Phytoglobin overexpression promotes barley growth in the presence of enhanced level of atmospheric nitric oxide

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Abstract
To investigate the effect of high atmospheric NO concentrations on crop plants and the role of phytoglobins under these conditions, we performed a long-term study on barley ‘Golden Promise’ wild type (WT), class 1 phytoglobin knockdown (HvPgb1.1−) and class 1 phytoglobin overexpression (HvPgb1.1+) lines. Plants were cultivated with nitrogen-free nutrient solution during the entire growth period and were fumigated with different NO concentration (ambient, 800, 1500, and 3000 ppb). Analysis of fresh weight, stem number, chlorophyll content, and effective quantum yield of PSII showed that NO fumigation promoted plant growth and tillering significantly in the HvPgb1.1+ line. After 80 d of NO fumigation, dry matter weight, spikes number, kernel number, and plant kernel weight were significantly increased in HvPgb1.1+ plants with increasing NO concentration. In contrast, yield decreased in WT and HvPgb1.1− plants the higher the NO level. Application of atmospheric 15NO and 15NO2 demonstrated NO specificity of phytoglobins. 15N from 15NO could be detected in RNA, DNA, and proteins of barley leaves and the 15N levels were significantly higher in HvPgb1.1+ plants in comparison with HvPgb1.1− and WT plants. Our results demonstrate that overexpression of phytoglobins allows plants to more efficiently use atmospheric NO as N source.

Keywords: 15N, barley, fumigation, nitrate, nitric oxide, nitrogen, phytoglobin.

Introduction
Nitric oxide (NO) is an air pollutant that contributes to the formation of smog and acid rain together with its oxidation product nitrogen dioxide (NO2). NO also plays an important role in atmospheric chemistry by affecting the production and destruction of tropospheric ozone thereby influencing the oxidation capacity of the atmosphere (Pilegaard, 2013). Since NO was identified as a mediator of defense responses in plants (Delledonne et al., 1998; Durner et al., 1998), many studies have demonstrated its ubiquitous signaling function in different physiological process in plants, including hormonal signaling, germination, flowering, biotic and abiotic stresses (Neill et al., 2002; García-Mata et al., 2003; He et al., 2004; Huang et al., 2004; Sokolovski et al., 2005; Bethke et al., 2006, 2007; Grün et al., 2006; Corpas et al., 2011). Anthropogenic (from combustion of fossil fuels) and biogenic (from soils) NO emissions (Davidson and Kingerlee, 1997; Pilegaard, 2013; Huang and Li, 2014) are the major sources of atmospheric NO. Dependent on location (city–land...
site), soil fertilization, activity of soil bacteria, season of the year, temperature, soil water content, etc., atmospheric NO can reach concentrations up to 100 ppb—in exceptional cases (e.g. smog) up to 200 ppb (Roberts-Semple et al., 2012; Homyak and Sichman, 2013; Huang and Li, 2014; Kramer et al., 2015; Xu et al., 2015). Atmospheric NO has long been discussed as either detrimental or beneficial for plant growth and development. High concentration of NO was found to impair plant growth in several species. In tomato, more than 400 ppb NO treatment caused an inhibition of photosynthesis and a reduction of plant biomass (Capron and Mansfield, 1976; Anderson and Mansfield, 1979; Bruggink et al., 1988). In ryegrass, long-term exposure to NO leads to around 30% reduction of shoot growth (Lane and Bell, 1984). Low concentrations of NO, however, can stimulate plant growth by affecting plant biochemical processes. A shoot biomass increase was observed in Arabidopsis plants exposed to 50 ppb NO (Takahashi et al., 2014), and positive effects on vegetative growth were also found in pea leaf discs and spinach (Leschem and Haramaty, 1996; Jin et al., 2009). The shoot biomass of soil-cultivated spinach plants became significantly increased after treatment with additional low concentrations (200 ppb) of NO. In addition to the atmospheric concentration, the biological effect of NO on plants also depends on exposure time, plant species, and soil fertility (Anderson and Mansfield, 1979; Wellburn et al., 1980). Until now, little is known about the effect of atmospheric NO on important crops, e.g. barley.

Phytoglobins are plant proteins with globular structure containing a heme as a prosthetic group. Phytoglobins were discovered in 1939 by Kubo after spectroscopic and chemical analysis of the red pigment of soybean root nodules (Kubo, 1939). The isolation of the phytoglobin gene from *Trema tomentosa* was the first demonstration of the presence of phytoglobins in a non-nodulating plant (Bogusz et al., 1988). After that, phytoglobins were identified in many evolved and primitive plants, including monocots such as maize, teosinte (Aréchaga-Ocampo et al., 2001), and wheat (Larsen, 2003), dicots such as soybean (Andersson et al., 1996), Arabidopsis (Trevaskis et al., 1997), chicory (Hendriks et al., 1998), and tomato (Wang et al., 2003), and bryophytes (Garrocho-Villegas and Arredondo-Peter, 2008; Vázquez-Limón et al., 2012). The presence of phytoglobins is widespread in the plant kingdom suggesting that these proteins have an important role in the metabolism of plants. The different types consist of phytoglobin class 0 (Pgb0), phytoglobin class 1 (Pgb1), phytoglobin class 2 (Pgb2), symbiotic phytoglobin (Spgb), legphytoglobin (Lb), and phytoglobin class 3 (Pgb3) (Hill et al., 2016). Pgb0 consists of primitive phytoglobins found in algae, bryophytes, and gymnosperms. Spgb is specifically localized in N2-fixing nodules of actinorhizal plants or any other non-leguminous land plant, whereas Lb is specifically localized in N2-fixing nodules (Hill et al., 2016). Pgb1 and Pgb2 are both found in any plant organ of angiosperms. Pgb1 has an extremely high affinity for O2 (Km in the order of 2 nM; Hargrove et al., 2000; Smaghe et al., 2009), while Pgb2 varies between a moderate and high affinity (Km of the order of 100–200 nM; Dordas, 2009; Vigeolas et al., 2011). Pgb3 is structurally similar to the bacterial truncated globins and is found in algae and land plants, with a very low similarity to Pgb1 and Pgb2, and having low affinity to O2 (Km 1500 nM; Watts et al., 2001). The evolution of different types of phytoglobins and new functions have been shown to parallel major transitions in plant evolution (Vázquez-Limón et al., 2012).

