefa). Plates were kept for 4 d at 4 °C in darkness and exposed to 200 mmol m^{-2} s^{-1} light for 4 h before packing in aluminium foil. Thereafter the plates were left in darkness for 5 d at 20 °C. After 5 d, hypocotyls were photographed and their length measured using ImageJ software. The results observed were not due to toxic effects of ACC as the ethylene-insensitive *ein2* mutant had an elongated phenotype at all concentrations.

Hyponastic response assay

Hyponastic growth was measured using an automated time-lapse camera system, as described (Millenaar *et al*., 2005). Plants were placed in glass cuvettes with the petiole under study perpendicular to the axis of the camera, 1 d before the start of measurements to allow acclimation. Pictures of two petioles per plant were taken every 10 min using petioles that were marked at the petiole–lamina junction with orange paint (Decofin Universal; Apeldoorn, The Netherlands). Angles were measured between the marked point and a fixed basal point of the petiole, compared with the horizontal, using KS400 software (Carl Zeiss Vision, Hallbergmoos, Germany). To enable continuous photography, no dark period was included during the experiments. All other conditions were kept identical to those in the cultivation room (see above).

To rule out diurnal and/or circadian effects on petiole movement, a pair-wise subtraction (Benschop *et al*., 2007) was performed by calculating the difference in angle between treated and control plants for each time point.

Ethylene (Hoek Loos, Amsterdam, The Netherlands) mixed with humidified normoxic air [70% (v/v)] was supplied in a saturating concentration of 5 μl l^{-1}, in continuous flow-through to the glass cuvettes containing the plant at a flow rate of 75 l h^{-1} and then vented away. Anoxic conditions during hyponastic growth measurements were generated by replacing the flow-through normoxic air in the closed cuvettes containing the plants by a flow-through mixture of N_2 and 0.03% CO_2, but lacking O_2. For hypoxic conditions, pure oxygen was added to the flow-through to the required concentration.

Results

Haemoglobin gene expression modulates NO and ethylene emission

To establish that NO and ethylene emission were under Hb control in *Arabidopsis*, their release from *Arabidopsis* wild-type, GLB2 and GLB3 knock-out lines (*glb2* and *glb3*), the GLB1 silencing line (Hg:Glb1), as well as lines overexpressing *GLB1* and *GLB2* (35S:Glb1 and 35S:Glb2) was measured (Hebelstrup *et al*., 2006, 2007). The rate of NO emission from whole rosettes was strongly increased in the *GLB1* silencing line (Hg:Glb1) and in the *glb2* mutant (10- and 4-fold, respectively), whereas in the *glb3* mutant line the emission rate was similar to that of the wild type (Fig. 1A). A significantly lower NO emission was measured from the leaves of the 35S:Glb1 and 35S:Glb2 overexpression lines. The rate of ethylene emission, as measured from whole rosettes, was strongly increased in Glb1 silencing (Hg:Glb1) and *glb2* mutant lines (~14- and ~2-fold, respectively), whereas the rate of ethylene emission from 35S:Glb1, 35S:Glb2, and the *glb3* mutant line was unaltered (Fig. 1B). Similar results were seen when the experiment was repeated for the lines Hg:Glb1, Col-0, 35S:Glb1, and 35S:Glb2 to measure internal ethylene by squeezing leaves from the plants in a syringe and immediately measuring the ethylene content as described by Millenaar *et al*. (2005); see Supplementary Fig. S2 at *JXB* online.

The sensitivity to ethylene was tested by exposing *Arabidopsis* seedlings to the ethylene precursor ACC in the dark. This classical triple-response assay (Guzman *et al*., 1990; Kende, 1993; Hua *et al*., 1998) revealed no differences between the Glb1 overexpression line and Hg:Glb1 compared with the wild type as all were equally sensitive to hypocotyl growth inhibition by ACC (Fig. 1C). As expected, the *ethylene insensitive-2* (*ein2*) mutant, used as a negative control, was insensitive to ACC.

