Nitric oxide-fixation by non-symbiotic hemoglobin proteins in *Arabidopsis thaliana* under N-limited conditions

Running title: Nitric oxide-fixation in plants

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Abstract

Nitric oxide is an important signalling molecule which is involved in many different physiological processes in plants. Here we report about a NO-fixing mechanism in *Arabidopsis*, which allows the fixation of atmospheric NO into nitrogen metabolism. We fumigated *Arabidopsis* plants cultivated in soil or as hydroponic cultures during the whole growing period with up to 3 ppmv of NO gas. Transcriptomic, proteomic and metabolomic analyses were used to identify non-symbiotic hemoglobin proteins as key components of the NO-fixing process. Overexpressing non-symbiotic hemoglobin 1 or 2 genes resulted in four-fold higher nitrate levels in these plants compared to NO-treated wild-type. Correspondingly, rosettes size and weight, vegetative shoot thickness and seed yield were 25%, 40%, 30%, and 50% higher, respectively, than in wild-type plants. Fumigation with 250 ppbv ^{15}NO confirmed the importance of non-symbiotic hemoglobin 1 and 2 for the NO-fixation pathway and we calculated a daily uptake for non-symbiotic hemoglobin 2 overexpressing plants of 250 mg N/kg dry weight. This mechanism is probably important under conditions with limited N supply via the soil. Moreover, the plant-based NO uptake lowers the concentration of insanitary atmospheric NOx and in this context NO-fixation can be beneficial to air quality.

Keywords: Nitric oxide-fixation, non-symbiotic hemoglobin, nitrogen, *Arabidopsis thaliana*

Summary statement

This study reports about a NO-fixing mechanism in *Arabidopsis*, which allows the fixation of atmospheric NO into nitrogen metabolism. Non-symbiotic hemoglobin class 1 and class 2 were identified as key proteins of the NO-fixation pathway converting NO to nitrate, which is further introduced into the N-metabolism. This mechanism is probably important under conditions with limited N supply via the soil. Moreover, the plant-based NO uptake lowers the concentration of insanitary atmospheric NOx and in this context NO-fixation can be beneficial to air quality.

Introduction

Nitric oxide (NO) is a signaling molecule that coordinates many physiological processes in plants. In the late 90th it was identified as mediator of plant defense responses (Delledonne et al., 1998; Durner et al., 1998). Since then, many studies demonstrated the ubiquitous signaling function of NO in different physiological processes in plants, such as germination (Bethke et al., 2006; Belenghi et al., 2007), stomatal closure (Neill et al., 2002; Garcia-Mata et al., 2003; Sokolovski et al., 2005), flowering (He et al., 2004), senescence (Corpas et al., 2004; Guo and Crawford, 2005), wounding responses (Huang et al., 2004), and abiotic stresses (Grun et al., 2006; Corpas et al., 2011). Due to its instable nature, NO has a very rich chemistry. Besides direct binding to metal ions NO can also react with superoxide and molecular oxygen, resulting in the formation of peroxynitrite and dinitrogen trioxide N_2O_3 (or higher oxides like NO_2), respectively. Moreover, adding or removing one electron from the antibonding highest occupied molecular orbital by reducing or oxidizing chemicals yields nitroxyl anion (NO^-) and nitrosonium cation (NO^+). All these species are referred to as reactive nitrogen species each having distinct chemical properties leading to numerous reactions with biological molecules like lipids, carbohydrates, nucleic acids and proteins (Mengel et al., 2013).

Plant hemoglobin (Hb) proteins were described first in the early 20th century and it was assumed that they have a function in oxygen binding similar to animal Hb. However, 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, where classes 1 and 2 are structurally similar to human/animal myoglobins and Hbs (Trevaskis et al., 1997). 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). Moreover, plant Hbs may also be classified as either symbiotic or non-symbiotic. Symbiotic Hbs are produced in high concentration in root nodules, whereas non-symbiotic Hbs 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).

Medicago sativa, for instance, contains five class 2 Hbs and one class 1 Hb (Hebelstrup et al., 2007). The class 1 is non-symbiotic - which is usually the case for legumes, where the symbiotic ones, which facilitates oxygen diffusion in symbiotic N_2 fixing root nodules

(leghemoglobins) are of the class 2 type. For other plant families with symbiotic N_2 fixation it may be different - e. g. in *Myrica gale* there is a class 1 Hb, which is expressed in leaves and nodules (Heckmann et al., 2006). Moreover, it cannot be excluded that one or more of the class 2 Hbs in *Medicago sativa* are in fact non-symbiotic. It may be that some of them are expressed in leaves or other 'non-symbiotic' tissues. In general, all plant families contain Hb encoding genes, many of which are not related to symbiotic root nodules, but are expressed at a low concentration in other tissues, where they do not contribute to facilitate oxygen diffusion. Several studies have demonstrated a role for plant Hbs in catalysing the turnover of nitric oxide (NO) to nitrate and modulating NO signalling (Perazzolli et al., 2004; Hebelstrup et al., 2006). Moreover, it was shown that Hbs are influencing the generation of the defence hormones salicylic acid, ethylene, and jasmonic acid, most probably acting via modulation of NO production (Mur et al., 2012).

Here we report about the NO-fixing function of non-symbiotic hemoglobin proteins, which allows plants to use atmospheric NO as a source of nitrogen. Using ^{15}NO we could demonstrate that NO-fixation is enhanced considerably in plants overexpressing *AtGLB1* or *AtGLB2* genes. NO uptake resulted in four-fold higher nitrate levels in these plants compared to NO-treated WT plants. Correspondingly, the growth parameters like rosettes size and weight, vegetative shoot thickness and also seed yield were significantly higher in the overexpression lines in comparison to WT plants. The NO-fixing capability of plants might be of physiological importance for plants growing under low soil N availability. Moreover, the plant-based uptake of NO lowers the level of toxic atmospheric NOx, which would have a positive effect on air quality.

Materials and Methods

Plant material and NO treatment conditions

Wassilewskija (Ws) and Columbia 0 (Col-0) ecotypes of *Arabidopsis thaliana* were used in this study. The non-symbiotic hemoglobin (often abbreviated with GLB for globin) expression levels in plant lines with altered *GLB* expression were quantified in a previous study (Hebelstrup et al., 2006). Plants (3 plants/25 cm^2) were grown in Floragard B Seed containing 140 mg/l N, 80 mg/l P_2O_5 and 190 mg/l K_2O. The NO fumigation of the soil-grown plants was continuous, starting on the 5^{th} day after germination, and was performed in climatic fumigation chambers whose internal NO levels were constantly monitored (Fig. S1). Air was purified using filter pads in combination with activated-carbon filters and silica

particles coated with permanganate (Purex International, Rotherham, UK) (ambient air, 5 +/- 2 ppbv) and supplemented with different concentrations of NO (0.8, 1.5 or 3.0 parts per million by volume [ppmv]). NO was obtained from Air Liquide (Düsseldorf, Germany) in cylinders containing 2% or 15% NO and 98% or 85% nitrogen, respectively. ^{15}N-NO (99 atom % isotopic enrichment) was obtained from Linde (Pullach, Germany) and diluted to 2% with nitrogen by Westfalen AG (Münster, Germany). In all experiments NO was injected via a mass-flow controller.

Growth conditions: light - 300 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR: 400–700 nm); temperature – day: 20 °C (14 h) and night: 16 °C (10 h); and relative humidity – 80%. Hydroponically grown plants germinated and grew in N-containing medium for twelve days (Gilbert et al., 1997). Then, they were transferred to medium without any N-source [1.25 mM KNO_3, $Ca(NO_3)_2$ and 0.075 µM $(NH_4)MoO_{24}$ were replaced by 1.5 mM $CaCl_2$, 1mM KCl and 0.075 µM Na_2MoO_4, respectively] and fumigated with 3 ppmv NO. Growth conditions: light - 155 µmol m^{-2} s^{-1} PAR (400–700 nm); temperature – day: 20 °C (14 h) and night: 16 °C (10 h); and relative humidity – 80%. In all experiments the NO levels in the chambers were monitored with an NO Analyzer AC32M (Ansyco, Karlsruhe, Germany).

Total RNA and protein isolation and quantification

Homogenized frozen rosette material (400 mg) was vortexed using 1 ml extraction buffer (100 mM Tris/HCl - pH 8.0, 10 mM EDTA, 1 mM $MgCl_2.H_2O$, 1 mM L-Ascorbic acid, 12 mM 2-mercaptoethanol - freshly added), 1 Complete mini EDTA-free protease inhibitor cocktail/ 10 ml buffer). Homogenate was centrifuged (12,000 g for 20 min at 4 °C) and supernatant filtered using 70 µm nylon membrane. Protein extract was then desalted using PD-10 desalting columns (GE Healthcare, Germany) according to the manufacture's instruction. Bradford reagent (Bio-Rad Laboratories, Munich, Germany) was used to photometrically determine the concentration of proteins after extraction as described earlier (Harlow and Lane, 2006). In order to measure and plot a standard curve of protein concentration versus absorbance at 595 nm, a series of dilutions of bovine serum albumin (BSA) protein standard stock solution was prepared. 1 ml of reaction mixture contained 790 µl of water, 200 µl of Bradford reagent and 10 µl of known concentration of BSA. A standard curve was plotted and used as a reference to quantify protein extracts with unknown concentrations.

Homogenized frozen rosette material (100 mg) was incubated (5 min, RT) with TRIZOL (1 ml). 200 µl of chloroform was added, vortexed (2 min) and centrifuged (18,000 g for 15 min

at 4 °C). The aqueous phase was further purified using Qiagen's RNeasy® Mini Kit (according to manufacturer's instruction) with on-column DNA digest (Qiagen's RNase-free® DNase). Purified total RNA was quantified using the Nanodrop ND-1000 spectrophotometer.

cDNA Synthesis and polymerase chain reaction

First cDNA synthesis was performed using Invitrogen's Superscript II Reverse Transcriptase (200 U/μl) (according to given in the manufacturer's manual) with 1 μg of total RNA. Gene-specific primers (see supplementary information) were used to amplify specific gene of desired quantity. cDNA is used as template (100 ng). Amplification is used by Phusion high-fidelity DNA polymerase (2 U/μl) (according to given in the manufacturer's manual – New England Biolabs).

Gene expression profiling of *Arabidopsis thaliana* fumigated with NO

An expression profiling of 4-week-old *Arabidopsis thaliana* nuclear transcripts in both ambient and 3 ppm NO fumigated plants were performed. Around 70 to 80 mg of frozen and homogenized plant tissue material per sample was used as the starting material. Total RNA from the rosettes were isolated using Qiagen's RNeasy Plant Mini kit according to the instructions given in the manufacturer's manual. An on-column DNase digests to remove DNA contamination was performed as recommended by the manufacturer. Quality and quantity of the RNA was analyzed using Nanodrop ND-1000 spectrophotometer. RIN (RNA integrity number) was used to assess the quality of the RNA with a lower threshold value of 7. Microarray analysis was carried out on Agilent platform using the technique 'One-Color Microarray-Based Gene Expression Analysis' with 'Low Input Quick Amp Labeling' technology and 60K microarray chip. Microarray analysis was carried out according to the protocol described in the manual provided by Agilent (G4140-90040). Following cRNA synthesis, chip hybridization, scanning and feature extraction, raw expression data of the genes was analyzed using GeneSpring GX software tool.