Most studies have focused on class 1 and 2 phytoglobins related to their function in NO turnover in response to stress, development, and programmed cell death (Perazzolli et al., 2004; Hebelstrup et al., 2006; Hill et al., 2013; Huang et al., 2014; Mira et al., 2017). In Arabidopsis, overexpression of Pgb1 induces the onset of flowering (Hebelstrup and Jensen, 2008). In barley, overexpression of Pgb1 also leads to changes in development associated with the modulation of NO levels (Hebelstrup et al., 2014). However, the developmental effect of overexpression of phytoglobin in barley was different from that in Arabidopsis (Hebelstrup et al., 2014).

During hypoxia, NO produced in plant tissues can be oxidized to NO3– by oxy-phytoglobin, thereby contributing to increased homeostasis of cellular redox and energy potentials during hypoxia in the biochemical cycle, which is known as the phytoglobin–NO cycle (Igamberdiev and Hill, 2004; Igamberdiev et al., 2006). Since phytoglobins can scavenge endogenous NO via the phytoglobin–NO cycle, it is interesting to investigate possible roles of phytoglobin in plants exposed to NO.

Previously, we reported a NO-fixing function of phytoglobins in Arabidopsis (Kuruthukulangara et al., 2017), which allows the fixation of atmospheric NO into nitrogen metabolites. Overexpressing Pgb1 or Pgb2 resulted in an altered phenotype with increases in rosette size and weight, vegetative shoot thickness, and seed yield compared with WT plants under NO fumigation (Kuruthukulangara et al., 2017). Moreover, the NO-fixing capacity of phytoglobins could also represent a kind of N-recycling process by preventing loss of N due to NO release. Therefore, an obvious step is to investigate the relevance of this mechanism in cultivated plants such as barley.

In this research, we performed a long-term study with the barley cultivar ‘Golden Promise’ wild type, and genetically modified class 1 phytoglobin knockdown (HvPgb1.1−) and overexpression (HvPgb1.1+) lines. The plants were fumigated under controlled environmental conditions with different NO concentration during the entire growth period. We showed that overexpression of the HvPgb1.1 gene promoted barley growth and increased yield after long-term exposure to NO concentrations higher than 800 ppb. Moreover, overexpression of this gene protects plants from toxic effects of high NO concentrations (≥800 ppb). Short-term exposure to naturally occurring levels of 15NO and 15NO2 (90 ppb) demonstrated that both molecules are taken up and can be used as additional N source, but only NO is fixed by phytoglobins. NO-derived nitrogen could be detected in protein, RNA, and DNA. Improving this mechanism through classical breeding methods or biotechnological approaches could pave the way for a new generation of crops that are better able to cope with nitrogen-limited conditions by using atmospheric NO as an additional N source and by reducing loss of N due to NO release.
Materials and methods

Plant growth and NO/NO2 fumigation

Transgenic barley (Hordeum vulgare L. var. Golden Promise) with overexpression of phytoglobin (HvPgb1.1+) and knockdown phytoglobin (HvPgb1.b) lines were generated at Aarhus University as described previously (Høbelstrup et al., 2010, Høbelstrup et al., 2014, Sorensen et al., 2019). Arabidopsis overexpressing phytoglobin 1 (AtPgb1+) or phytoglobin 2 (AtPgb2+), and Arabidopsis with reduced phytoglobin 1 (AtPgb1−) or knocked out phytoglobin 2 (AtPgb2) used in this study have been described previously (Kuruthukulangarakoola et al., 2017).

Barley plants (one plant per pot, size 10×10×11 cm) were grown in a soil matrix composed of Floragard B, Meteorite, and sand (floragard B: vermiculite: sand=2:2:1). Floragard B contains 140 mg 1 N, 80 mg 1 P2O5, and 190 mg 1 K2O. The soil-grown plants were continuously fumigated with NO, starting on the fourth day after germination in gas-tight climate chambers where their internal NO levels were constantly monitored (Supplementary Fig. S1 at JXB online). Inlet air was purified using filter pads in combination with activated-carbon filters and silica particles coated with permanganate (Purex International, Rotherham, UK) (ambient air) and supplemented with different concentrations of NO (800, 1500, or 3000 ppb, Supplementary Fig. S1). NO was obtained from Air Liquide (Düsseldorf, Germany) in cylinders containing 2 or 15% NO in N2. During the experiment, 100 ml modified Hoagland nutrient solution without any N-source (KNO3 and Ca(NO3)2 were replaced by CaCl2 and KCl, respectively) was added to the plants every 2 weeks.

Detailed growth conditions are shown in Supplementary Fig. S1 with a photosynthetically active radiation (PAR: 400–700 nm) <300 μmol m−2 s−1 and diurnal shifts of air temperature (night: 15 °C, 8 h; day: 15–20 °C, 16 h) and humidity (60–90%).

For 15NO15NO2 fumigation, 20-day-old barley (four plants per pot) and 28-day-old Arabidopsis (five plants per pot, size 5×5×5 cm) grown in the soil matrix (floragard B: vermiculite: sand=3:1:1) were used. 15NO15NO2 (99 % atom isotopic enrichment) was obtained from Linde (Pullach, Germany) and diluted to 2% with N2 by Westfalen AG (Münster, Germany). Plants were fumigated with 90 ppb 15NO15NO2 for 7 d during the 12 h of the photoperiod (i.e. 08.00–20.00 h). For controls, 90 ppb natural abundance NO/NO2 was used. Growth conditions were as follows: light—300 µmol m−2 s−1, PAR (400–700 nm); temperature—day: 20 °C (14 h) and night: 16 °C (10 h); relative humidity—80%. In all experiments, the NO levels in chambers were monitored with an AC32M NO analyser (Ansyo, Karlsruhe, Germany).