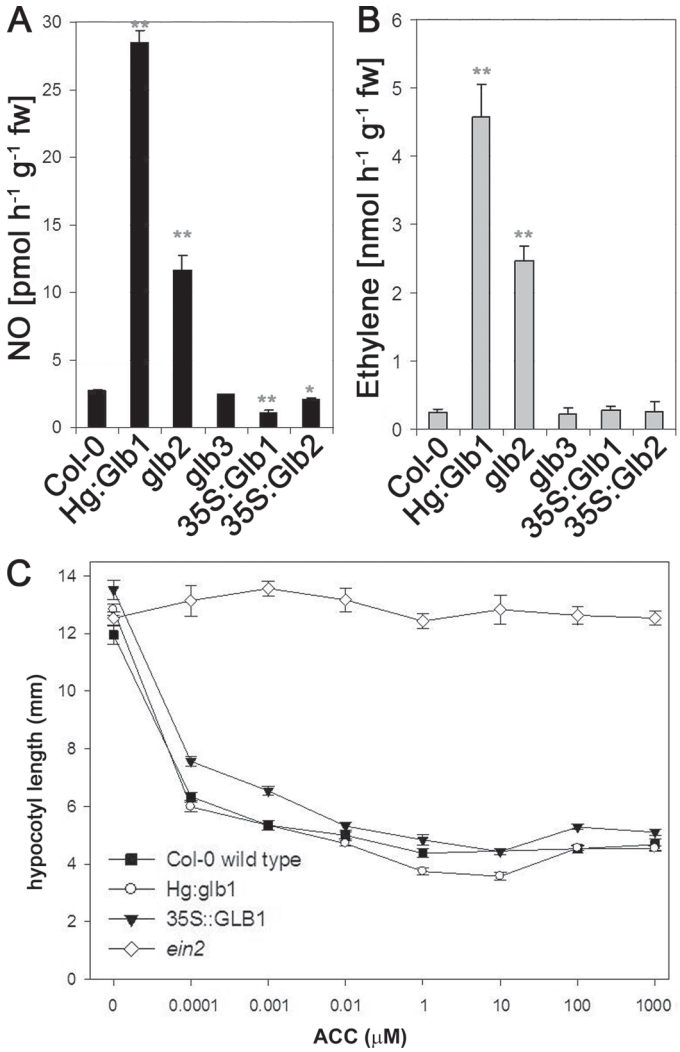

Fig. 1. (A) NO emission and (B) ethylene emission from Hb overexpression and mutant/silencing lines. Significant differences from the wild type are indicated by asterisks: $^{**}P < 0.001$; $^{*}P < 0.05$ (A, n=12; B, n=6). (C) Ethylene triple-response curves of hypocotyl growth in etiolated Hb overexpression (filled triangles) and mutant/silencing lines (open circles), wild-type Col-0 (filled squares), and *ethylene insensitive-2* (*ein2*) (open diamonds) mutant seedlings. Error bars indicate the SE ($n \geq 10$).

These results indicate that *GLB1* and *GLB2* but not *GLB3* control NO and ethylene emission rates in *Arabidopsis*. Moreover, *Arabidopsis* Hb controls ethylene release without altering ethylene sensitivity.

NO emission and formation of S-nitrosothiols increase under hypoxia and are controlled by haemoglobin

Online measurements of NO emission were derived from *Arabidopsis* rosette leaves incubated at a range of low (0–1%) oxygen concentrations in the dark (Fig. 2). At 1% oxygen (Fig. 2A), the rate of NO emission from wild-type plants was similar to that observed at normal oxygen concentrations (Fig. 1A). Interestingly, the NO emission rate increased rapidly when the plants were incubated at 0.43% oxygen (100-fold increase after 60 min) (Fig. 2B). When oxygen concentrations were gradually lowered, the NO emission rates of wild-type plants became progressively higher (Fig. 2C, 2D, 2F). When the 0.1% oxygen treatment was continued overnight, it was observed that this rate of NO emission was maintained for >21 h of hypoxia (results not shown).

Under hypoxia, Hg:Glb1 silenced plants showed higher NO emission rates compared with the wild type (Fig. 2B–D, F). This effect was strongest under anoxic (0% O_2) conditions, where rates of production were >500 times those observed under normoxia (21% O_2) and 4-fold that of wild-type plants (Figs 1A, 2E). Consistent with a role for GLB1 in inhibiting NO formation under hypoxia, 35S:Glb1 showed significantly lower rates of NO emission than the wild type at 0.1–0.43% oxygen (Fig. 2B–D), however, not under anoxia (Fig. 2E, 2F) where NO production did not significantly differ from that of wild-type plants.

One well-established means through which NO can rapidly modify protein function is via *S*-nitrosylation (Astier *et al*., 2011). Thus, total *S*-nitrosothiol content was measured after 24 h of oxygen depletion (0.1% oxygen). The total *S*-nitrosothiol content was increased ~1000-fold in oxygen-depleted tissues compared with normoxic tissues (Fig. 2G, 2H). Hg:Glb1 silenced plants accumulated more *S*-nitrosothiols during both normoxia and hypoxia, whereas 35S:Glb1 plants accumulated less (Fig. 2G, 2H). These observations correlated with the NO emission rates under identical experimental conditions (Figs 1A, 2D).