Chemiluminescence determination of the nitrosothiol, nitrate, and nitrite contents and measuring of NO levels

The total nitrite, nitrate, and nitrosothiol contents were estimated using a Sievers 280i nitric oxide analyser (GE Analytical Instruments, Boulder CO, USA). Rosette proteins were extracted using extraction buffer (137 mM NaCl, 0.027 mM KCl, 0.081 mM $Na_2HPO_4.2H_2O$,

0.018 mM NaH_2PO_4). 100 µl of leaf protein extract was injected into the purging vessel of NOA containing 3.5 ml of acidified KI/I_3 solution (reducing agent) at 30 °C. The recorded mV signals were plotted against a calibration curve produced using known concentrations of sodium nitrite solution to quantify the nitrite level. To estimate the S-nitrosothiol content (RSNO), the above protocol was repeated by pre-treating the leaf protein extract with 20 mM sulphanilamide (in 1 M HCl) at the ratio of 9:1. For nitrate quantification, the reducing agent was replaced with vanadium chloride at 95 °C. The recorded mV signals were plotted against a calibration curve produced using known concentrations of sodium nitrate solution to quantify the nitrate levels.

Pots with plants were placed in a closed system/cuvette and levels of accumulated NO were measured after 30 min. Afterward the plants were cut and the levels of accumulated NO was determined again after 30 min. The difference between the levels with and without plants reflects the amount of NO taken up in 30 min by the different genotypes. Gaseous NO was measured using a CLD 88 CY p analyzer (ECO PHYSICS, Germany).

Phenylalanine ammonia lyase (PAL) activity assay

PAL activity was performed according to previously published protocol (Yin et al., 2012). PAL enzyme converts phenylalanine into trans-cinnamic acid and ammonium. Formed trans-cinnamic has been determined photometrically at 290 nm at 37 °C. Activity has been determined by incubating 100 µg of total protein in 1 ml of assay mix containing PAL activity buffer (100 mM Tris-HCl, pH – 8.8) and 5 mM L-phenylalanine. After 2 h the reaction was terminated by adding 50 µl of 5 M HCl. Mixture was then centrifugation at 14000 g for 15 min. The absorbance was recorded and quantified against that of control samples without L-phenylalanine.

HPLC quantification of secondary metabolites

Quantification of sinapinic acid, kaempferol and quercitin was performed according to previously published protocol (Yin et al., 2012). 100 mg of leaf tissue homogenate was added to 1 ml of methanol and incubated on a shaker for 1 h at RT in the dark. The mixture was then centrifuged at 10,000 g for 5 minutes. 75% of the supernatant was mixed with 25% double distilled water and centrifuged for 5 min at 10,000 *g*. The clear supernatant was transferred into a HPLC micro-vial. Samples were analyzed using Beckman Gold 7.11 HPLC system at a flow rate of 1 ml/min with sample injection volume of 10 µl. Solvent A (double distilled water and 5 % ammonium formate in formic acid; mixed in 98**:**2 ratio respectively)

and solvent B (methanol, double distilled water and ammonium formate; mixed in 88.2**:**9.8**:**2 ratio respectively) were used for the separation. The separation was isocratic with 100% solvent A in first 5 min; linear gradient to 100 % solvent B in 40 min; isocratic with 100% solvent B for 5 min; linear gradient to 0% solvent B in 5 min; isocratic with 100% solvent A for 5 min. The separation was completed in 60 min. Flavonols were separated with Bischoff Prontosil Spheribond ODS2 Type NC column (5 µm – 250 mm x 4.6 mm) and pre-columned at 20 °C. Absorbance at 280 nm was recorded using Beckman diode-array detector model 168 and scan mode was set between 250 to 450 nm. The flavonol aglycone and sinapate ester derivatives were identified by the diode array spectra and retention time in comparison with authentic standards.

Colorimetric determination of ammonia content in soil and rosette leaves

20 g of soil was shaken with 50 ml of Milli-Q (2 h) centrifuged, filtered using black ribbon filter paper and the filtrate is used for downstream ammonia analysis. For, ammonia estimation in the rosette, 1 g of frozen leaf material was shaken with 5 ml of Milli-Q water (2 h), centrifuged (8,000 *g*) and the supernatant is used for downstream ammonia analysis. Ammonia content was analyzed using Skalar colorimetric segmented continuous flow analyzer. To complex the cations, the samples were mixed (flow rate set to 0.42 ml/min) with buffer A (117 mM potassium sodium tartrate, 82 mM trisodium citrate dehydrate, 0.1% of 30% Brij 35 (v/v)) (flow rate set to 0.80 ml/min). Ammonia ions react with hypochlorite ions generated by the alkaline hydrolysis of sodium dichloroisocyanurate (flow rate set to 0.32 ml/min) to form monochloramine which in turn reacted with the salicylate ions of sodium salicylate solution (625 mM sodium hydroxide and 500mM sodium salicylate; flow rate set to 0.32 ml/min) in the presence of sodium nitroprusside (flow rate set to 0.16 ml/min) to form a green coloured complex with ammonium ion. The absorbance of this compound was determined photometrically at 660 nm at 40 °C and was related to the ammonia concentration by means of a calibration curve using DiscreteAccess software.

Ion-chromatographic determination of nitrate and nitrite in the soil

A mixture of 20 g of soil sample was shaken with 50 ml of Milli-Q water (2 hours), centrifuged and the supernatant was filtered using black ribbon filter paper. 25 µl of the filtered supernatant was analyzed for total nitrite and nitrate content using Dionex ICS 1500 Ion chromatography with a flow rate of 1.2 ml/min. A mixture of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ was used as eluent. Dionex AG 4 anion exchange pre-column and Dionex AG

4 anion exchange column were used for separation of anions. Nitrite and nitrate were determined based on their conductivity with respect to the certified standard solutions (SPEX Certiprep, Metuchen, NJ, USA) used for calibration of the device.

Determination of ^{15}N content

Plant material was dried at 60 °C for overnight and grounded to a homogenous powder using a ball mill (Tissue Lyser II, Qiagen, Venlo, Netherlands). Aliquots of about 1.5 to 2 mg were transferred into tin capsules (IVA Analysentechnik, Meerbusch, Germany). ^{15}N abundance was determined with an Isotope Ratio Mass Spectrometer/IRMS (delta V Advantage, Thermo Fisher, Dreieich, Germany) coupled to an Elemental Analyzer (Euro EA, Eurovector, Milano, Italy). Isotope ratio mass spectrometry measurements always need the comparison with one or more standards with known isotope composition in the same range of the analysed samples. For that reason a lab standard (acetanilide) was part of every sequence in intervals. It was also used in different weights to determine isotope linearity of the system. The lab standard itself is calibrated against several unlabelled international isotope standards (IAEA; Vienna). Calibration curves for enriched samples were generated using suitable enriched standards (Fischer Analysen Instrumente, Leipzig, Germany) for the correct evaluation of ^{15}N contents.

Two dimensional difference gel electrophoresis (2D-DIGE)

2D-DIGE experiment and MALDI-TOF/TOF MS were performed according to previously published protocol (Holzmeister et al., 2011). Protein extracts from the treated samples were labelled with Cy3 and Cy5 dyes. An internal standard (equal amounts of all biological samples in the experiment) was labelled with Cy2. A 2D-DIGE gel contains two different samples – one labelled with Cy3, the other one labelled with Cy5. Moreover, the internal standard was loaded on each gel, allowing a comparison of all samples. One dimensional isoelectric focusing (*p*H 4 - 7) and second dimension SDS gel electrophoresis separated the proteins according to their *p*I and molecular weight respectively. Each gel was scanned at three different wavelengths using the following excitation/emission conditions: 473/520 nm for Cy2, 532/580 nm for Cy3 and 635/670 nm for Cy5. Acquired images were analyzed by Ettan 2D DeCyder software to identify regulated protein spots. These spots were analyzed using MS/MALDI-TOF to identify regulated proteins.

HPLC analysis to quantify the chlorophyll content in *Arabidopsis* leaf extract

100 mg of homogenized frozen leaf material was suspended in 0.7–1.5 ml of DMF. 1 mg of calcium carbonate was added to each sample to prevent acidic pH, which would result in loss of Mg^{2+} from chlorophyll. It was further centrifuged at 10,000 *g* for 10 min at 4 °C. Centrifugation step was repeated and the supernatant was then added with half volume of DMF in 50% methanol making the samples more hydrophilic. Afterwards the samples were filtered through a 0.45 µm PTFE filter. The HPLC unit for pigment analysis consisted of two Model 515 pumps, a 717 cooled Autosampler, a Model 2996 photo diode array detector, a Model 447 fluorescence detector and the Empower 2 chromatographic software. The pump flow rate was set to 0.9 ml/min. With the sample injection volume of 50 µl, the pigment separations were performed on Merck LichroCART 125-4 Cartridge (LiChrospher 100 RP-18, 5 µm) that was protected with a LichroCART 4-4 Guard pre-column insert. Separation was carried out at 30 °C. Solvent A (acetonitrile, methanol and 0.2 M tris-buffer of pH 8.0, mixed in 74:6:1 ratio respectively) and solvent B (methanol and hexane, mixed in 5:1 ratio respectively) were used for separation. The separation was isocratic with 100% solvent B in first 3.5 min; linear gradient to 100% solvent B in 4 min; isocratic with 100% solvent B for 5.5 min; linear gradient to 0% solvent B in 2 min. The separation was completed in 15 min. The peaks were identified and quantified using calibration standards (Chl a and Chl b from spinach: Sigma-Aldrich, Taufkirchen, Germany).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries or the Arabidopsis Genome Initiative database under the following accession numbers: NP_179204 (AT2G16060), NP_187663 (AT3G10520).

Results

Treatment with gaseous NO improves plant growth and delays senescence

The initial aim of this experiment was the analysis of the plant stress response to high NO concentrations. Therefore, the dicotyledonous model plant *A. thaliana* (ecotype Wassilewskija) was fumigated during the whole growth phase season with different concentrations of NO gas. Air was purified using filter pads in combination with activated-carbon filters and silica particles coated with permanganate (ambient, ca. 5 ppbv) and supplemented with 0.8 ppmv (parts per million by volume), 1.5 ppmv, and 3.0 ppmv of NO.