15NO tracer application

Barley plants were germinated and grown in a matrix composed of vermiculite and sand (4:1, four plants per pot, size 10×10×11 cm). Seven days after sowing, 50 ml nutrient solutions with 0.3 mM 15NO3 was added for each pot every day. The nutrient solution contained 1 mM K2HPO4, 0.5 mM K15NO3 (60 atom % 15N, Sigma-Aldrich, Deisenhofen, Germany), 0.5 mM Ca(NO3)2, 0.9 mM MgSO4, 50 μM Fe-EDTA, 16 μM H3BO3, 0.3 μM ZnSO4, 0.3 μM CuSO4, and 0.4 μM Na2MoO4. Leaf samples were taken after 2, 9, and 12 d for 15N measurements.

Growth and yield parameters

After 20, 30 and 45 d of NO fumigation, at least four plants were used for morphometric measurements (plant height, leaf number, stem number, and plant weight). After 80 d, 15 plants were harvested for the analysis of eight traits: dry matter weight per plant (DWP), plant height (PH), spike length excluding awns (SL), spikes per plant (SP), spike weight (SW), kernel number per plant (KNP), kernel weight (KW), and kernel weight per plant (KWP).

Chlorophyll ratio and chlorophyll fluorescence measurement

An optical sensor (Dualex Scientific-T™, FORCE-A, Paris, France) was used to measure the chlorophyll ratio. The second leaves of 10 plants per accession were measured at the middle surface for both sides. Chlorophyll fluorescence was measured with a MINI-PAM-II Photosynthesis Yield Analyzer (Walz GmbH, Effeltrich, Germany). The effective quantum yield of PSII (ΔF/ΦFm′) was determined according to Genty et al. (1989). ΔF/ΦFm′ was calculated as ΔF/Fm′=(Fm′−F′)/Fm′. F is the fluorescence yield of the irradiation-adapted sample and Fm′ is the maximum irradiation adapted fluorescence yield when a saturating pulse of 800 ms duration is superimposed upon the prevailing natural photosynthetic photon flux density (PPFD). All measurements were performed between 13.00 and 15.00 h.

Nitrate and nitrite in leaves

The total nitrate and nitrite concentration were measured using a Sievers280 nitric oxide analyser (NOA, GE Analytical Instruments, Boulder CO, USA). Leaf proteins were extracted with an extraction buffer (137 mM NaCl, 0.027 mM KCl, 0.081 mM NaH2PO4, 2H2O and 0.018 mM NaH2PO4). One hundred microliters of leaf extract was injected into the purging vessel of NOA containing 3.5 ml of acidified KI/I2 solution (reducing agent) at 30 °C. The recorded mV signals were plotted against a standard curve produced using known concentrations of sodium nitrite solution to quantify the nitrite level. For nitrate quantification, the reducing agent was replaced with vanadium chloride at 95 °C. The recorded mV signals were plotted against a standard curve produced using known concentrations of sodium nitrate solution to quantify the nitrate levels.

Real time PCR

One hundred milligrams of frozen plant material was ground to powder, followed by RNA extraction using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany, cat no. 74904) according to the manufacturer’s instructions. RNA concentration and quality were determined spectrophotometrically (NanoDrop 1000). One microgram of total RNA was used for cDNA synthesis with the Quantitect Rev Transcription Kit (Qiagen, cat no. 205311). A qRT-PCR reaction comprised 10 μl of Sybr Green (Bioline, Luckenwalde, Germany, cat no. QT625–05), 5 μl of ddH2O, 0.5 μl of 10 μM specific primers, and 4 μl of 1:20 diluted cDNA template. Cycling conditions were 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 45 s. Each sample was run in triplicate. GAPDH and actin were used as housekeeping genes. Primers are listed in Supplementary Table S1.

Nitrate and ammonium measurement in soil samples

After totally removing roots, a mixture of 5 g of soil was shaken with 20 ml of 0.01 M CaCl2 for 2 h. After centrifugation for 20 min at 4200 g (Rotanta 460R, Hettich AG, Bäch, Schweiz), the supernatant was filtered using black ribbon filter paper. The concentrations of ammonium and nitrate were determined simultaneously with an N-autoanalyser (Skalar 5100, Skalar Analytic GmbH, Erkelenz), which operates in continuous flow. Specific chemical reactions produce soluble dyes from ammonium or nitrate, respectively, which are photometrically quantified. Ammonium forms a green indophenol dye after the Berthelot reaction with salicylate. Nitrate is first reduced to NO2 and detected as a red-colored azo complex.

DNA, RNA, and protein extraction for 15N measurement

Genomic DNA was extracted with a modified CTAB method according to Krizman et al., 2006. TRizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract RNA from the leaves, following the manufacturer’s instructions. Purified total DNA and RNA were quantified using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For protein extraction, homogenized frozen plant material (400 mg) was mixed using 1 ml extraction buffer (100 mM Tris–HCl pH 8.0, 10 mM EDTA, 1 mM MgCl2, H2O). Homogenate was centrifuged (12 000 g for 20 min at 4 °C) and the supernatant was filtered using a 70 μm nylon membrane. Protein extracts were then desalted using a PD-10 desalting column (GE Healthcare, Freiburg,
Determination of $^{15}$N content in leaves and total N content in soil

Plant and soil material were dried at 60 °C for 48 h and ground to a homogeneous powder using a ball mill (Tissue Lyser II, Qiagen, Venlo, Netherlands). Aliquots of about 2 mg leaf material and 5 mg soil material were transferred to tin capsules (IVA Analysentechnik, Meerbusch, Germany). $^{15}$N abundance and N content were determined with an isotope ratio mass spectrometer (deltaV Advantage, Thermo Fisher, Dreieich, Germany) coupled to an elemental analyser (Euro EA, Eurovector, Milano, Italy).

Isotope ratio MS measurements always require 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), being part of every sequence in intervals, was used. A series of lab standards of different weight were measured to determine isotope linearity of the system. All lab standard measurements were also the base for the calibration of the N content calculation. The lab standard itself was calibrated against several suitable international isotope standards (International Atomic Energy Agency, Vienna, Austria). International and lab isotope standards were also part of every sequence to create a final correction of $^{15}$N covering all $^{15}$N results of this sequence.

Different from solid (plant or soil) samples, aliquots of DNA, RNA, and protein samples, which exist in solution, were transferred to 4×6 mm tin capsules and dried overnight at 60 °C. Volume of sample aliquots was chosen depending on their expected N concentrations to get about 5–20 µg N for each single measurement. In this case BSA was used as the lab standard for calculating $^{15}$N abundance, also in different amounts. BSA and all other isotope standards were applied in solution.