Haemoglobin expression under waterlogging conditions

In nature, a hypoxic environment is often experienced in roots during waterlogging (Agarwal *et al*., 2006). Therefore, experiments were conducted to test whether Hb expression was modulated in roots and/or shoots of *Arabidopsis* plants exposed to waterlogged conditions, where only the roots were submerged in water. *GLB1* expression was increased (>10-fold) in the shoot as well as in the root (Fig. 3) in both the relatively flooding-tolerant C24 accession and the less tolerant, standard Col-0 accession (Vashisht *et al*., 2011). This shows that *GLB1* expresssion in the shoots responded to flooding, even when only the roots are flooded. No significant differences were found in *GLB1* expression between the two accessions Col-0 and C24. It should be noted that *GLB2* expression levels were only slightly increased in the roots, whereas no change was observed in the shoots. This suggests that *GLB2* gene expression plays no role in modulating responses to anoxia.

Haemoglobin modulates leaf movement in response to ethylene

To relate Hb effects to a mechanism of avoiding the effects of flooding, the effects of Hb expression on hyponastic growth, which is a prominent flooding escape response induced by ethylene (Millenaar *et al*., 2005, 2009; Van Zanten *et al*., 2010), were examined. Ethylene induced a hyponastic response with maximum leaf angle after ~12 h in wild-type plants (Fig. 4A). In the Hg:Glb1 silenced line, the amplitude of the leaf movement response was further increased compared with wild-type