Plants were grown in climate chambers under highly controlled conditions (light - 300 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR: 400–700 nm); temperature – day: 20 °C (14 h) and night: 16 °C (10 h); and relative humidity – 80%) without additional fertilization (Fig. S1). After four weeks of treatment a phenotype was observed (Fig. 1a). Plants grown under ambient conditions showed a red senescence phenotype. However, there was a proportional delay in the development of the red senescence phenotype and also a greater amount of lateral shoot development with increased concentrations of NO fumigation (Fig. 1a and Fig. S2). Even at the highest NO concentration used no toxic effect of NO was observed. The delay in senescence was verified by analysing *SAG12*. *SAG12* encodes a cysteine protease (Lohman et al., 1994) and is a well-known senescence marker gene (Noh and Amasino, 1999). We observed that *SAG12* expression was clearly delayed in those plants fumigated with NO gas, and the delay correlated with the NO concentration applied (Fig. 1b). Moreover, total protein (Fig. 1c), RNA (Fig. 1d) and chlorophyll *a* and *b* (Fig. S3) content is lower in plants fumigated with ambient air in comparison to NO-treated plants.

NO-treatment results in activation of primary metabolism and inhibition of phenylpropanoid metabolism

To identify key processes responsible for the phenotype of the NO treated *Arabidopsis* plants, transcriptomic and proteomic studies were performed. An expression profiling of more than 27,000 *A. thaliana* transcripts in four weeks old NO-treated WT plants was done using microarray technique based on Agilent One Color Microarray-based Gene Expression Analysis platform. Plants treated with ambient air were used as control. Total RNA of rosette leaves was isolated and their quality was assured using Agilent RNA 6000 Nano kit on Agilent 2100 BioAnalyzer. Following cRNA synthesis, chip hybridization, scanning and feature extraction, raw expression data of the genes was analyzed using GeneSpring GX software tool. In total, 1534 genes were differentially regulated in NO-fumigated WT plants in comparison to plants treated with ambient air (Table S1) - 1097 genes were up-regulated and 437 genes were down-regulated. The vast majority of the up-regulated genes are involved in primary metabolic processes, such as photosynthesis and protein biosynthesis (Table S1 and S2 and Fig. S4).

Similar results were obtained within the proteomic studies. Protein extracts from rosettes of four weeks old WT plants (ambient, 0.8 ppmv, 1.5 ppmv and 3 ppmv NO) were analysed using two dimensional difference gel electrophoresis (2D-DIGE). 2D-DIGE was carried out on Ettan DIGE platform. According to the DIGE-technology proteins were labelled with

different fluorescent dyes to enable quantification. After 2D separation the protein spot intensity was analysed and differentially accumulated protein spots were identified. In total, 57 protein spots differentially accumulated due to NO fumigation. These spots were numbered (Fig. S5) and proteins in each spot were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy analysis. The proteins and their regulation pattern after NO-fumigation is summarized in Table S3. A total of 93 proteins were identified from these 57 protein spots. Accumulation of 71 proteins was higher in NO fumigated plants, while accumulation of 10 proteins was lower under these conditions. In general, mainly proteins involved in plant primary metabolism accumulated after NO fumigation (Fig. S6).

It is a well known phenomenon that the higher the N-content, the lower the content in phenolic compounds (Lea et al., 2007). Higher N-content significantly reduces phenylpropanoid metabolism in plants (Lea et al., 2007). Sinapinic acid, quercetin, kaempferol and anthocyanin are important phenolic compounds that are believed to function as antioxidants capable of regulating the cellular levels of toxic reactive intermediates during stress responses (Dixon and Paiva, 1995; Winkel-Shirley, 2002). We observed that these secondary metabolites accumulated in plants during aging, and this accumulation was delayed in plants fumigated with NO gas in a concentration-dependent manner (Fig. 2a). Correspondingly, the activity of phenylalanine ammonia lyase (PAL), the central player and the branching point that induces the biosynthesis of flavonoids, was significantly lower in the NO-fumigated plants than in plants grown under ambient conditions (Fig. 2b).

NO-fumigation enhances nitrate, nitrite, ammonia and S-nitrosothiol levels in leaves

The uptake of NO by plants through stomata was proposed in the 1990s (Wellburn, 1990; Stulen et al., 1998), and this hypothesis was supported by a study that showed the expansion of the leaf disc in pea plants after NO fumigation (Leshem et al., 1998). The S-nitrosothiol (RSNO) levels in plants fumigated with NO were 2.5 times higher than those in plants grown under ambient conditions, suggesting a rapid increase in the intrinsic NO levels after fumigation (Fig. 3a). The increase in the RSNO levels was accompanied by significant increases in the levels of nitrate, nitrite, and ammonia (Fig. 3b-d). Among these analytes, nitrate showed the most significant increase, more than 6-fold, after NO fumigation (Fig. 3b). However, the increased nitrate levels cannot be explained by non-enzymatic mechanisms (Wellburn, 1990; Stulen et al., 1998). To assess the possible effect of soil NO deposition during fumigation, which would help plants acquire more N from the soil, we analysed the

soil from pots with plants (used) and without plants (unused) from both the ambient and NO treatment groups. However, no significant difference in the levels of inorganic N-containing ions – nitrate, nitrite, and ammonia – was found between the ambient and NO-treated samples (Fig. 3e). Therefore, we hypothesized that plants directly convert NO enzymatically to nitrate in leaves.

NO-fixation by non-symbiotic hemoglobin proteins

Interestingly, we observed an increase in the expression levels of non-symbiotic hemoglobin 1 (*AtGLB1* for *Arabidopsis* globin 1) along with nitrate reductase (*AtNIA2*) and nitrite reductase (*AtNiR1*) in the plants fumigated with NO in comparison with plants grown under ambient conditions (Fig. 4a). In plants, non-symbiotic hemoglobins are known to mediate the enzymatic conversion of NO into nitrate, which is subsequently reduced to nitrite by nitrate reductase, as part of the metabolic pathway known as the hemoglobin/NO cycle (Igamberdiev et al., 2005). There are three classes of non-symbiotic hemoglobins in *Arabidopsis* - Class 1 (GLB1), Class 2 (GLB2), and Class 3 (GLB3) (Smagghe et al., 2009), whereas GLB1 and GLB2 exhibit NO dioxygenase (NOD) activity (Perazzolli et al., 2004; Hebelstrup et al., 2012). Based on our observations, we concluded that non-symbiotic hemoglobin might mediate NO fixation, resulting in enhanced N-assimilation in *Arabidopsis* plants (Fig. 4b).

To test this, *Arabidopsis* plants with altered *GLB1* and *GLB2* expression were treated with 3 ppmv NO. Plants overexpressing *GLB1* (*GLB1-Ox*) or *GLB2* (*GLB2-Ox*) as well as plants, with reduced (*glb1-RNAi*) or knocked out (*glb2-KO*) *GLB* expression were used. All transgenic mutants were generated in the Columbia-0 (Col-0) background (Hebelstrup et al., 2006). The growth of all different plant lines except *glb2-KO* was enhanced after NO fumigation in comparison with the control treatment (Fig. 4c). The rosette sizes of both *GLB1-Ox* and *GLB2-Ox* plants fumigated with NO gas were significantly larger than the sizes of the rosettes of fumigated WT control plants. The diameter, fresh weight, and dry weight of the rosettes were also greater in both overexpression lines after NO fumigation in comparison with WT Col-0 plants (Table 1). Moreover, the vegetative shoot length, shoot thickness, and lateral (secondary) shoot formation were more pronounced in *GLB1-Ox* and *GLB2-Ox* plants than in WT plants after NO fumigation. *GLB1-Ox* and *GLB2-Ox* plants showed significant differences in shoot development after NO fumigation. In *GLB2-Ox* plants, the shoots were thicker and more laterals were formed than in *GLB1-Ox* plants (Table 1). Interestingly, we could not observe differences in shoot number or shoot morphology in plants grown under ambient NO conditions. Furthermore, the net yield of seeds produced per

plant was significantly higher in WT plants and both overexpression lines after NO treatment, in the order of *GLB2-Ox* > *GLB1-Ox* > WT.

The RSNO levels in all the plant lines were analysed to determine the effect of altered hemoglobin expression on the intrinsic NO levels. The intrinsic RSNO levels were significantly higher in all plant lines fumigated with NO than in plants grown under ambient conditions (Fig. 5a). However, the increase in the RSNO level was much lower in *GLB1-Ox* and *GLB2-Ox* plants than in WT plants (Fig. 5a). The results indicated an enhanced NO metabolism in *GLB1-Ox* and *GLB2-Ox* plants. Correspondingly, plants with reduced *GLB* expression (*glb1-RNAi* and *glb2-KO*) have a reduced capability to metabolize accumulating NO resulting in significant increase in the RSNO levels in comparison to WT Col-0 plants (Fig. 5a). Significantly higher nitrate levels in NO fumigated *GLB-Ox* plants compared to WT plants confirmed the role of non-symbiotic hemoglobins in converting NO to nitrate (Fig. 5b and Fig. S7). Increased N-assimilation in these lines is evident from the higher levels of nitrite and ammonia after NO fumigation (Fig. 5c,d and Fig. S7). However, the nitrate and ammonia levels of *glb1-RNAi* and *glb2-KO* plant lines were also higher than their ambient counterparts and were not significantly different from WT Col-0 plants (Fig. 5b,d).

To demonstrate NO uptake by leaves, NO fumigation experiments were performed with hydroponic cultures. *Arabidopsis* WT plants and plants with altered *GLB1* and *GLB2* expression were hydroponically cultivated in N-containing medium for twelve days (Gilbert et al., 1997). Then, these plants were transferred to medium without any N-source and fumigated with NO. Also here a positive growth effect of the NO-treatment was observed (Fig. 6 and Fig. S10 and S11). Similar to soil grown plants rosette size, shoot length, number of siliques and seed yield was increased in NO-treated *GLB1*- and/or *GLB2*-overexpressing plants in comparison to the untreated controls (Fig. 6a-d). Moreover, the levels of RSNO, nitrite and nitrate are higher in leaves of NO-fumigated plants (Fig. S12). Similar to the soil grown plants nitrate levels were highest in NO-fumigated *GLB1*- and/or *GLB2*-overexpressing plants.

To further demonstrate NO uptake and the importance of non-symbiotic hemoglobin proteins for N accumulation, we fumigated hydroponic cultures of *Arabidopsis* with 250 ppbv ^{15}NO. Again the plants were first cultivated in N-containing medium and transferred to N-free medium before ^{15}NO fumigation. Samples were harvested after 4, 6 and 11 days of fumigation and ^{15}N content was determined in leaves (Fig. 7a). ^{15}N accumulation could be observed in all lines during the treatment. The highest accumulation was detected in *GLB2-Ox* plants (up to almost 7% of total N content after 11 days of ^{15}NO fumigation). After 6 days

of treatment WT plants accumulated the same amount of ^{15}N as the *GLB1-Ox* plants, most likely due to NO-induced expression of *GLB1* (Fig. 4a). The lowest ^{15}N accumulation was observed in *glb1-RNAi* and *glb2-ko* lines. These results demonstrate that overexpression of *GLB1* or *GLB2* positively affects ^{15}N accumulation in plants and that both non-symbiotic hemoglobin isoforms promote the use of NO as N source. Based on the ^{15}N data after 4 days of ^{15}NO-treatment we calculated a daily uptake for GLB2 overexpressing plants of 250 mg N/kg dry matter, which is almost 50% more than in WT plants (Fig. 7b).