Stomatal conductance

Stomatal conductance (µmol m$^{-2}$ s$^{-1}$) of 20-day-old barley and 28-day-old Arabidopsis leaves was measured with a portable leaf porometer (SC-1 leaf porometer, Decagon Devices, Pullman, WA, USA) during midday (10.00–12.00 h). Measurements were made in the auto mode using the first 30 s of stomatal conductance data to predict the final stomatal conductance under true steady state conditions.

Results

Plant phytoglobins—relationships and expression pattern

In barley, the phyglobin gene ($HvPgb1.1$) was discovered by Taylor’s group (Taylor et al., 1994). By using NCBI BLAST and IPK barley BLAST Server, we could identify another phyglobin gene in barley (Access number: HORVU1HR1G076460.3 in IPK and AK376331.1 in NCBI). A phylogenetic tree was generated with phyglobin proteins of other plant species (Supplementary Table S2) by the neighbor-joining method using MEGA 6.06. Based on the phylogenetic tree (Fig. 1A) and the rules for phyglobin genes (Hill et al., 2016), the new gene was named $HvPgb1.2$. The amino acid sequence alignment analysis revealed a 74.7% similarity of $HvPgb1.2$ to barley $Pgb1.1$ and a 70.1% similarity to Arabidopsis $Pgb1$ (Fig. 1B).

We compared the expression patterns of the $HvPgb1.1$, $HvPgb1.2$, and $HvPgb3$ in different tissues according to the collected data from the morexGenes-Barley RNA-seq Database. In general, the expression levels of $HvPgb1.2$ and $HvPgb3$ were much higher in all plant tissues compared with $HvPgb1.1$ (Fig. 1C). Highest expression levels for $HvPgb1.1$ were found in roots (ROO1 and ROO2), etiolated seedlings (ETI), and shoots (LEA). $HvPgb1.2$ showed a high expression level in senescing leaves (SEN), roots (ROO1 and ROO2), embryos (EMB), shoots (LEA), and epidermal strips (EPI). In contrast, the expression of $HvPgb3$ was relatively balanced in all tissues (Fig. 1C).

Nitric oxide fumigation enhances expression level of $HvPgb1.1$

To analyse whether phyglobin genes respond to NO fumigation, we examined the gene expression level in barley leaves collected from WT plants exposed to different NO concentrations for 20 d. NO fumigation significantly enhanced the $HvPgb1.1$ expression level. Concentrations up to 1500 ppb NO resulted in an 8-fold increase in transcript abundance of $HvPgb1.1$, whereas a concentration of 3000 ppb did not further enhance the expression level. In contrast, the expression of $HvPgb1.2$ and $HvPgb3$ genes was only slightly or not affected by NO fumigation, respectively (Fig. 2). The expression level of $HvPgb1.2$ increased only 1.5-fold in the presence of 800 and 1500 ppb of NO (Fig. 2) and decreased to the control level if plants were fumigated with 3000 ppb. The transcript levels of $HvPgb3$ decreased in the presence of NO concentrations higher than 800 ppb. Although accumulation of $HvPgb1.1$ transcripts was enhanced after NO fumigation, its transcript levels were still clearly lower than the levels of $HvPgb1.2$ and $HvPgb3$ (Fig. 2). These results indicated that $HvPgb1.1$ might play an important role in conditions with enhanced levels of NO.

NO fumigation promotes growth of barley overexpressing $HvPgb1.1$

Since $HvPgb1.1$ might play a role in NO metabolism, growth and development of $HvPgb1.1$ overexpressing ($HvPgb1.1+$) and knockdown ($HvPgb1.1−$) barley lines were analysed in the presence of different NO concentrations. For the NO treatment, air was purified using filter pads in combination with activated-carbon filters and silica particles coated with permanganate (ambient, ca. 5 ppb) and supplemented with 800, 1500, and 3000 ppb of NO. Plants were grown in climate chambers under highly controlled conditions (Supplementary Fig. S1). During the whole growth phase season, nutrient solutions without N were added every 2 weeks. After 20 d of treatment, there were no obvious phenotypic differences—not only among the three different lines, but also among the different NO conditions (Fig. 3A). According to leaf number analysis during the first 16 d of growth, no obvious difference in development of the different barley lines could be seen (Supplementary Fig. S2). The expression levels of $HvPgb1.2$ and $HvPgb3$ in the $HvPgb1.1−$ and $HvPgb1.1+$ plants were...
Phytoglobin-dependent NO-fixation promotes growth

**Fig. 1.** Plant phytoglobins—relationships and expression pattern. (A) Phylogenetic tree of phytoglobins. The tree was constructed with the neighbor-joining method (1000 replications of bootstrap test, JTT model+Gamma distribution using MEGA 6.06. The NCBI accessions of labelled phytoglobins are listed in Supplementary Table S2. (B) Amino acid sequence comparison of HvPgb1.1, HvPgb1.2, and AtPgb1. HvPgb1.2 shared 74.7% similarity to HvPgb1.1 and 70.1% similarity to AtPgb1. (C) Expression patterns of HvPgb1.1, HvPgb1.2, and HvPgb3 in different tissues. Data were collected from morexGenes-Barley RNA-seq Database: HvPgb1.1 (HORVU4Hr1G066200.1), HvPgb1.2 (HORVU1Hr1G076460.3), and HvPgb3 (HORVU0Hr1G021640.3). FPKM, fragments per kilo base million; EMB: 4-day embryos; ROO1, roots from seedlings (10 cm shoot stage); LEA, shoots from seedlings (10 cm shoot stage); INF1, young developing inflorescences (5 mm); INF2, developing inflorescences (1-1.5 cm); NOD, developing tillers, 3rd internode (42 days after planting (DAP)); CAR5, developing grain (5 DAP); CAR15: developing grain (15 DAP); ETI, etiolated seedling, dark condition (10 DAP); LEM, inflorescences, lemma (42 DAP); LOD, inflorescences, lodicule (42 DAP); PAL, dissected inflorescences, palea (42 DAP); EPI, Epidermal strips (28 DAP); RAC, inflorescences, rachis (35 DAP); ROO2: roots (28 DAP); SEN: senescing leaves (56 DAP).
only slightly different from the expression levels in WT plants (Supplementary Fig. S3). Expression of both genes was reduced by ca. 25% in HvPgb1.1+ plants under ambient conditions, whereas no differences could be observed when plants were fumigated with 3000 ppb of NO (Supplementary Fig. S3). At 30 and 45 d after exposure to NO, both stem number and plant weight increased in correlation with the increasing NO concentration in HvPgb1.1+ plants, while at the same time, no significant differences were observed in WT and HvPgb1.1− plants (Fig. 3B). In the presence of 3000 ppb NO, the stem number and weight of HvPgb1.1+ plants increased about 2-fold in comparison with ambient conditions (Fig. 3B). These results demonstrated that NO fumigation significantly promoted growth of HvPgb1.1+ plants, while the same NO concentration had no obvious effect on growth of WT and HvPgb1.1− plants.