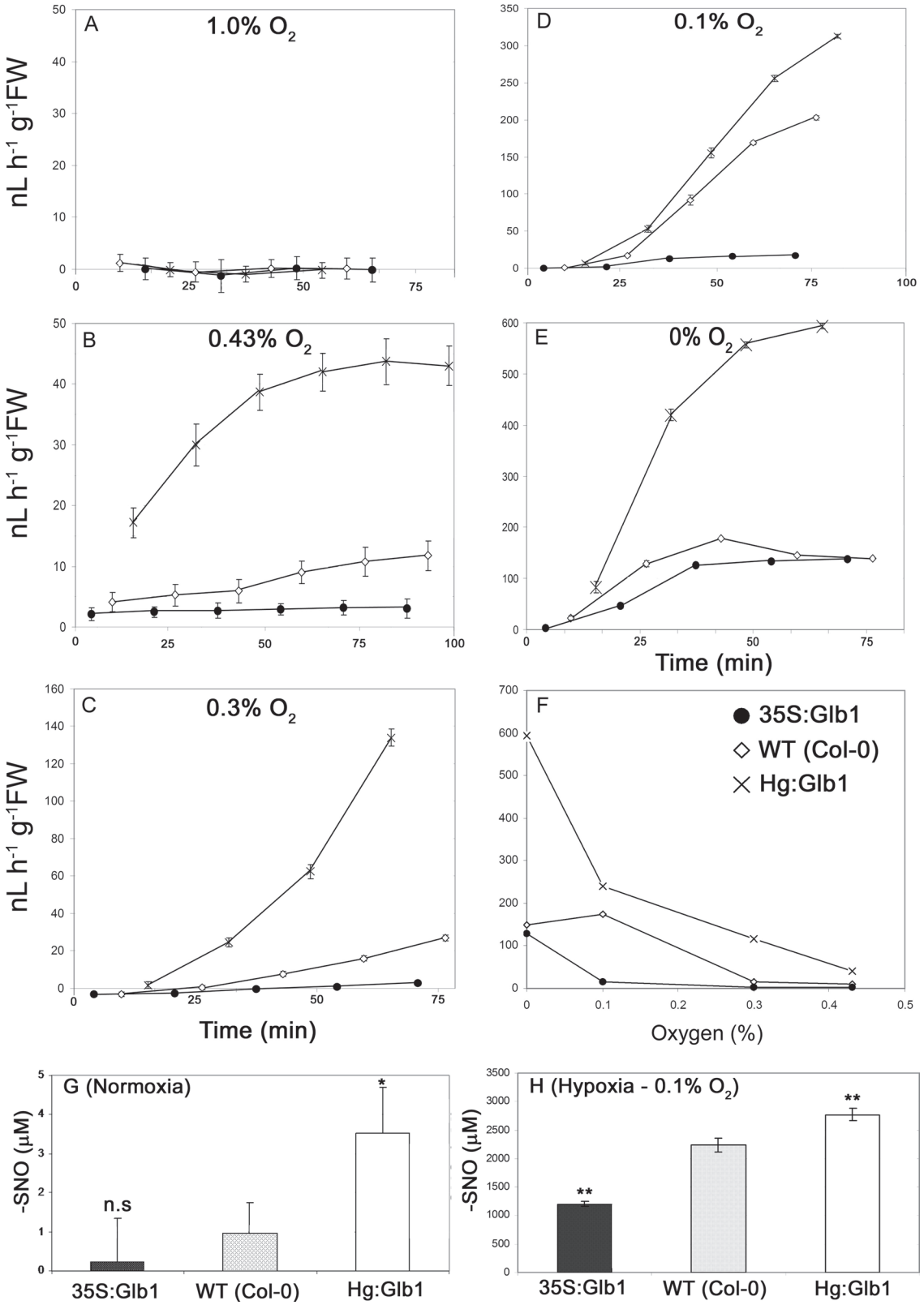

Fig. 2. NO emission from Glb1 overexpression (35S:Glb1, filled circles), wild type (Col-0, open diamonds), and silencing lines (Hg:Glb1, crosses) under hypoxia at 1% O_2 (A), 0.43% O_2 (B), 0.3% O_2 (C), 0.1% O_2 (D), and anoxia (E). *Arabidopsis* rosettes were placed in dark cuvettes and flushed with gas mixtures of O_2 and N_2 or pure N_2 (0% O_2). Bars indicate the SD. The rate of NO production after 60 min at different oxygen concentrations is shown in (F) where data points are taken from (A–E). (G and H) *S*-Nitrosothiol (–SNO groups) levels in *Arabidopsis* rosette leaves with Hb silencing (Hg:Glb1), overexpression (35S:Glb1), or the wild type (Col-0) under normoxic conditions (G) or after 24 h hypoxic (0.1% O_2) treatment (H). Error bars indicate the SD. Significant differences from the wild type: $^{**}P < 0.01$; $^{*}P < 0.05$. NS, not significant. Note the 1000-fold different scales on the *y*-axis of G and H.

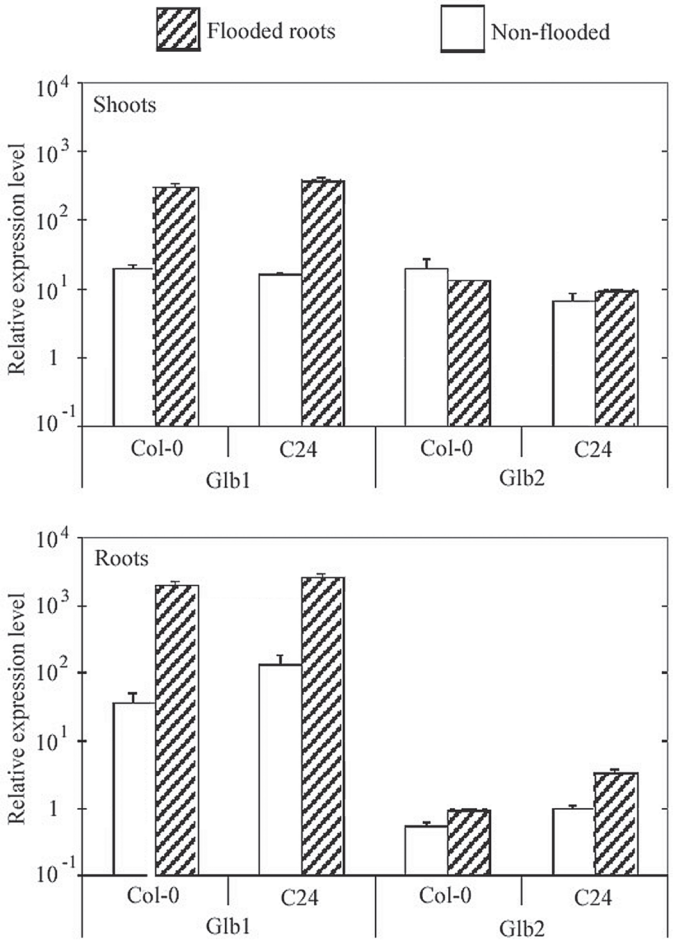

Fig. 3. Gene expression of *Glb1* and *Glb2* in wild-type plants under waterlogging of the roots. Top, shoots; bottom, roots in cases of both non-flooded plants (open bars) and plants with permanently flooded roots (hatched bars) of the accessions C24 and Col-0. Note the log scale on the *y*-axis for both figure parts. Error bars indicate the SE.

controls and the maximum angle was observed 22 h after the start of treatment (Fig. 4C). As a slightly increased response was noted in 35S:Glb1 (Fig. 4B), it seems likely that there are Hb/NO-independent mechanisms of hyponastic regulation. To test if hyponasty is affected by oxygen depletion, wild-type Col-0 plants were subjected to an anoxic environment. However, the plants were under light and therefore able to photosynthesize during the experiment. Consequently, the petioles were probably not completely anoxic in these conditions (Lee *et al*., 2011). In the absence of additional ethylene, a modest increase in leaf angle compared with normoxic (21% O_2) control plants was observed (Fig. 4D). Beyond 13 h, the plants could not maintain high leaf angles and exhibited severe wilting (not shown). A 5% O_2 treatment also resulted in a modest increase in petiole angle, but this was now maintained for at least 24 h (Fig. 4E). In the presence of ethylene, 5% O_2-treated plants showed a strongly increased hyponastic growth response (~10 ° more) compared with ethylene-treated plants under normoxic conditions, which was maintained for at least 24 h (Fig. 4F). The 5% oxygen is above the threshold level at which increased NO formation was measured (Fig. 2). These data therefore indicate that the effect of hypoxia is additive to ethylene-induced hyponastic growth and partially independent of increased NO formation.