The NO uptake by the plants was further demonstrated by "scavenging" NO released from soil (Fig. 7c). In a similar experiment, reduction of soil-emitted NO has been already demonstrated in WT *Arabidopsis* plants (Mur et al., 2011). The experiment was performed in a closed system/cuvette. When placing pots with soil and *Arabidopsis* rosettes in the cuvette, we detected lower NO levels than when the plants were cut and removed. When the excised plants were reapplied to the surface of the soil, NO levels were again reduced. In our experimental system ca. 100 ppbv NO accumulated in the closed cuvette within 30 min, when the plants were cut and removed (soil-released NO). *GLB2-Ox* plants reduced the levels of soil-released NO up to 4 ppbv per gram fresh weight within 30 min. This is more than the double amount of NO removed by WT plants and four-times more than by *glb2-ko* plants.

Discussion

Nitrogen is an essential component of plant growth and reproduction and plays a vital role in the development of healthy foliage. It is required for biosynthesis of chlorophyll and it is also the basic element of proteins and genetic material, such as DNA and RNA. NO is an important signalling molecule which is involved in many different physiological processes in plants. Here we describe the NO-fixing function of non-symbiotic hemoglobin proteins. Experiments with ^{15}NO demonstrated that NO-fixation is enhanced considerably in plants overexpressing *AtGLB1* or *AtGLB2* genes. NO uptake resulted in four-fold higher nitrate levels in these plants compared to NO-treated WT plants. Correspondingly, the growth parameters like rosettes size and weight, vegetative shoot thickness and also seed yield were significantly higher in the overexpression lines in comparison to WT plants. However, we have to emphasize that in our experiments the plants were treated with 0.25 – 3.0 ppm NO. These are relatively high concentrations, which are normally not found in the natural environment. In most towns, where the air is rather polluted, NO is on average at 0.1 ppm.

Nevertheless, our results demonstrate the NO-fixing capability of non-symbiotic hemoglobin proteins, which allows a channelling of atmospheric NO into the plant N metabolism.

Growth promoting effect of NO-treatment

The dicotyledonous model plant *A. thaliana* (ecotype Wassilewskija) was fumigated during the whole growth phase season with different concentrations (ambient, 0.8 ppmv, 1.5 ppmv and 3.0 ppmv) of NO gas. Plants treated with NO showed a delay in the development of the red senescence phenotype in comparison to plants treated with ambient air (Fig. 1a). Moreover, total protein (Fig. 1c), RNA (Fig. 1d) and chlorophyll *a* and *b* (Fig. S3) content is lower in plants fumigated with ambient air in comparison to NO-treated plants. Since these compounds are the major N-sinks in plants, the senescence phenotype is probably triggered by under-supply of N. This would be also in line with the decrease in total protein, RNA and chlorophyll content during age-dependent senescence in plants (Lohman et al., 1994). Since NO fumigation delayed age-dependent senescence in *A. thaliana*, we hypothesized that the plants were able to use the supplied NO as N-source. A positive effect of NOx on plants growth and fruit yield has been already described previously (Leshem et al., 1998; Takahashi et al., 2005; Takahashi et al., 2011; Takahashi et al., 2014). In presence of up to 200 ppbv nitrogen dioxide (NO_2) shoot biomass and total leaf area is increased in many different plants species (Takahashi and Morikawa, 2014). Moreover, cell proliferation and enlargement seems to be regulated by NO_2. A shoot biomass increase was also observed in *Arabidopsis* plants exposed to 50 ppbv NO (Takahashi et al., 2014) and positive effects on vegetative growth was demonstrated in pea leaf discs and spinach (Leshem and Haramaty, 1996; Jin et al., 2009). Furthermore, vegetative growth could be enhanced in *Arabidopsis* seedlings treated with the NO donor sodium nitroprusside (He et al., 2004). However, the molecular mode of action underlying these effects has often remained elusive.

It is a well known phenomenon that an enhanced N-content significantly reduces phenylpropanoid metabolism and as consequence the content of phenolic compounds in plants (Lea et al., 2007). In plants, phenylalanine ammonia lyase (PAL) serves as a gateway from primary plant metabolism to phenylpropanoid metabolism (Hahlbrock and Scheel, 1989). PAL catalyzes the formation of trans-cinnamic acid by removing ammonia from L-phenylalanine. Downstream to cinnamic acid is a wide variety of secondary metabolites that belongs to different classes of phenylpropanoid products like anthocyanins, flavonoids, ultraviolet (UV) protectants, antimicrobial furanocoumarins, isoflavonoid phytoalexins, lignins and wound phenolic esters (Dixon and Paiva, 1995; Ritter and Schulz, 2004). We

observed that the accumulation of several different phenolic metabolites was delayed in plants fumigated with NO gas in a concentration-dependent manner (Fig. 2a). Correspondingly, the PAL activity in the NO-fumigated plants was significantly lower than that in plants grown under ambient conditions (Fig. 2b). This is in line with the observation that reduced N-nutrition in plants induces PAL activity and downstream secondary metabolism in plants (Kovacik et al., 2007). Moreover, NO-fumigation can also result in S-nitrosylation of PAL and inhibition of its activity. In poplar, a physiological function of S-nitrosylation of PAL is described (Vanzo et al., 2014). The isoform PAL2 is de-nitrosylated in response to acute ozone and enhanced PAL activity was observed. The authors hypothesized that S-nitrosylation/de-nitrosylation represents a mechanism to regulate enzyme activities and in this way the metabolic flux through the phenylpropanoid pathways. All these findings suggested that there was greater carbon and nitrogen assimilation (Lawlor, 2002) in the NO-fumigated plants, which positively influenced the growth and development of the plants.

The RSNO, levels and the content in N-containing ions were significantly higher in NO fumigated plants than those in plants grown under ambient conditions, suggesting a rapid increase in the intrinsic NO levels after fumigation (Fig. S7 and S12). In soil grown plants and hydroponic cultures the nitrate levels were highest in NO-fumigated *GLB1*- and *GLB2*-overexpressing plants.

The rosette sizes, fresh weight, and dry weight of the rosettes of *GLB1-Ox* and *GLB2-Ox* plants fumigated with NO gas were significantly larger/higher than that of the fumigated WT control plants (Table 1). Moreover, the vegetative shoot length, shoot thickness, and lateral (secondary) shoot formation were more pronounced in *GLB1-Ox* and *GLB2-Ox* plants than in WT plants after NO fumigation. Furthermore, in hydroponic cultures rosette size, shoot length, number of siliques and seed yield was increased in NO-treated *GLB1*- and/or *GLB2*-overexpressing plants in comparison to the ambient control plants (and Fig. 6a-d). The differences are not as clear as in soil grown plants (Table 1) since plant growth is limited in our hydroponic system – mainly because of the missing N-source in the medium, concluding that atmospheric NO alone cannot substitute N-uptake through the roots. But especially in hydroponic cultures of *GLB2-Ox* plants the red senescence phenotype was delayed (Fig. S10) further demonstrating a nitrogen supply effect of NO fumigation. In sum, all these observations support the existence of a NO-fixation mechanism, resulting in enhanced N-assimilation in *Arabidopsis* plants and better growth and development.

However, it is also known that NO can interact with other plant hormones or proteins involved in plant hormone metabolism (Freschi, 2013). In this way NO can modulate plant hormone function/signaling and affect plant growth and development and we cannot exclude that the observed effects are at least partly due to interaction of NO with plant hormones. For instance, a direct role of NO in cytokinin signal transduction is under discussion and both synergistic and antagonistic interactions have been described often dependent on the experimental approach, physiological response or plant species (Freschi, 2013). Moreover, it should be considered, that nitrate induces transcription of cytokinin biosynthesis genes and that there is a well-described interaction of nitrate and cytokinin (Sakakibara, 2003; Sakakibara et al., 2006).

On the other side, the effects of intrinsic NO signalling in plant development are usually antagonised by hemoglobin overexpression and are increased by hemoglobin knock-out (Hebelstrup et al., 2013). However, in the present study, hemoglobin overexpression enhanced the effect and hemoglobin knock-out limited the effect of NO treatment, suggesting that the delayed senescence and enhanced growth and lateral shoot development induced by NO fumigation are not due to NO acting as an intrinsic signalling molecule.

Non-symbiotic hemoglobin proteins allow the fixation of atmospheric NO into nitrogen metabolism

The uptake of NO by plants through stomata was proposed in the 1990s and highlighted a possible pathway of assimilating atmospheric NO that involves the non-enzymatic conversion of NO into nitrite in the apoplast (Wellburn, 1990; Stulen et al., 1998). Nitrite was thought to be transported across the plasma membrane into the chloroplast, where it is reduced to ammonia (Wellburn, 1990). However, the increased nitrate levels, which we measured in the NO fumigated plants, could not be explained by non-enzymatic mechanisms proposed by Wellburn (1990) and Stulen et al. (1998) concluding that plants directly convert NO enzymatically to nitrate in leaves.

We observed an increase in the expression levels of non-symbiotic hemoglobin 1 (*AtGLB1*) in the plants fumigated with NO in comparison with plants grown under ambient conditions (Fig. 4a). The microbial heme protein NO dioxygenase (NOD) is known to oxidise NO to yield nitrate (Gardner et al., 1998). The primary function of this enzyme is to maintain proper cellular NO levels. Interestingly, the expression of bacterial NOD in *Arabidopsis* resulted in delayed senescence after NO fumigation (Mishina et al., 2007). In mammals, both hemoglobin and myoglobin exhibit NOD activity (Ouellet et al., 2002). In *Arabidopsis* GLB1

and GLB2 exhibit NOD activity (Perazzolli et al., 2004; Hebelstrup et al., 2012). This NOD activity of non-symbiotic plant hemoglobins has already been demonstrated to be important for limiting the loss of cellular N through NO gas emission from plants under hypoxic conditions (Hebelstrup et al., 2006; Hebelstrup et al., 2012). Moreover, the role of non-symbiotic plant hemoglobins in modulating NO metabolism/signalling by functioning as NO scavenger has been already discussed in different contexts such as seed germination, bolting, and nitrogen-fixing symbiosis (Hebelstrup et al., 2013; Hebelstrup and Jensen, 2008; Shimoda et al., 2009).

Because of its pentacoordinated heme iron GLB1 is known to convert NO to NO_3^-. Reducing equivalents were supplied by NADPH (Gupta et al., 2011). Metabolism of NO by *GLB2-Ox* plants is surprising, because GLB2 is not known for its NO metabolizing function because of its low oxygen affinity (Gupta et al., 2011). But also GLB2, which contains a hexacoordinated heme iron, seems to be able to interact with NO, because an effective NO scavenging activity was already described for this protein (Hebelstrup and Jensen, 2008). Moreover, enhanced NO metabolism mediated by S-nitrosylation of GLB2 cannot be ruled out. A similar function is suggested for hemoglobin proteins in animals (Foster et al., 2003).