NO fumigation increases barley yield in HvPgb1.1 overexpressing lines

To analyse the effect of high atmospheric NO concentrations on the yield of barley expressing different levels of HvPgb1.1, we measured eight biomass and yield parameters including DWP, PH, SL, SP, SW, KNP, KW, and KWP (Fig. 4). After 80 d of NO fumigation no differences in PH and SL were observed, either among the three barley lines (WT, HvPgb1.1+ and HvPgb1.1−), or among the different NO conditions (Fig. 4B). However, fumigation with 3000 ppb of NO significantly increased the DWP, KNP, and KWP level of HvPgb1.1+ plants up to 2-fold. In contrast, the SW and KW levels were decreased with increasing NO concentrations (Fig. 4B). Such a negative correlation between the spike number and kernel weight has often been observed (Dorostkar et al., 2015). In WT and HvPgb1.1− plants, 800 ppb fumigation had a promoting effect on DWP and KNP, while 3000 ppb fumigation led to a reduction of both parameters (Fig. 4B).

Enhanced atmospheric NO affects nitrogen metabolism in barley plants

To analyse whether atmospheric NO affects the nitrogen metabolism, we measured the nitrate and nitrate contents in leaves of WT, HvPgb1.1−, and HvPgb1.1+ plants fumigated for 30 d with 3000 ppb of NO. Under ambient conditions, no significant differences among the three different lines were detected. Fumigation with 3000 ppb of NO, however, caused an increase in nitrite and nitrate content in all three lines (Fig. 5). The nitrate level in HvPgb1.1+ plants was increased 3.5-fold, while in HvPgb1.1− and WT plants the nitrate levels were only 1.4- and 1.7-fold increased, respectively (Fig. 5). Such an increase in N metabolites activated also genes of N metabolism. Especially in HvPgb1.1+ plants, expression of nitrate reductase, nitrite reductase, glutamine synthetase and ferredoxin-dependent glutamate–oxoglutarate aminotransferase was upregulated in NO-fumigated plants in comparison with plant grown under ambient level of NO (Fig. 6). In WT and HvPgb1.1− plants, transcript levels of nitrite reductase and glutamine synthetase were increased by NO treatment.

Since N supply correlates with leaf chlorophyll concentration (Ercoli et al., 1993), we measured the chlorophyll content in leaves after 20 and 35 d of NO fumigation. Twenty days of NO treatment did not affect the chlorophyll content (Fig. 7A). However, since the plants were cultivated already under N-limited conditions, the chlorophyll content decreased over time under ambient conditions from 35 µg cm⁻² (20 d of fumigation) to 22 µg cm⁻² (35 d of treatment). In plants fumigated with NO for 35 d, the chlorophyll content correlated with the NO concentration applied (Fig. 7B). Especially in plants overexpressing HvPgb1.1 and treated with 3000 ppb NO, the chlorophyll content in the older plants (35 d of fumigation) was still as high as in the younger plants (20 d of fumigation). In WT and HvPgb1.1− plants the chlorophyll content was lower in older plants in comparison with younger plants, but with increasing NO concentrations the decrease was less pronounced (Fig. 7B).

The effective quantum yield of PSII (ΔF/F′) gives the actual efficiency of energy conversion in PSII (Björkman and Demmig-Adams, 1995), which is proportional to reduce photosynthetic efficiency and provides a link to diminished photosynthetic carbon fixation (Genty et al., 1989; Wilkinson et al., 2015). Using a MINI-PAM-II Photosynthesis Yield Analyzer, we measured the chlorophyll fluorescence and calculated the effective quantum yield of PSII. Similar to chlorophyll content, the effective quantum yield of PSII (ΔF/F′m) in the HvPgb1.1+ line increased with NO concentration in older plants (35 d of fumigation) (Fig. 7B).

To exclude that the enhanced N content in the HvPbg1.1+ plants was a result of better absorption of N metabolites from the soil, we performed a 15NO3− tracer experiment. Barely plants were grown in a soil-less matrix with additional nutrient solution containing 15NO3−. 15N level in barley leaves after 2, 9, and 12 d were compared among the three lines. We observed no significant differences in 15N levels between WT, HvPgb1.1+, and HvPgb1.1− plants (Fig. 8). We also measured the NO3− and NH4+ content in soil of plants fumigated...
Phytoglobin-dependent NO-fixation promotes growth for 30 d. Compared with the unused bare soil (control), both NO$_3^-$ and NH$_4^+$ contents decreased to a very low level in the soil of plants cultivated under ambient and 3000 ppb NO conditions with no significant difference between the treatments (Supplementary Fig. S4). Soil N content was also decreased after 30 d of treatment in all samples compared with