Discussion

Haemoglobin modulates NO production during hypoxia and may act as a N-scavenging mechanism

Most plant species contain members of each of the three known Hb classes, suggesting that non-redundant functions exist among them (Hebelstrup *et al*., 2007). It is well documented that turnover of NO is a central function of class 1 Hb to maintain energy status in plants under hypoxic stress (Sowa *et al*., 1998; Dordas *et al*., 2003a, b; Igamberdiev *et al*., 2005). However, to date, no systematic assessments of the roles of difference forms of Hb during hypoxia/anoxia and flooding have been undertaken. Also, as a preliminary to these studies, real-time, *in planta* measures of loss of nitrogen by NO emission during hypoxia were carried out, which facilitated the direct comparison of the effects of differing degrees of hypoxia and complete anoxia. Hypoxic NO production was triggered when the O_2 concentration was <1%. Although not assessed in this study, this NO is most probably generated by the mitochondrial electron transport chain which, under hypoxic conditions, can generate NO by reducing nitrite (Gupta *et al*., 2011). The released NO is in turn oxidized to nitrate by Hb to establish the Hb/NO cycle (Igamberdiev and Hill, 2004). This hypoxic/anoxic generation of NO can be sustained for many

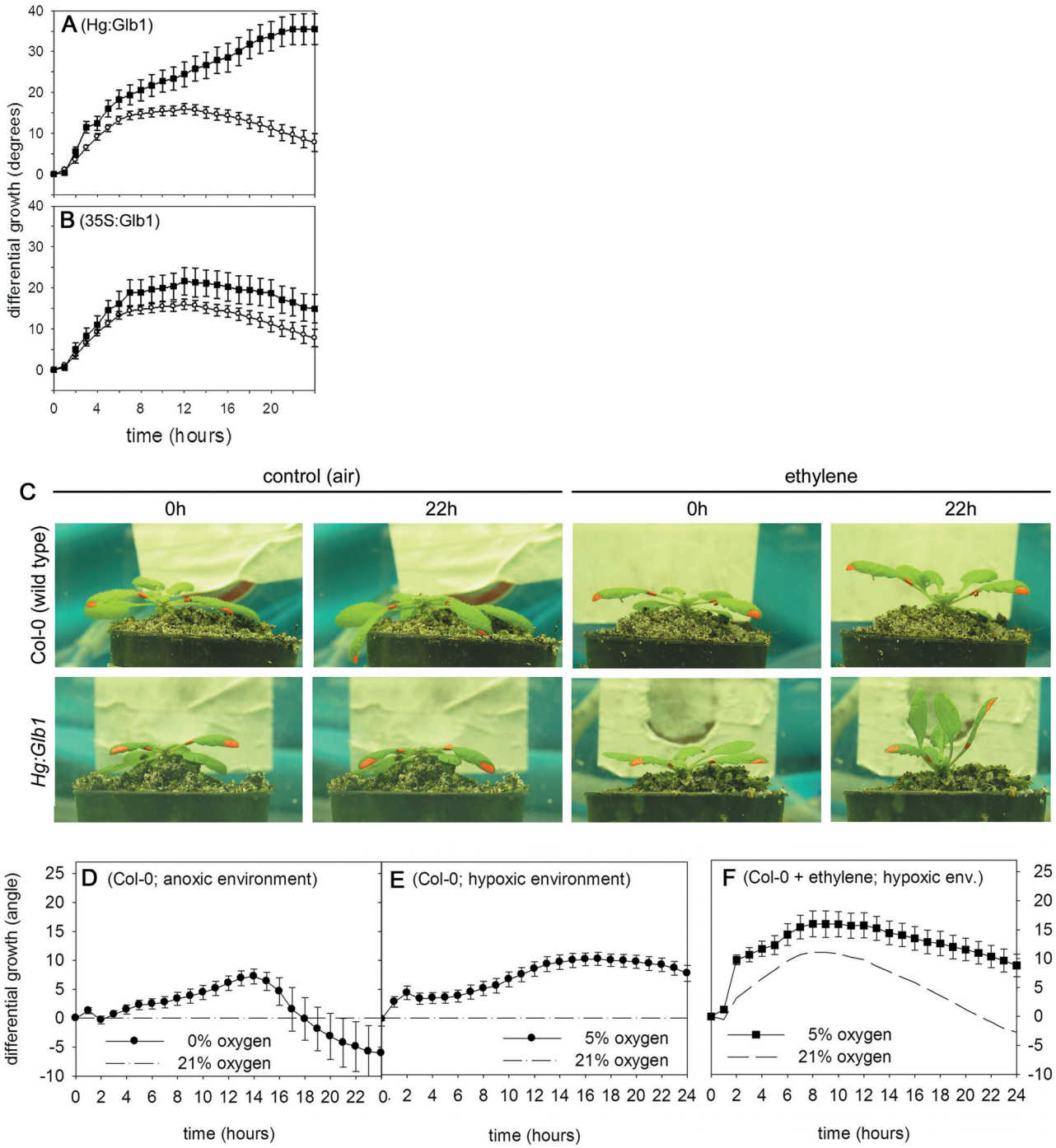

Fig. 4. Ethylene-induced upward leaf movement phenotypes of (A) Hg:Glb1 (filled squares) and (B) 35S:Glb1 (filled squares) compared with the wild type (open circles). Error bars represent the SE, n >12. Data are pair-wise subtracted to give the difference between the angles of treated and control plants for each time point (Benschop *et al*., 2007). (C) Visualization of rosette leaf angle in wild-type (Col-0) or Hb silencing (Hg:Glb1) plants treated with ethylene (5 ppm) or control (air). The pictures were taken at 0 h and 22 h after the beginning of treatment. The red spots on the leaves are markers for the software to recognize the leaf angle. (D–F) Petiole angles of hypoxia- and anoxia-treated Col-0 plants. Petiole movement kinetics of (D) anoxia-grown (0% O_2; circles) and (E) hypoxia-grown plants (5% O_2; circles) relative to normoxic plants (21% O_2; dash-dotted line). (F) Ethylene- (5 μl l^{-1}) treated plants under hypoxic conditions (circles) compared with the ethylene-induced hyponastic growth response under normoxic conditions (dash-dotted line). Growth conditions, treatments, data acquisition, and analysis were as described in Millenaar *et al*. (2005). Error bars represent the SE; n >12.