In NO-fumigated *GLB-Ox* plants significantly higher nitrate levels were detected compared to WT plants confirming that non-symbiotic hemoglobins converted NO to nitrate (Fig. 5b and Fig. S7). Surprisingly, the nitrate and ammonia levels of *glb1-RNAi* and *glb2-KO* plant lines were also higher than their ambient counterparts and were not significantly different from WT Col-0 plants (Fig. 5b,d). This can be due to presence of either one of the functional GLB in these mutant lines; GLB2 is functional in *glb1-RNAi* plants and GLB1 is functional in *glb2-KO* plants. The nitrite content in the *glb1-RNAi* plants fumigated with NO was much higher than in all the other plant lines (Fig. 5c). A possible explanation might be the conversion of accumulated NO into nitrite in the apoplast (Wellburn, 1990), if GLB1 level is decreased. NO can be converted to nitrite non-enzymatically in aerobic aqueous solution (Ignarro et al., 1993). However, this high level of nitrite accumulation was not observed in *glb2-KO* plants after fumigation with NO (Fig. 5c). This shows that the functional GLB1 in *glb2-KO* is more capable in metabolizing NO than functional GLB2 in *glb1-RNAi*. This was also evident in our gene expression analyses where induction of *GLB1* expression was more prominent than that of *GLB2* after fumigation with NO (Fig. 4a). In sum, these results lead to the assumption that not only GLB1, but also GLB2 can oxidize NO into nitrate. Moreover,

the reduced flavonoid biosynthesis and PAL activity in the *GLB-Ox* lines in comparison with WT plants confirmed the role of NO fixation in delaying senescence (Fig. S8 and S9).

The importance of non-symbiotic hemoglobin proteins for NO uptake was demonstrated by experiments using ^{15}NO (Fig. 7a) and by analysing the "scavenging" of NO released from soil (Fig. 7c). The highest ^{15}N uptake was observed in *GLB2-Ox* plants. However, also WT plant accumulated already quite high amounts of ^{15}N, which was in the range of that in *GLB1-Ox* plants. Probably this is due to the NO-induced expression of *GLB1* in WT plants (Fig. 4a). Of course, that would be also expected in *GLB-Ox* plants, but maybe the induction is higher in WT plants, because GLB levels are already "boosted" in *GLB-Ox* plants.

The uptaken ^{15}N can be present in both inorganic (non-assimilated) and organic (assimilated) forms. Especially the composition of the N-containing organic form is very complex since it includes different types of compounds, such as amino acids/proteins, nucleic acids, secondary metabolites, and pigments. Therefore we presented here the total ^{15}N content. However, in future we want to analyse the metabolic fluxes of ^{15}N in ^{15}NO-treated plants to identify the exact pathway of incorporation of N from uptaken NO into metabolites/proteins. This will be done in a combination of targeted (analyzing the compounds of the N-assimilation pathway) and non-targeted approaches (metabolomics and fluxomics).

Based on the ^{15}N data after 4 days of ^{15}NO-treatment we calculated a daily uptake for *GLB2-Ox* plants of 250 mg N/kg dry matter, which is almost 50% more than in WT plants (Fig. 7b). We tried to extrapolate these results to field conditions. The average total N content in well grown healthy plants is ca. 2% of the plant dry matter (Epstein, 1965). This corresponds to 50 kg total N in 2500 kg plant dry matter - an amount, which can be harvested per year on one hectare grassland. Based on the NO-fixing capacity of *GLB2-Ox* plants (250 mg N/kg dry matter) we calculated a NO-based N-uptake of 0,625 kg N/ha/year (250 mg N/kg dry matter x 2500). This is in the range of the N-fixation capacity of free living bacteria (ca. 1–3 kg N/ha/year), whereas plant associated N-fixing bacteria fix 100-300 kg N/ha/year. Of course until now, we do not know which portion of total N-uptake can be covered by NO-fixation under conditions with optimal NO_3^- and NH_4^+ supply. Under such circumstances N-uptake via NO_3^- and NH_4^+ might be the dominant way, but under N-limited conditions N-uptake via NO might be of greater importance.

Conclusion

In sum, we demonstrated that fumigation with up to 3 ppm NO has no negative/toxic effects on plant growth and development. In contrast, the NO-fixing capability of non-symbiotic

hemoglobin proteins allows a channelling of atmospheric NO into the plant N metabolism. This pathway is probably important under conditions with limited N supply via the soil. Moreover, such a mechanism is likely to decrease loss of NO by emissions from agricultural fields and soils, which presently represents - together with industrial combustion - an increasing contributor to emission of nitrous greenhouse gases. In this way, the plant-based NO-fixation lowers the concentration of atmospheric NOx and in this context plants have a beneficial effect on air quality and human health. Furthermore, preventing loss of soil-emitted NO and making it available for N assimilation processes displays also a kind of N-recovery mechanism. Which portion of the total N-uptake is covered by NO-uptake will be analysed in the future. Moreover, analyses of metabolic fluxes in ^{15}NO treated plants should allow us to identify the exact pathway of incorporation of N from uptaken NO into metabolites/proteins.

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References

Belenghi, B., Romero-Puertas, M.C., Vercammen, D., Brackenier, A., Inze, D., Delledonne, M. & Van Breusegem, F. (2007) Metacaspase activity of *Arabidopsis thaliana* is regulated by S-nitrosylation of a critical cysteine residue. *Journal of Biological Chemistry* **282**,1352-1358.

Bethke, P.C., Libourel, I.G.L. & Jones, R.L. (2006) Nitric oxide reduces seed dormancy in *Arabidopsis*. *Journal of Experimental Botany* **57**, 517-526.

Corpas, F.J., Leterrier, M., Valderrama, R., Airaki, M., Chaki, M., Palma, J.M. & Barroso, J.B. 2011. Nitric oxide imbalance provokes a nitrosative response in plants under abiotic stress. *Plant Science* **181**, 604-611.

Corpas, F.J., Barroso, J.B., Carreras, A., Quiros, M., Leon, A.M., Romero-Puertas, …del Rio, L.A. (2004) Cellular and subcellular localization of endogenous nitric oxide in young and senescent pea plants. *Plant Physiology* **136**, 2722-2733.

Delledonne, M., Xia, Y., Dixon, R.A. & Lamb, C. (1998) Nitric oxide functions as a signal in plant disease resistance. *Nature* **394**, 585-588.

Dixon, R.A. & Paiva, N.L. (1995) Stress-induced phenylpropanoid metabolism. *Plant Cell* **7**, 1085-1097.

Durner, J., Wendehenne, D. & Klessig, D.F. (1998) Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proceedings of the National Academy of Science USA* **95**, 10328-10333.

Epstein, E. (1965). Typical concentrations sufficient for plant growth. *Plant Biochemistry* :438-466.

Foster, M.W., McMahon, T.J. & Stamler, J.S. (2003) S-nitrosylation in health and disease. *Trends in Molecular Medicine* **9**, 160-168.

Freschi, L. (2013) Nitric oxide and phytohormone interactions: current status and perspectives. *Frontiers in Plant Science* **4**, 398.

Garcia-Mata, C., Gay, R., Sokolovski, S., Hills, A., Lamattina, L. & Blatt, M.R. (2003) Nitric oxide regulates K^+ and Cl^- channels in guard cells through a subset of abscisic acid-evoked signaling pathways. *Proceedings of the National Academy of Science USA* **100**, 11116-11121.

Gardner, P.R., Gardner, A.M., Martin, L.A. & Salzman, A.L. (1998) Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. *Proceedings of the National Academy of Science USA* **95**, 10378-10383.

Gilbert, G., Wilson, C. & Madore, M. (1997) Root zone salinity alters raffinose oligosaccharide metabolism and transport in Coleus. *Plant Physiology* **115**, 1267-1276.

Grun, S., Lindermayr, C., Sell, S. & Durner, J. (2006) Nitric oxide and gene regulation in plants. *Journal of Experimental Botany* **57**, 507-516.

Guo, F.Q. & Crawford, N.M. (2005) *Arabidopsis* nitric oxide synthase1 is targeted to mitochondria and protects against oxidative damage and dark-induced senescence. *Plant Cell* **17**, 3436-3450.

Gupta, K.J., Hebelstrup, K.H., Mur, L.A. & Igamberdiev, A.U. (2011) Plant hemoglobins: important players at the crossroads between oxygen and nitric oxide. *FEBS Letters* **585**, 3843-3849.

Hahlbrock, K. & Scheel, D. (1989) Physiology and molecular biology of phenylpropanoid metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**, 347-369.

Harlow, E. & Lane, D. (2006) Bradford assay. *Cold Spring Harbor Protocols* 2006, doi:10.1101/pdb.prot4644.

He, Y.K., Tang, R.H., Hao, Y., Stevens, R.D., Cook, C.W., Am, S.M., …Pei, Z.M. (2004) Nitric oxide represses the *Arabidopsis* floral transition. *Science* **305**, 1968-1971.

Hebelstrup, K.H. & Jensen, E.O. (2008) Expression of NO scavenging hemoglobin is involved in the timing of bolting in *Arabidopsis thaliana*. *Planta* **227**, 917-927.

Hebelstrup, K.H., Igamberdiev, A.U. & Hill, R.D. (2007) Metabolic effects of hemoglobin gene expression in plants. *Gene* **398**, 86-93.

Hebelstrup, K.H., Shah, J.K. & Igamberdiev, A.U. (2013) The role of nitric oxide and hemoglobin in plant development and morphogenesis. *Physiologia Plantarum* **148**, 457-469.

Hebelstrup, K.H., Hunt, P., Dennis, E., Jensen, S.B. & Jensen, E.O. (2006) Hemoglobin is essential for normal growth of *Arabidopsis* organs. *Physiologia Plantarum* **127**, 157-166.

Hebelstrup, K.H., van Zanten, M., Mandon, J., Voesenek, L.A., Harren, F.J., Cristescu, S.M., …Mur, L.A. (2012) Haemoglobin modulates NO emission and hyponasty under hypoxia-related stress in *Arabidopsis thaliana*. *Journal of Experimental Botany* **63**, 5581-5591.

Heckmann, A.B., Hebelstrup, K.H., Larsen, K., Micaelo, N.M. & Jensen, E.O. (2006) A single hemoglobin gene in Myrica gale retains both symbiotic and non-symbiotic specificity. *Plant Molecular Biology* **61**, 769-779.

Holzmeister, C., Frohlich, A., Sarioglu, H., Bauer, N., Durner, J. & Lindermayr, C. (2011) Proteomic analysis of defense response of wildtype Arabidopsis thaliana and plants with impaired NO- homeostasis. *Proteomics* **11**, 1664-1683.

Huang, X., Stettmaier, K., Michel, C., Hutzler, P., Mueller, M.J. & Durner, J. (2004) Nitric oxide is induced by wounding and influences jasmonic acid signaling in *Arabidopsis thaliana*. *Planta* **218**, 938-946.

Igamberdiev, A.U., Baron, K., Manac'h-Little, N., Stoimenova, M. & Hill, R.D. (2005) The Haemoglobin/Nitric Oxide Cycle: Involvement in Flooding Stress and Effects on Hormone Signalling. *Annals of Botany* **96**, 557-564.