Fig. 3. Phenotypical differences of barley plants fumigated with different concentrations of NO for 20, 30, and 45 d. Barely WT, HvPgbl.1.1 overexpressing (HvPgbl.1.1+), and knockdown (HvPgbl.1.1−) plants were exposed to ambient, 800, 1500, and 3000 ppb NO and phenotypical parameters were analysed after 20, 30, and 45 d. The plants are shown in (A). The plant height, plant weight, and leaf/stem number are shown in (B). Each bar represents mean ±SE of at least four plants. Different letters indicate significant differences among treatments at $P<0.05$, according to Tukey’s test. Two independent measurements were performed.
Fig. 4. Yield differences of barley after 80 d NO fumigation. (A) The barley plants fumigated after 80 d. (B) The dry matter weight (DWP), kernel weight (KW), plant height (PH), kernel number per plant (KNP), spikes per plant (SP), spike length (SL), spike weight (SW), and kernel weight (KW). Each bar represents mean ±SE (n=15). Different letters indicate significant differences among treatments at P<0.05, according to Tukey’s test. Two independent measurements were performed.
Fig. 5. Nitrite and nitrate content of barley plants after 30 d of NO fumigation. Nitrite and nitrate contents were determined in leaves of barley WT, HvPgb1.1+ and HvPgb1.1− plants fumigated for 30 d with 3000 ppb of NO. Ambient conditions were used as control. The total nitrite and nitrate concentrations were measured using a Sievers280i NO analyser. The number above the bars indicates the ratio of 3000 ppb NO and ambient NO fumigated plants. Each bar represents mean ±SE (n=4). Two independent measurements were performed.

Fig. 6. Transcription levels of HvNR, HvNiR, HvGS2, and HvFd-GOGAT in barley leaves after NO fumigation. Leaf samples were taken after 30 d of NO fumigation. HvGADPH and HvACTIN were used as housekeeping genes. Each bar represents mean ±SE (n=4). Different letters indicate significant differences among treatments at P<0.05, according to Tukey’s test. NR, nitrate reductase; NiR, nitrite reductase; GS, glutamine synthetase; Fd-GOGAT, ferredoxin-dependent glutamate–oxoglutarate aminotransferase. Two independent measurements were performed.
unused soil. Interestingly, in the soil of the HvPgb1.1+ plants fumigated with 3000 ppb, the N content was higher than in the other samples (Supplementary Fig. S4).

Atmospheric NO can be used as nitrogen source

As shown above, long-term exposure to enhanced concentrations of NO promoted growth and yield of HvPgb1.1+ plants by using NO as an additional N source. To further demonstrate the importance of phytoglobin 1.1 in NO fixation and N accumulation in barley, we fumigated 20-day-old plants with 90 ppb of 15NO for 7 d and determined the 15N level in leaves of WT, HvPgb1.1+, and HvPgb1.1−. Although such NO values are not present in the atmosphere continuously, they can be reached in the course of a day (Supplementary Fig. S5) dependent on weather conditions, season of the year, and/or activity of soil bacteria. We observed that in 15NO-fumigated barley leaves, significantly more 15N was detected in all three lines compared with the control fumigation with the natural abundance of 15NO (Fig. 9A). The highest 15N levels were measured in HvPgb1.1+ plants, whereas no difference in 15N levels could be observed between WT and HvPgb1.1− plants. We calculated a daily uptake for HvPgb1.1+ barley of 90 mg N kg⁻¹ dry matter, which is around 2.5 times higher than in WT and HvPgb1.1− plants (Fig. 9B).

DNA, RNA, and protein are typical N-containing biological molecules. To demonstrate that the uptaken 15N has been channeled into the plants’ global N metabolism, we measured
the $^{15}$N levels in DNA, RNA, and protein in leaves. We detected an increased incorporation of $^{15}$N in RNA, DNA, and protein in plants fumigated with $^{15}$NO. Consistent with the $^{15}$N level in leaves, the $^{15}$N content in RNA, DNA, and protein of HvPgb1.1+ plants was much higher than in WT and HvPgb1.1− plants (Fig. 9C–E).

Furthermore, we compared the $^{15}$NO uptake by barley with the uptake rates of Arabidopsis. Consistent with barley, Arabidopsis plants overexpressing class 1 (AtPgb1+) or class 2 (AtPgb2+) phytoglobins contained more $^{15}$N in DNA, RNA, and protein in comparison with WT plants (Supplementary Fig. S6). Interestingly, in Arabidopsis most of the $^{15}$N was found in proteins, whereas in barley only low amounts of $^{15}$N were detected in this fraction. Here most of the $^{15}$N was found in DNA.

Atmospheric NO is mainly taken up by plants through the stomata. To analyse whether phytoglobin-dependent NO uptake was associated with changes in stomatal opening, stomatal conductance was measured in WT and the different transgenic barley as well as in Arabidopsis plants. No differences in stomatal conductance among the three barley lines could be observed (Supplementary Fig. S7). In Arabidopsis, there were also no differences in stomatal opening observed, except in the class 1 knockdown line (AtPgb1−), which had a higher stomatal conductance than all other lines.

Plants can take up not only NO, but also NO$_2$ (Takahashi et al., 2014). To analyse, whether phytoglobins can also promote the uptake of NO$_2$ as N source, barley and Arabidopsis plants with different phytoglobin expression levels were fumigated with 90 ppb of $^{15}$NO$_2$, and $^{15}$N content in leaves was determined after 3 and 7 d of fumigation (Supplementary Fig. S8). In general, the $^{15}$N contents in leaves were up to 6-fold higher when plants were fumigated for 7 d with NO$_2$ in comparison with NO (see Supplementary Figs S6, S8). But phytoglobin overexpression did not enhance the NO$_3$ uptake demonstrating that the phytoglobin-dependent NO-fixation mechanism is NO-specific.

**Discussion**

After the barley phytoglobin 1.1 (HvPgb1.1) gene was cloned in 1994 (Taylor et al., 1994), many studies focused on the function of this gene in barley (Nie et al., 2006; Igamberdiev et al., 2004, 2006). We found another barley class 1 phytoglobin gene, HvPgb1.2 (Fig. 1A, B). The expression pattern of HvPgb1.2 in different tissues differed from that of HvPgb1.1 and in general the expression levels of HvPgb1.2 were much higher than those of HvPgb1.1 (Fig. 1C). This indicates that HvPgb1.2 may have a different function in barley. Since little is known about HvPgb1.2, further research is needed to analyse the function of HvPgb1.2 and its relationship to HvPgb1.1 and HvPgb3.