hours, even in the absence of the main Hb—as shown by the measurements with the Hg:*Glb1* line (Fig. 2). The difference in NO emission between wild-type and Hg:*Glb1 Arabidopsis* plants at 60 min after onset of full anoxia is ~20 nmol g FW^{-1} h^{-1}, which corresponds to NO turnover rates by Hb measured in cell cultures (Dordas *et al*., 2004).

The value of the accurate assessments of NO emission is highlighted by considering the potential cost to the plant of

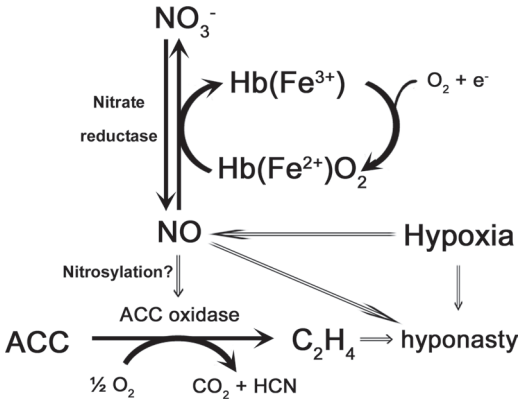

Fig. 5. Schematic model for the interaction between Hbs, NO, ethylene, and hyponastic growth. Nitrate reductase and Hb activities can allow redox shuttling between NO_3^- and NO in the Hb/NO cycle, which can maintain ATP generation under conditions of low oxygen. NO generation is increased with hypoxia—most probably through the reduction of nitrite in the mitochondrial electron transport chain (Gupta *et al*., 2011). A hypoxic increase in NO will activate ethylene biosynthesis, possibly through post-translational modification of key enzymes such as ACC synthase and oxidase by *S*-nitrosylation. Ethylene will promote hyponasty via well-characterized mechanisms (Voesenek *et al*., 1997; Rijnders *et al*., 2000). The data suggest that Hb/NO can also influence hyponasty via ethylene-independent mechanisms. Thus, modification of Hb expression by plants represents a key regulatory node in a plant's responses to hypoxia/flooding. Closed arrows indicate a direct chemical reaction. Open arrows indicate a (indirect) stimulatory effect.

such high and persistent NO production. Although the Hb/NO cycle does not consume nitrate because NO is cycled back (Igamberdiev *et al*., 2005), the NO emission observed under hypoxia and anoxia represents a major net loss of leaf nitrogen. The rate of NO emission at 0.1% oxygen was 8.3 nmol g FW^{-1} h^{-1} (Fig. 2D) equivalent to 0.2 mM (0.2 mmol g FW^{-1}) nitrate lost over the 24 h period given the sustained high NO emission rates that were recorded. This represents a significant nitrogen loss from the plant through NO emission. The Hb/NO cycle has been suggested to be important for maintaining energy status during hypoxia (Igamberdiev *et al*., 2005). However, the above data suggest that increased Hb levels under hypoxia may also be important for limiting nitrogen loss through restriction of NO emission via Hb-dependent NO turnover.