Ignarro, L.J., Fukuto, J.M., Griscavage, J.M., Rogers, N.E. & Byrns, R.E. (1993) Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. *Proceedings of the National Academy of Science USA* **90**, 8103-8107.

Jin, C.W., S.T., D., Zhang, Y.S., Tang, C. & Lin, X.Y. (2009) Atmospheric nitric oxide stimulates plant growth and improves the quality of spinach (Spinacia oleracea). *Annals of Applied Biology* **155**, 113-120.

Kovacik, J., Klejdus, B., Backor, M. & Repcak, M. (2007) Phenylalanine ammonia-lyase activity and phenolic compounds accumulation in nitrogen-deficient *Matricaria chamomilla* leaf rosettes. *Plant Science* **172**, 393-399.

Lawlor, D.W. (2002) Carbon and nitrogen assimilation in relation to yield: mechanisms are the key to understanding production systems. *Journal of Experimental Botany* **53**, 773-787.

Lea, U.S., Slimestad, R., Smedvig, P. & Lillo, C. (2007) Nitrogen deficiency enhances expression of specific MYB and bHLH transcription factors and accumulation of end products in the flavonoid pathway. *Planta* **225**, 1245-1253.

Leshem, Y. & Haramaty, E. (1996) The characterization and contrasting effects of the nitric oxide free radical in vegetative stress and senescence of *Pisum sativum Linn*. foliage. *Journal of Plant Physiology* **148**, 258-263.

Leshem, Y.Y., Wills, R.B.H. & Ku, V.V. (1998) Evidence for the function of the free reduced gas – nitric oxide (·NO) as an endogenous maturation and senescence regulating factor in higher plants. *Plant Physiology and Biochemistry* **36**, 825-826.

Lohman, K.N., Gan, S., John, M.C. & Amasino, R.M. (1994) Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. *Physiologia Plantarum* **92**, 322-328.

Mengel, A., Chaki, M., Shekariesfahlan, A. & Lindermayr, C. (2013) Effect of nitric oxide on gene transcription - S-nitrosylation of nuclear proteins. *Frontiers in Plant Science* **4**, 293.

Mur, L.A., Mandon, J., Cristescu, S.M., Harren, F.J. & Prats, E. (2011) Methods of nitric oxide detection in plants: a commentary. *Plant Science* **181**, 509-519.

Mur, L.A., Sivakumaran, A., Mandon, J., Cristescu, S.M., Harren, F.J. & Hebelstrup, K.H. (2012) Haemoglobin modulates salicylate and jasmonate/ethylene-mediated resistance mechanisms against pathogens. *Journal of Experimental Botany* **63**, 4375-4387.

Neill, S.J., Desikan, R., Clarke, A. & Hancock, J.T. (2002) Nitric oxide is a novel component of abscisic acid signaling in stomatal guard cells. *Plant Physiology* **128**, 13-16.

Noh, Y.S. & Amasino, R.M. (1999) Identification of a promoter region responsible for the senescence-specific expression of SAG12. *Plant Molecular Biology* **41**, 181-194.

Ouellet, H., Ouellet, Y., Richard, C., Labarre, M., Wittenberg, B., Wittenberg, J. & Guertin, M. (2002) Truncated hemoglobin HbN protects *Mycobacterium bovis* from nitric oxide. *Proceedings of the National Academy of Science USA* **99**, 5902-5907.

Perazzolli, M., Dominici, P., Romero-Puertas, M.C., Zago, E., Zeier, J., Sonoda, M., …Delledonne, M. (2004) Arabidopsis nonsymbiotic hemoglobin AHb1 modulates nitric oxide bioactivity. *Plant Cell* **16**, 2785-2794.

Ritter, H. & Schulz, G.E. (2004) Structural basis for the entrance into the phenylpropanoid metabolism catalyzed by phenylalanine ammonia-lyase. *Plant Cell* **16**, 3426-3436.

Sakakibara, H. 2003. Nitrate-specific and cytokinin-mediated nitrogen signaling pathways in plants. *Journal of Plant Research* **116**, 253-257.

Sakakibara, H., Takei, K. & Hirose, N. (2006) Interactions between nitrogen and cytokinin in the regulation of metabolism and development. *Trends in Plant Science* **11**, 440-448.

Shimoda, Y., Shimoda-Sasakura, F., Kucho, K., Kanamori, N., Nagata, M., Suzuki, …Uchiumi, T. (2009) Overexpression of class 1 plant hemoglobin genes enhances symbiotic nitrogen fixation activity between Mesorhizobium loti and Lotus japonicus. *The Plant Journal* **57**, 254-263.

Smagghe, B.J., Hoy, J.A., Percifield, R., Kundu, S., Hargrove, M.S., Sarath, G., …Appleby, C.A. (2009) Correlations between oxygen affinity and sequence classifications of plant hemoglobins. *Biopolymers* **91**, 1083-1096.

Sokolovski, S., Hills, A., Gay, R., Garcia-Mata, C., Lamattina, L. & Blatt, M.R. (2005) Protein phosphorylation is a prerequisite for intracellular Ca^{2+} release and ion channel control by nitric oxide and abscisic acid in guard cells. *The Plant Journal* **43**, 520-529.

Stulen, I., Perez-Soba, M., De Kok, L.J. & Van der Eerden, L. (1998) Impact of gaseous nitrogen deposition on plant functioning. *New Phytologist* **139**, 61-70.

Takahashi, M., & Morikawa, H. 2014. Nitrogen dioxide is a positive regulator of plant growth. *Plant Signaling & Behavior* **9**, e28033.

Takahashi, M., Sakamoto, A., Ezura, H. & Morikawa, H. (2011) Prolonged exposure to atmospheric nitrogen dioxide increases fruit yield of tomato plants. *Plant Biotechnology* **28**, 485–487.

Takahashi, M., Nakagawa, M., Sakamoto, A., Ohsumi, C., Matsubara, T. & Morikawa, H. (2005) Atmospheric nitrogen dioxide gas is a plant vitalization signal to increase plant size and the contents of cell constituents. *New Phytologist* **168**, 149-154.

Takahashi, M., Furuhashi, T., Ishikawa, N., Horiguchi, G., Sakamoto, A., Tsukaya, H. & Morikawa, H. (2014) Nitrogen dioxide regulates organ growth by controlling cell proliferation and enlargement in Arabidopsis. *New Phytologist* **201**; 1304-1315.

Trevaskis, B., Watts, R.A., Andersson, C.R., Llewellyn, D.J., Hargrove, M.S., Olson, …Peacock, W.J. (1997) Two hemoglobin genes in Arabidopsis thaliana: the evolutionary origins of leghemoglobins. *Proceedings of the National Academy of Science USA* **94**, 12230-12234.

Vanzo, E., Ghirardo, A., Merl-Pham, J., Lindermayr, C., Heller, W., Hauck, S.M., …Schnitzler, J.P. (2014) S-nitroso-proteome in poplar leaves in response to acute ozone stress. *PLoS One* **9**, e106886.

Watts, R.A., Hunt, P.W., Hvitved, A.N., Hargrove, M.S., Peacock, W.J. & Dennis, E.S. (2001) A hemoglobin from plants homologous to truncated hemoglobins of microorganisms. *Proceedings of the National Academy of Science USA* **98**, 10119-10124.

Wellburn, A.R. (1990) Why are atmospheric oxides of nitrogen usually phytotoxic and not alternative fertilizers? *New Phytologist* **115**, 395-429.

Winkel-Shirley, B. (2002) Biosynthesis of flavonoids and effects of stress. *Current Opinion in Plant Biology* **5**, 218-223.

Yin, R., Messner, B., Faus-Kessler, T., Hoffmann, T., Schwab, W., Hajirezaei, M.R., …Schaffner, A.R. (2012) Feedback inhibition of the general phenylpropanoid and flavonol biosynthetic pathways upon a compromised flavonol-3-O-glycosylation. *Journal of Experimental Botany* **63**, 2465-2478.

Table 1 - Analysis of growth parameters in plants fumigated with NO gas

Growth Parameter	NO treatment	WT Col-0	*glb1-RNAi*	*GLB1-Ox*	*glb2-KO*	*GLB2-Ox*
Rosette Diameter	Ambient NO [a]	4.28 ± 0.17	4,28 ± 0.13	4.38 ± 0.10	3.90 ± 0.26	4.25 ± 0.21
(cm) / Week 4	3 ppmv NO [b]	4.88 ± 0.17	4.93 ± 0.32	5.98 ± 0.39	3.30 ± 0.63	6.13 ± 0.36
(n = 10)	[c]Ratio [b/a]	1.14***	1.15	1.37***	0.85**	1.44***
Rosette Fresh Weight	Ambient NO [a]	1.05 ± 0.01	0.80 ± 0.01	1.07 ± 0.01	1.04 ± 0.02	1.07 ± 0.11
(g) / Week 4	3 ppmv NO [b]	1.15 ± 0.01	0.89 ± 0.01	1.46 ± 0.07	0.56 ± 0.03	1.64 ± 0.07
(n = 8 / 5 rosettes)	[c]Ratio [b/a]	1.10***	1.11***	1.37**	0.54***	1.53***
Rosette Dry Weight	Ambient NO [a]	0.15 ± 0.01	0.12 ± 0.02	0.15 ± 0.03	0.13 ± 0.02	0.16 ± 0.03
(g) / Week 4	3 ppmv NO [b]	0.13 ± 0.00	0.11 ± 0.01	0.18 ± 0.03	0.05 ± 0.00	0.20 ± 0.03
(n = 8 / 5 rosettes)	[c]Ratio [b/a]	0.88	0.93	1.20	0.40**	1.25
Vegetative Shoot thic-	Ambient NO [a]	0.88 ± 0.09	0.85 ± 0.10	0.94 ± 0.11	0.94 ± 0.12	0.91 ± 0.09
kness (mm) / Week 6	3 ppmv NO [b]	1.00 ± 0.12	0.97 ± 0.08	1.12 ± 0.11	0.99 ± 0.15	1.30 ± 0.16
(n = 20)	[c]Ratio [b/a]	1.13**	1.14***	1.20***	1.05	1.43***
Vegetative shoot len-	Ambient NO [a]	17.38 ± 2.90	10.90 ± 2.52	17.43 ± 3.25	17.93 ± 2.60	16.48 ± 1.78
gth (cm) / Week 6	3 ppmv NO [b]	17.33 ± 2.34	10.83 ± 1.82	19.90 ± 2.42	14.40 ± 2.68	17.85 ± 3.06
(n = 20)	[c]Ratio [b/a]	1.00	0.99	1.14**	0.80***	1.08
Number of secondary	Ambient NO [a]	0.35 ± 0.67	0.55 ± 0.69	0.30 ± 0.57	0.25 ± 0.55	0.15 ± 0.37
shoot / Week 6	3 ppmv NO [b]	1.15 ± 0.93	1.35 ± 0.75	2.50 ± 0.83	1.25 ± 0.91	3.80 ± 0.89
(n = 20)	[c]Ratio [b/a]	3.29**	2.45**	8.33***	5.00***	25.33***
Total Seed Yield	Ambient NO [a]	37.91	25.36	37.74	34.22	32.84
(mg) / Week 9	3 ppmv NO [b]	43.17	23.01	51.05	39.27	64.2
	[c]Ratio [b/a]	1.14	0.91	1.35	1.15	1.95

[c]Average values of a and b without standard error were used to calculate the ratios. 'n' represents either the total number of plants or number of groups with each having fixed number of plants used for the estimation. Asterisks indicate statistical significant differences from WT (Student´s t-test; **P*<0.05, ***P*<0.01, ****P*<0.001).

