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% O₂), 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
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 Voenesenek, 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 hypostatic growth, followed by petiole elongation to reach above the water surface and restore (e.g. oxygen) gas exchange. Ethylene also induces a marked hypostatic 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 hypostatic 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 what oxygen concentration NO production is triggered and the estimation 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 hypostatic 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 Arabidopsis 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 hypostatic growth experiments were grown on a fertilized mixture of pot-soil and vermiculite (1:2; v/v) as described previously by Millenaar et al. (2005), under the following conditions: 20 °C, 70% 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–Skooog 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 antisera 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% O₂, 99.9% N₂). 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 mecopeuroine—which inhibits dinitrosylation) 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-nitrosoglutathione (GSNO) in water.

Results

NO and ethylene emission measurements
NO and ethylene emissions were measured online using laser-based spectroscopic detectors (Senser 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 N₂ and O₂ (0, 0.1, 0.3, 0.43, and 1.0% O₂). 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 (Hoernum complete fertilizer; P. Braste 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–Skooog (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.
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% O₂) conditions, where rates of production were >500 times those observed under normoxia (21% O₂) 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 expression 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% O₂ (A), 0.43% O₂ (B), 0.3% O₂ (C), 0.1% O₂ (D), and anoxia (E). Arabidopsis rosettes were placed in dark cuvettes and flushed with gas mixtures of O₂ and N₂ or pure N₂ (0% O₂). 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% O₂) 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.
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% O2) 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% O2 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% O2-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 different 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 O2 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
Hb modulates NO emission and hyponasty under hypoxia-related stress in A. thaliana

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
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 gfb3 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 accesses used here were the flooding-tolerant C24 and the less tolerant Col-0 (Vashishth et al., 2011). Since the expression level of Glb1 was the same in the two accesses, 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 hypoxia 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 hypoxia 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 hypoxia response, which is additive to that of the applied ethylene. As hypoxia (5% O2) induced a hypoxia response in the absence of added ethylene (Fig. 4E), low oxygen probably also controls hypoxia 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% O2, no increased NO emission was detected, suggesting that NO synthesis was not stimulated at oxygen concentrations above this level of hypoxia. Thus, the hypoxia response observed at 5% O2; oxygen must occur independently of increased NO. At lower levels of O2 (<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 show 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-adenosylmethionine (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 Arabidopsis 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).

Acknowledgements

This work was supported by EU-FP6-Infrastructures-5 programme, project FP6-026183 ‘Life Science Trace Gas Facility’ and by The Danish Council for Independent Research Technology and Production Sciences – Fund no. 274-08-0366 to KHH, and a VENI grant (863.11.008) from the Netherlands scientific organization to MvZ.

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