Within the context of flooding and hypoxia, Hb class one (Glb1) has been shown to scavenge NO and is up-regulated under hypoxia and flooding in various plant species (Hebelstrup *et al*., 2007). Similarly, Hb gene expression was strongly enhanced in flooded roots (Fig. 3) in *Arabidopsis*, consistent with the view that plant Hbs act during root hypoxia induced by reduced oxygen diffusion. A parallel strong up-regulation of Hb gene expression in the normoxic shoots of the flooded plants could indicate that this was responding to signals transmitted from root to shoot, possibly ethylene (Jackson and Campbell, 1975). These observations were in agreement with recent transcriptomic experiments which identified *Glb1* as an anaerobically induced gene during hypoxia and shoot/root submergence (Lee *et al*., 2011). The role of systemically induced Hb in the shoot could be to limit the effects of NO and ethylene—by suppressing their production—on the flooded organs. In contrast, it was found that class 2 Hb was not up-regulated by hypoxia in *Arabidopsis* but plants overexpressing this gene did exhibit increased scavenging of endogenous NO (Fig. 1) and increased survival under hypoxia (Hebelstrup *et al*., 2006). This indicates that class 2 Hb plays a role in NO scavenging mainly under normoxic conditions and that effects on hypoxia seen with 33S-*Glb2* overexpression lines were most probably artefactual. Little is known about the function of class 3 Hbs. The present results showed that *glb3* plants do not have increased NO emission under control conditions (Fig. 1), which suggests that Glb3 is not the main contributor to NO scavenging as compared with Glb1. Further, Glb3 expression is not regulated by hypoxia (Watts *et al*., 2001). Taking all of these data together, it appears that Glb1 is the major Hb involved in scavenging NO under hypoxic conditions.

An important part of this work was to link these observations to hypoxia tolerance. Long-term exposure of the roots of *Arabidopsis* plants to hypoxia during waterlogging led to the same strong up-regulation of Glb1 in the roots and shoots of two different *Arabidopsis* accessions (Fig. 3). The two accessions used here were the flooding-tolerant C24 and the less tolerant Col-0 (Vashisht *et al*., 2011). Since the expression level of Glb1 was the same in the two accessions, this indicates that variation in *Glb1* expression, and probably NO levels, is unlikely to explain the difference in submergence survival during complete submergence, at least in these accessions.

NO, haemoglobin, and ethylene interact in the hyponastic response to hypoxia

In seeking to integrate hypoxic NO generation establishing physiological responses to submergence, a link with ethylene was obviously implied. It has been demonstrated in cell cultures that Hb expression can modulate ethylene production and ACO enzyme activity without affecting its protein or gene expression level, and that NO can directly stimulate ACO enzyme activity (Manac'h-Little *et al*., 2005). In the present work, the effect of Hb gene expression in intact plants on both the physiological response to ethylene and its biosynthesis was studied. In agreement with previous work (Manac'h-Little *et al*., 2005), it was found that *Arabidopsis* plants with reduced expression of Hb, and an increased NO emission rates as a result, also had increased rates of ethylene emission (Fig. 1). When examining hyponastic growth, it was observed that this was enhanced in the silenced Hg:Glb1 line (Fig. 4). This implies that non-symbiotic Hb contributes to the control of this important adaptation to restore gas exchange in flooded plants. The silenced Hg:Glb1 line had a significantly increased ethylene emission (Fig. 1B). However, because saturating ethylene concentrations were applied (Polko *et al*., 2012), the observed differences in leaf angle cannot be explained by the additive effects of increased endogenous and applied ethylene. Moreover, ethylene sensitivity was unaffected in the Hb overexpression lines (Fig. 1C). It is therefore concluded that Hb-mediated effects on ethylene-induced leaf angle are independent from the observed Hb effects on ethylene (emission).