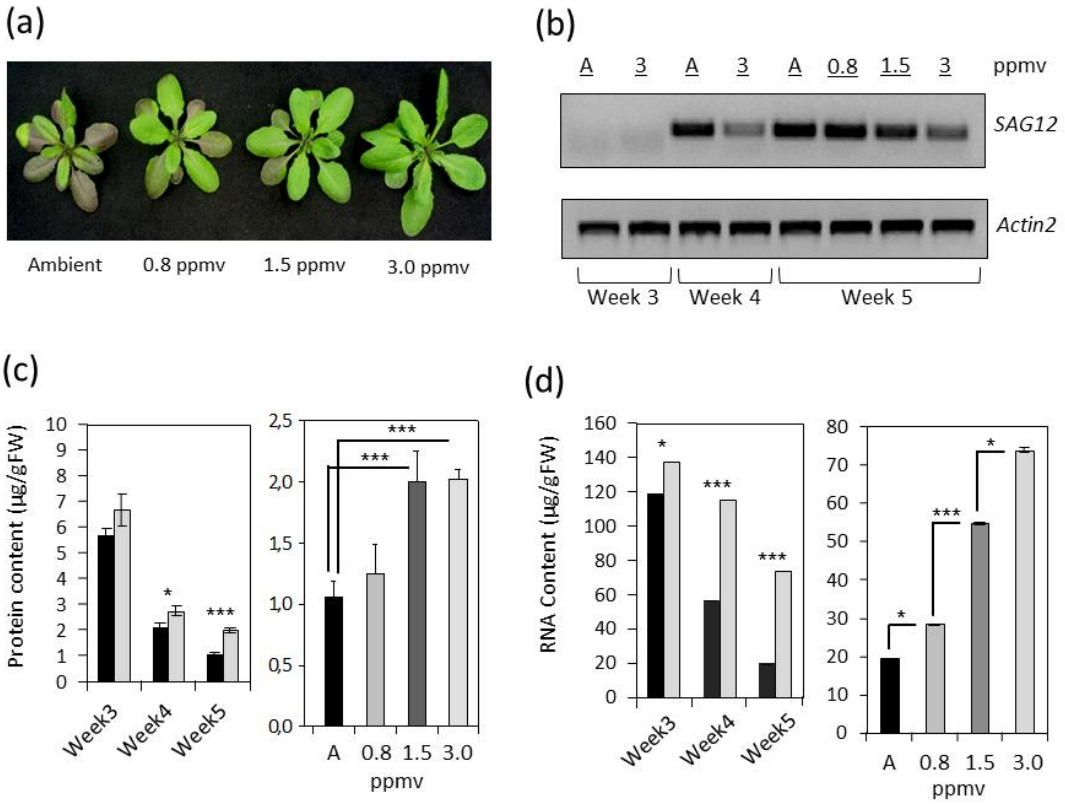

Fig. 1 Physiological effects of NO fumigation on the model plant *Arabidopsis thaliana*. (a) Rosettes of 4-week-old WT Ws plants fumigated with different NO gas concentrations. (b) Semi-quantitative reverse transcriptase PCR analysis. Agarose gels (1%) loaded with amplified *SAG12* gene transcripts (27 PCR cycles). Amplified *AtActin2* (AT3G18780) gene transcripts (27 cycles) were used as the loading control. Total protein (c) and RNA (d) contents of the ambient and 3 ppmv NO-fumigated plants at different ages (left) and of plants treated with four different concentrations of NO gas in Week 5 (right). Asterisks indicate statistical significant differences from WT (Student´s t-test; **P*<0.01, ***P*<0.001, ****P*<0.0001).

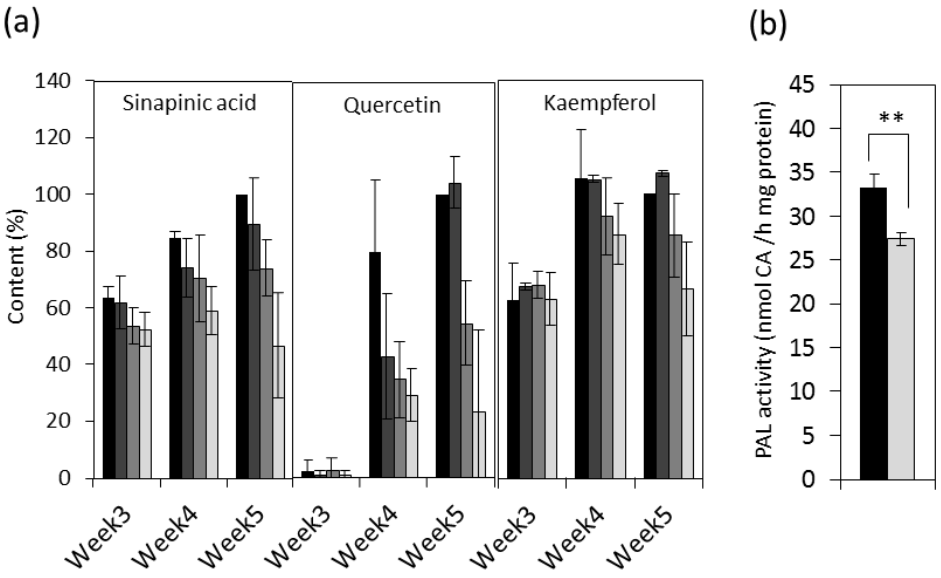

Fig. 2 Quantification of content of phenolic compounds. (a) The total sinapinic acid, kaempferol, and quercetin aglycone contents were quantified after acid hydrolysis of the methanol extracts from the rosette leaves using HPLC. The maximum detected levels of sinapinic acid, kaempferol, and quercetin were 1354, 2540, and 451.9 nmol/ gFW, respectively. (b) Quantification of PAL activity in ambient and 3 ppmv NO-fumigated plants. Error bars, SD from experiments repeated four times. $^{**}P<0.001$; unpaired two-tailed *t*-test. The colour scale from dark to light represents ambient conditions and 0.8, 1.5, and 3 ppmv NO fumigation, respectively.

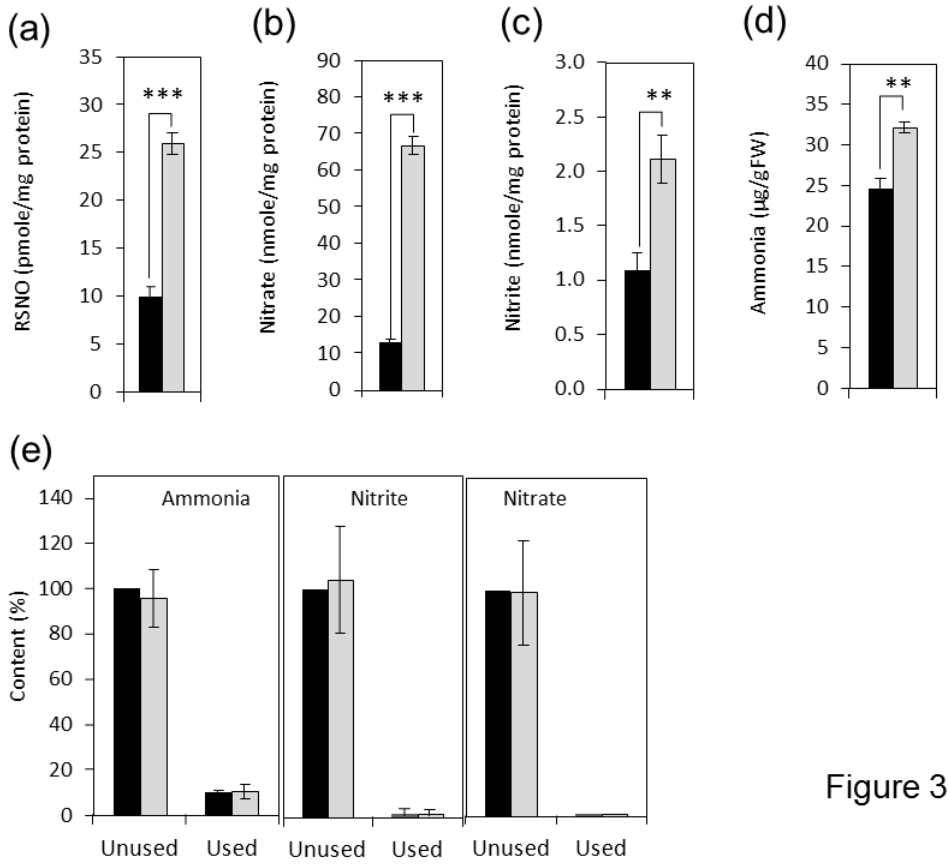

Figure 3

Fig. 3 Analysis of the RSNO and N-containing ion levels in the soil and in plants fumigated with NO. The RSNO (a), nitrate (b), and nitrite (c) levels in four-week-old rosette leaves were estimated using a chemiluminescence detection method. (d) The ammonia contents of four-week-old rosette leaves were determined calorimetrically. (e) The total nitrate, nitrite, and ammonia contents in the extracts of five-week-old unused and used soil samples were determined colorimetrically. The detected values (ambient NO; unused soil) for nitrate, nitrite, and ammonia were 337, 194, and 41.4 mg/kg, respectively and were set to 100%. Error bars, SD from experiments repeated three times. $^{**}P<0.001$, $^{***}P<0.0001$; unpaired two-tailed *t*-test. Black: Ambient NO; Light grey: 3 ppmv NO.