In this study we observed that the expression level of HvPgb1.1 was strongly increased by NO fumigation (Fig. 2), which is consistent with other studies in different plant species (Ohwaki et al., 2005; Qu et al., 2006; Sasakura et al., 2006; Bustos-Sanmamed et al., 2011; Kuruthukulangarakoola et al., 2017). The enhanced phytoglobin synthesis might be a common mechanism in plant to protect differentiated plant cells from the cellular damage caused by excess NO. But phytoglobins are also involved in plant development. In a previous study, overexpression of HvPgb1.1 in barley showed a delayed growth and flowering phenotype and reduced yield (Hebelstrup et al., 2014). This is different from Arabidopsis, where 5-week-old plants overexpressing class 1 phytoglobin flower earlier and have inflorescences showing more progress than WT plants (Hebelstrup and Jensen, 2008; Hebelstrup et al., 2013). Surprisingly, we did not observe significant developmental differences among WT, HvPgb1.1−, and HvPgb1.1− plants, when growing the plants under a controlled climate (see growth parameter under ambient conditions in Fig. 3 and Supplementary Fig. S2), probably because of differences in the environmental conditions of the two experiments. This is also similar to Arabidopsis, where the effect of phytoglobin overexpression was only very weak in a short-day regime (Hebelstrup and Jensen, 2008) in comparison with a longer day regime (Hebelstrup et al., 2013).

NO promotes barley growth via phytoglobin-dependent NO fixation

The effect of atmospheric NO on barley depends on the NO concentration. Fumigation with 800 ppb of NO had a slight promoting effect on the DWP, KW, and KNP of WT plants (Fig. 4). In contrast, treatment with 1500 and 3000 ppb NO did not increase or even decrease dry matter, kernel, and spike development (Fig. 4). These results indicate that different developmental stages of WT and HvPgb1.1− plants showed different sensitivity to high concentrations of NO, whereas at least no harmful effects were observed during the vegetative phase (Figs 3, 4). The decrease in DWP and KWP at high NO (1500 and 3000 ppb) levels in WT and HvPgb1.1− plants could be explained by the toxic effect of NO at these treatments at <0.05, according to Tukey’s test.
concentrations. Interestingly, when fumigated with 3000 ppb of NO for 9 weeks, the total seed yield of Arabidopsis WT plants increased by 14% in comparison with ambient conditions (Kuruthukulangarakoola et al., 2017), which means that barley is more sensitive to high concentration of NO than Arabidopsis. Maybe the expression level of HvPgb1.1 or the NO-fixing activity of the corresponding protein is too low (Fig. 1C) to protect plants from such a high concentration of NO.

Based on the results for plant weight and stem number, we observed a clear growth promoting effect after 30 and 45 d of NO fumigation in plants, especially in HvPgb1.1 overexpressing barley treated with 3000 ppb of NO (Fig. 3). This demonstrates that phytoglobin enabled better growth especially in the presence of high NO concentrations. However, the promoting effect was not observed in the early stage of development (20 d; Fig. 3). At this time, there was still enough nitrogen supply from the soil. However, after 30 d, nitrogen limitation in soil resulted in a nitrogen-deficient state of the plants (Supplementary Fig. S4). Therefore, the nitrogen supply by phytoglobin-dependent NO-fixation helps to overcome the N deficiency and promote plant growth. Probably a growth promoting effect could also be observed at earlier stages of development when plants are growing on an N-limited condition ab initio. In NO-fumigated HvPgb1.1+ barley, we observed increased stem number, plant weight, spikes and dry matter weight per plant, and kernel number and weight per plant compared with WT and HvPgb1.1− lines (Fig. 4), indicating that the NO promoting effect is phytoglobin-dependent. The increase appears to be more relevant for spike development than for the other parameters. Surprisingly, we could not observe a significant difference in the NO-dependent response between WT and HvPgb1.1− plants. Although HvPgb1.1 expression is induced by NO, the expression level is generally very low in barley leaves (Figs 1, 2). Therefore, the absolute expression level of HvPgb1.1 in WT is not much higher than in the HvPgb1.1− line with knocked down transcript levels (Supplementary Fig. S3).

In NO-fumigated HvPgb1.1+ plants, higher nitrate levels were present compared with WT plants demonstrating that HvPgb1.1 converted NO to nitrate (Fig. 5). Previous studies demonstrated that NO is an important regulator of N assimilation (Frungillo et al., 2014). In spinach, enhanced nitrate assimilation in the presence of 200 ppb of NO contributes to biomass accumulation (Jin et al., 2009). Therefore, the increased...
Phytoglobin-dependent NO-fixation promotes growth

Phytoglobin-dependent NO uptake allows a channeling of atmospheric NO into plant N metabolites

The importance of phytoglobin for NO uptake was also demonstrated in the 15NO labeling experiments. The highest 15N uptake was observed in HvPgb1.1+ plants. In this plant line HvPgb1.1 expression is around 3000-fold stronger than in WT and HvPgb1.1− plants (Supplementary Fig. S3). However, also WT and HvPgb1.1− plants accumulated significant amounts of 15N above the background level (Fig. 9). This is probably due to the NO-induced expression of HvPgb1.1 in these plants (Fig. 2; Supplementary Fig. S3) and due to the deposition of 15NO in and on the plants. In the presence of O2 and H2O this deposited 15NO can be converted to nitrite, thus leading to an increased 15N level. Additionally, HvPgb1.2 might also be involved in NO fixation, which could contribute to the increased 15N level in leaves. At least expression of HvPgb1.2 is slightly increased in the presence of 800 and 1500 ppb of NO (Fig. 2). A possible NO-fixing ability of barley Pgb1.2 protein needs further investigation.