The Hg:Glb1 silencing line had a 20-fold increased NO emission level and a dramatically increased hyponastic response to ethylene compared with the wild type (Figs 1A, 4A). In contrast the overexpression line 35S:Glb1 had a slightly decreased NO level and a similar or slightly enhanced hyponastic response to ethylene compared with the wild type (Figs 1A, 4B). On the basis of this, it cannot be excluded that there is a direct effect of high NO on the hyponastic response, which is additive to that of the applied ethylene. As hypoxia (5% O_2) induced a hyponastic response in the absence of added ethylene (Fig. 4E), low oxygen probably also controls hyponastic leaf movement at least partly independently of ethylene action, which mirrors the observations made for flood-induced petiole elongation in the semi-aquatic species *Rumex palustris* (Voesenek *et al*., 1997; Rijnders *et al*., 2000). It was found that NO emission increases with the severity of hypoxia (Fig. 2). However, at 1% O_2, no increased NO emission was detected, suggesting that NO synthesis was not stimulated at oxygen concentrations above this level of hypoxia. Thus, the hyponastic response observed at 5% O_2 oxygen, must occur independently of increased NO. At lower levels of O_2 (< 1%), where NO emission is increased, a role for NO in this process is possible, and an additive effect of hypoxia, increased NO, and its stimulatory effect on ACO activity may result in enhanced leaf movement.

An important question arising from this and other work using pathogens (Mur *et al*., 2012) is how NO/Hb regulates ethylene production (Fig. 5). It has also been previously reported that plants with silenced Hb gene expression have delayed flowering and abnormal development of leaves and flowers (Hebelstrup *et al*., 2006, 2008). Since ethylene acts as a repressor of flowering in *Arabidopsis* (Tsuchisaka *et al*., 2009), the increased ethylene level in Hb-silenced lines (Fig. 1) is likely to contribute to the late flowering phenotypes. NO effects on ethylene production could come about through the modulation of the expression of the ethylene biosynthetic genes ACC synthase and/or ACO, as some evidence for this in tobacco has previously been provided (Mur *et al*., 2012). However, in *Arabidopsis*, expression of neither enzyme is directly affected by overexpression or silencing of Hb genes (Manac'h-Little *et al*., 2005), Modification of protein function through *S*-nitrosylation has been suggested as a mechanism for NO sensing in plants (Baudouin, 2011), and various biotic and abiotic stress treatments have been shown to induce *S*-nitrosylation of specific proteins (Abat and Deswal, 2009; Tanou *et al*., 2009). For example, the mechanism behind the effects of NO on disease resistance and hypersensitive responses has been reported to involve the modification of protein function by *S*-nitrosylation (Tada *et al*., 2008; Wang *et al*., 2009). Of direct relevance to the present experiments, *S*-nitrosylation of the enzyme *S*-adenosylmethione (SAM) synthetase (Lindermayr *et al*., 2005), which donates methyl groups to the ethylene biosynthetic cycle, and ACC synthase has been reported, and such modifications could directly influence ethylene production during flooding. It was observed that the >500-fold increase in NO emission (Fig. 2) with short-term hypoxia and anoxia was coupled to an increase in the amount of *S*-nitrosothiols (Fig. 2G, 2H). The concentration of *S*-nitrosothiol products (both high and low molecular weight) in the different lines correlated with the rate of NO emission from the same lines (Fig. 2). Thus, NO emission from plants using Quantum Cascade Lasers (QCL) would appear to be a good measure of the NO concentration in the tissue. More importantly, it further suggested that protein regulation by *S*-nitrosylation could be a means through which NO acts to confer submergence tolerance and regulation of ethylene production. Thus, the authors are currently investigating post-transcriptional (by *S*-nitrosylation) regulation of ethylene biosynthesis.

In addition, although Hb represents an important mechanism through which NO levels could be modulated, the formation of low molecular weight nitroso conjugates with glutathione is thought to be the first step in a glutathione-linked NO removal pathway (Liu *et al*., 2001). Thus, both Hb and low molecular weight *S*-nitrosothiols could limit the extent of NO and ethylene action in stressed plants.

Taking all of the data together, it is demonstrated in this work that overexpression of Hb in *A. thaliana* negatively affects NO and ethylene emission under normoxia, hypoxia, and anoxia. Therefore, Hb regulation is a mechanism through which plants modify these effects. A schematic model outlining this interaction is presented in Fig. 5 which is likely to also be relevant to pathogen attack (Mur *et al*., 2012) and developmental processes (Hebelstrup *et al*., 2006, 2008).

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Western blot showing the level of Glb2 (upper blot) or Glb1 (lower blot) in whole seedlings of wild types (C24 lane 1, Col-0 lane 2), 35S:Glb2 (lines 7, 6, 2, lanes 3, 4, 5), and 35S:Glb1 (line 5, lane 6). All the transgenic lines shown are derived from the Col-0 ecotype.

Figure S2. Internal ethylene was measured by squeezing leaves from the plants in a syringe and immediately measuring the ethylene content as described by Millenaar *et al*. (2005).

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