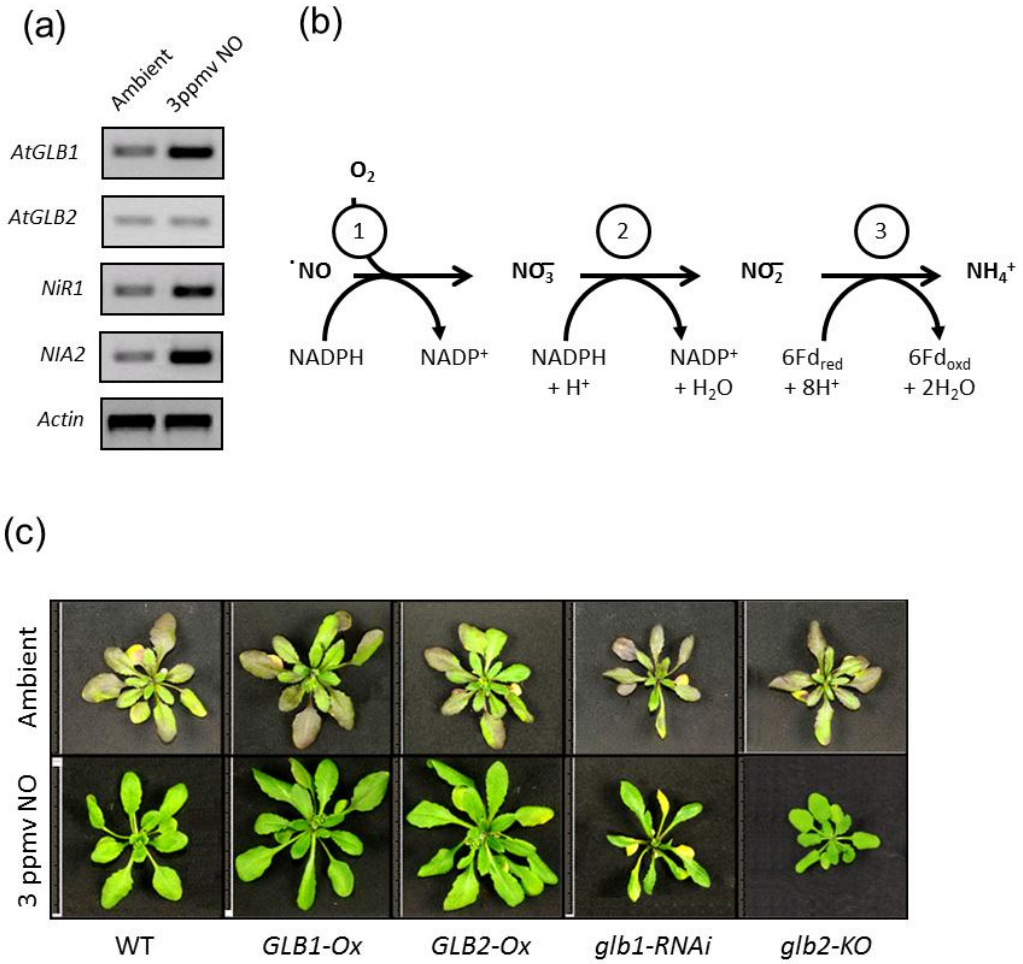

Fig. 4 Transcript analysis after NO fumigation and NO-fixation pathway. (a) Semi-quantitative reverse transcriptase PCR analysis. Agarose gels (1%) loaded with amplified *AtGLB1* (29 PCR cycles), *AtGLB2* (32 PCR cycles), *NiR1* (28 PCR cycles), *NIA2* (26 PCR cycles), and *actin* (control, 32 PCR cycles) gene transcripts. (b) Schematic of the proposed NO-fixation pathway. NO is oxidized enzymatically to nitrate by oxy-non-symbiotic hemoglobin①. In a two-step process, nitrate is reduced to ammonia by nitrate reductase② and nitrite reductase③. (c) Rosettes of 4-week-old plants fumigated with ambient and 3 ppmv NO gas concentrations.

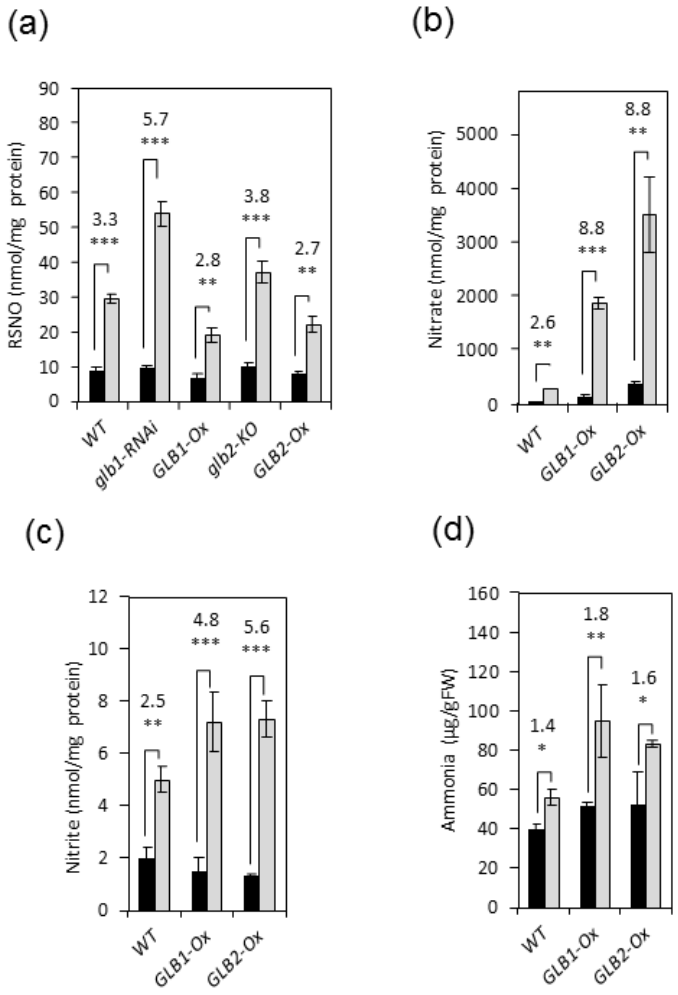

Fig. 5 Analysis of the RSNO and N-containing ion levels in plants fumigated with NO. The RSNO (a), nitrate (b) and nitrite (c) levels in the four-week-old rosette leaves were determined using chemiluminescence detection methods (see methods). Error bars, s.d. from experiments repeated four times. (d) The ammonia contents of the four-week-old rosette leaves were determined calorimetrically. Error bars, SD from experiments repeated three times. Dark: Ambient NO; Light grey: 3 ppmv NO. The number above the bars for each plant line represents the ratio of the estimated quantity for the plants fumigated with 3 ppmv NO gas to that for plants fumigated with ambient NO.

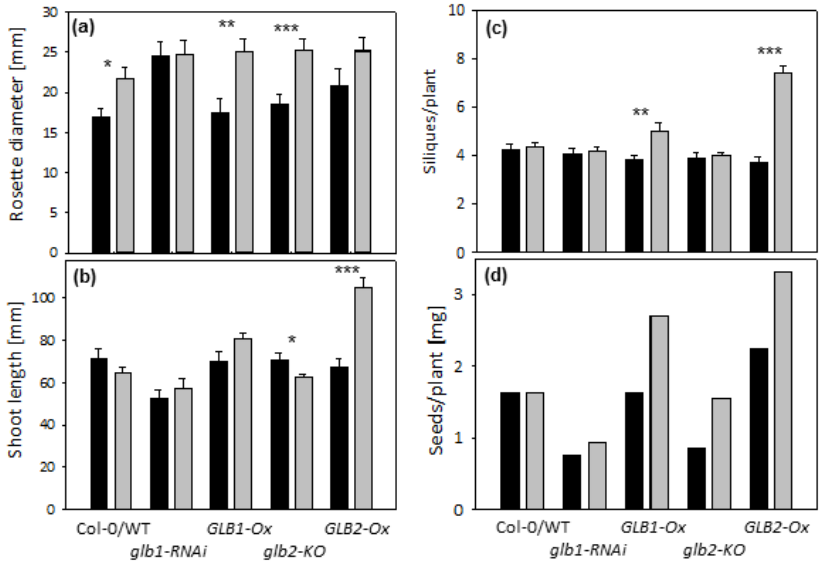

Fig. 6 Phenotypical parameters and N content of hydroponically cultivated *Arabidopsis* plants. Rosette size of 35 day old plants (a), shoot length (b), number of siliques (c) and seed yield (d) in hydroponically cultivated *Arabidopsis* plants with altered *AtGlb1*- or *AtGlb2*-expression. Plants were exposed to ambient (black) and 3 ppmv NO (grey). Data represent means ± SE of 15-20 plants for phenotypical parameters and 5 plants for N content. Asterisks indicate statistical significant differences from WT (Student´s t-test; **P*<0.05, ***P*<0.01, ****P*<0.001).

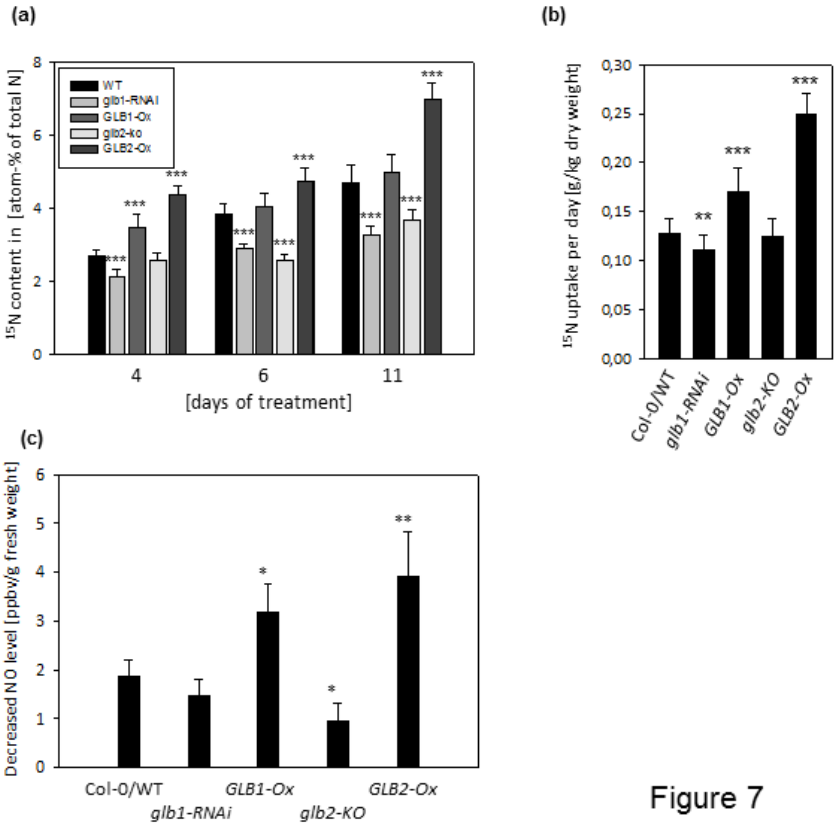

Figure 7

Fig. 7 NO uptake of *Arabidopsis* plants. Plants germinated and grew for twelve days in N-containing hydroponic medium. Afterwards, they were transferred in N-free medium. ^{15}N content was determined in plant exposed to 250 ppbv ^{15}NO for 4, 6 and 11 days (a). The ^{15}N uptake per day was calculated based on the ^{15}N data after four days of ^{15}NO fumigation (b). Data represent means ± SE of 8 plants. (c) Two to four pots with four weeks old *Arabidopsis* plants were placed in a closed reaction chamber and the NO level were measured after 30 min (L1). Then the plants were cut at the soil surface and the NO levels were determined again after 30 min (L2). The difference between both levels (L2-L1) reflects the amount of NO taken up in 30 min by the different genotypes. In all experiments asterisks indicate statistical significant differences from WT (Student´s t-test; **P*<0.05, ***P*<0.01, ****P*<0.001).