In the 15NO fumigation experiment, plants were fumigated with 90 ppb 15NO, a concentration which can be reached in nature (Corradi et al., 1998; Kuruthukulangarakoolal et al., 2017). The rate of 0.09 g 15N kg−1 leaf dry weight day−1 in HvPgb1.1+ barley is 2.6 times higher than the values in WT and HvPgb1.1− barley leaves (Fig. 9), and is even 2 times higher than the rate in the Pgb2+ Arabidopsis (Supplementary Fig. S6). This indicates that the phytoglobin-dependent NO-fixation mechanism is a quite promising trait in crop plants for using atmospheric NO as nitrogen source. Moreover, the NO-fixing process is also of importance in relation to climatic gas emissions of agricultural soil. Loss of NO from soil means on one side loss of nitrogen and on the other side an increase of the amount of greenhouse gases, such as N2O. The loss of NO could be limited by effective NO fixation. However, as already mentioned above, for practical application, improvement of the NO-fixing process is required. The 15N level in RNA, DNA, and protein demonstrated that the uptake NO was used as an additional N source in plants. The much higher 15N level in RNA, DNA, and protein of HvPgb1.1+ barley leaves further confirmed the importance of HvPgb1.1 for the NO-fixation process (Fig. 9). Consistent with barley, the total leaf 15N level and the 15N level in RNA, DNA, and protein of Arabidopsis leaves were significantly higher in phytoglobin overexpressing plants (Pgb1+ and Pgb2+) in comparison with WT plants (Supplementary Fig. S6). The 15N levels in the DNA, RNA, and protein fractions were not as high as in leaves. This is maybe related to the incorporation of the label into other N-containing compounds, not considered in our comparison. Interestingly, the
\(^{15}\text{N}/\text{N}_{\text{total}}\) ratio in barley protein was much lower compared with \(^{15}\text{N}/\text{N}_{\text{total}}\) in leaves, while the \(^{15}\text{N}/\text{N}_{\text{total}}\) in Arabidopsis protein was more similar to the leaf values (Supplementary Fig. S6). The differences of \(^{15}\text{N}\) levels in the protein fraction of barley and Arabidopsis could be explained by the different developmental stage for Arabidopsis and barley at the time point of the \(^{15}\text{NO}\) fumigation experiment. Young plant still undergo a strong vegetative growth with a need to allocate more N to chlorophyll and other biosynthetic processes, while in 4-week-old Arabidopsis rosettes, more N is demanded for the protein synthesis. In plants, diffusion and biochemical processes during photosynthetic CO\(_2\) assimilation lead to discrimination against heavier \(^{13}\text{C}\) isotope because the key photosynthetic enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase, favors more strongly \(^{12}\text{C}\) (Farquhar et al., 1989). Thus, the lower \(^{15}\text{N}\) level in barley protein could also be the result of the activity of a few key protein biosynthetic enzymes that might prefer \(^{14}\text{N}\) to \(^{15}\text{N}\). In general, it is suggested that fractionation of N during influx into cells is rather weak. In contrast, cytoplasmic pools of both NO\(_3^-\) and NH\(_4^+\) are commonly enriched with \(^{15}\text{N}\), largely due to fractionation during reduction of NO\(_3^-\) to NO\(_2^-\) by nitrate reductase, the reduction of NO\(_2^-\) to NH\(_4^+\) by nitrite reductase, and the subsequent assimilation into amino acids by the glutamine synthetase–glutamate synthase pathway (Needoba et al., 2004). Nitrate reductase and glutamine synthetase both fractionate strongly against \(^{15}\text{N}\) by ca. 15‰ and 17‰, respectively (Robinson, 2001).

As a gaseous molecule, NO is taken up by plant leaves via the stomata. NO also plays a significant role as a signaling intermediate in ABA-induced stomatal closure (Neill et al., 2008; Gayatri et al., 2013). Since the leaf internal NO levels are influenced by the NO–phytoglobin cycle in transgenic Arabidopsis and barley plants with altered phytoglobin expression (Hebelstrup et al., 2012, 2014; Cochrane et al., 2017), the stomatal opening could also be changed, affecting NO diffusion and hence NO fixation. The stomatal conductance in WT and transgenic barley was similar (Supplementary Fig. S7), indicating that the stomatal resistance is not a factor influencing the NO uptake. In Pgb1− Arabidopsis, the higher stomatal conductance did not result in a higher incorporation of \(^{15}\text{N}\) compared with WT Arabidopsis (Supplementary Figs S6, S7). This demonstrates that stomatal opening is not a key factor for the increased uptake of NO in leaves of phytoglobin overexpressing plants.

In conclusion, we demonstrated that the phytoglobin-dependent uptake of NO allows a channeling of atmospheric NO into the plant N metabolism in the crop plant barley (Fig. 10). In general, the effectiveness of this NO-fixation process depends on the availability of NO in the atmosphere, an increased content of phytoglobins and the plant developmental stage triggering the N allocation. Improving the NO-fixing process to enable plants to efficiently use low concentrations of atmospheric NO would be a promising approach allowing plants better growth and development under N-limited conditions. Such an improved NO-fixing capability would go along with improved N-recycling by preventing loss of N due to release of NO. In sum, these positive effects could make the NO-fixing pathway a new economically important breeding trait to enhance the nitrogen use efficiency of crops. However, it also has to be mentioned that NO is an important signaling molecule involved in plant growth, development, and stress response. Overexpression of \(HvPgb1.1\) might affect NO accumulation and signaling. Indeed compromised pathogen defense response or altered response to abiotic stress has already been reported in the \(HvPgb1.1\) overexpressing line (Gupta et al., 2014; Hebelstrup et al., 2014, Montilla-Bascón et al., 2017, Sørensen et al., 2019).

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Growth conditions for plants with long term NO fumigation treatment.
Fig. S2. Number of leaves during plant development.
Fig. S3. Transcription levels of \( \text{HvPgb1.1}, \text{HvPgb1.2}, \) and \( \text{HvPgb3} \) in barley leaves after NO fumigation.
Fig. S4. Nitrate and ammonium concentration and nitrogen content in soil after 30 d NO fumigation.
Fig. S5. NO and \( \text{NO}_2 \) measurements from 13 to 19 February 2019 at the Helmholtz Zentrum München.
Fig. S6. \( \text{^{15}N} \) level in barley and Arabidopsis leaves after \( \text{^{15}NO} \) fumigation.
Fig. S7. Stomatal conductance of barley and Arabidopsis plants.
Fig. S8. \( \text{^{15}N} \) level in barley and Arabidopsis leaves after \( \text{^{15}NO}_2 \) fumigation.
Table S1. Primers used for real-time PCR analysis.
Table S2. Accession numbers of hemoglobin sequences employed in the multiple alignments and used to generate the phylogenetic tree.

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