Journal of Experimental Botany, Vol. 70, No. 17 pp. 4521–4537, 2019
doi:10.1093/jxb/erz249 Advance Access Publication June 27, 2019
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RESEARCH PAPER

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

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Received 30 November 2018; Editorial decision 17 May 2019; Accepted 17 May 2019

Editor: Christine Foyer, University of Birmingham, UK

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 ^{15}NO and $^{15}NO_2$ demonstrated NO specificity of phytoglobins. ^{15}N from ^{15}NO could be detected in RNA, DNA, and proteins of barley leaves and the ^{15}N 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: ^{15}N, 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 oxide (NO_2). 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; Garcia-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 (Leshem 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 phytoglobin 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 N_2-fixing nodules of actinorhizal plants or any other non-leguminous land plant, whereas Lb is specifically localized in N_2-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 O_2 (K_m in the order of 2 nM; Hargrove *et al.*, 2000; Smagghe *et al.*, 2009), while Pgb2 varies between a moderate and high affinity (K_m 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 O_2 (K_m 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 NO_3^- 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 (Kuruthukulangarakoola *et al.*, 2017), which allows the fixation of atmospheric NO into nitrogen metabolites. Overexpressing *Pgb1* or *Pgb2* resulted in an alter phenotype with increases in rosettes size and weight, vegetative shoot thickness, and seed yield compared with WT plants under NO fumigation (Kuruthukulangarakoola *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 ^{15}NO and $^{15}NO_2$ (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/NO_2 fumigation

Transgenic barley (*Hordeum vulgare* L. Var. Golden Promise) with overexpression of phytoglobin (HvPgb1.1+) and knockdown phytoglobin (HvPgb1.1−) lines were generated at Aarhus University as described previously (Hebelstrup *et al.*, 2010, Hebelstrup *et al.*, 2014, Sørensen *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 l^{-1} N, 80 mg l^{-1} P_2O_5 and 190 mg l^{-1} K_2O). The soil-grown plants were continuously fumigated with NO, starting on the fourth day after germination in gas-tight climate chambers whose 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 N_2. During the experiment, 100 ml modified Hoagland nutrient solution without any N-source (KNO_3 and $Ca(NO_3)_2$ were replaced by $CaCl_2$ 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 $^{15}NO/^{15}NO_2$ 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: Meteorite: sand=3:1:1) were used. $^{15}NO/^{15}NO_2$ (99 % atom isotopic enrichment) was obtained from Linde (Pullach, Germany) and diluted to 2% with N_2 by Westfalen AG (Münster, Germany). Plants were fumigated with 90 ppb $^{15}NO/^{15}NO_2$ for 7 d during the 12 h of the photoperiod (i.e. 08.00–20.00 h). For controls, 90 ppb natural abundance NO/NO_2 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 (Ansyco, Karlsruhe, Germany).

$^{15}NO_3^-$ 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 $^{15}NO_3^-$ was added for each pot every day. The nutrient solution contained 1 mM KH_2PO_4, 0.5 mM $K^{15}NO_3$ (60 atom % ^{15}N, from Sigma-Aldrich, Deisenhofen, Germany), 0.5 mM $Ca(NO_3)_2$, 0.9 mM $MgSO_4$, 50 µM Fe-EDTA, 16 µM H_3BO_3, 0.3 µM $ZnSO_4$, 0.3 µM $CuSO_4$, and 0.4 µM Na_2MoO_4. Leaf samples were taken after 2, 9, and 12 d for ^{15}N 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+™, 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 ($\Delta F/F_m'$) was determined according to Genty *et al.* (1989). $\Delta F/F_m'$ was calculated as $\Delta F/F_m'=(F_m'-F)/F_m'$. F is the fluorescence yield of the irradiation-adapted sample and F_m' 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 nitrite and nitrate concentration were measured using a Sievers280i 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 $Na_2HPO_4{\cdot}2H_2O$ and 0.018 mM NaH_2PO_4). One hundred microliters of leaf extract was injected into the purging vessel of NOA containing 3.5 ml of acidified KI/I_2 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 spectrophotmetrically (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 ddH_2O, 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. GADPH 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 $CaCl_2$ 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 salycilate. Nitrate is first reduced to NO_2 and detected as a red-colored azo complex.

DNA, RNA, and protein extraction for ^{15}N 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 Fischer 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 $MgCl_2{\cdot}H_2O$). 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 columns (GE Healthcare, Freiburg,

Germany) according to the manufacturer's instructions. Bradford reagent (Bio-Rad Laboratories, Munich, Germany) was used to determine protein concentration. To measure and plot a standard curve of protein concentration versus absorbance at 595 nm, a series of dilutions of BSA protein standard stock solution was prepared. One milliliter 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 extraction.

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 exists 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 (g_s, mmol 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 phytoglobin 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 phytoglobin gene in barley (Access number: HORVU1Hr1G076460.3 in IPK and AK376331.1 in NCBI). A phylogenetic tree was generated with phytoglobin 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 phytoglobin 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 phytoglobin 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

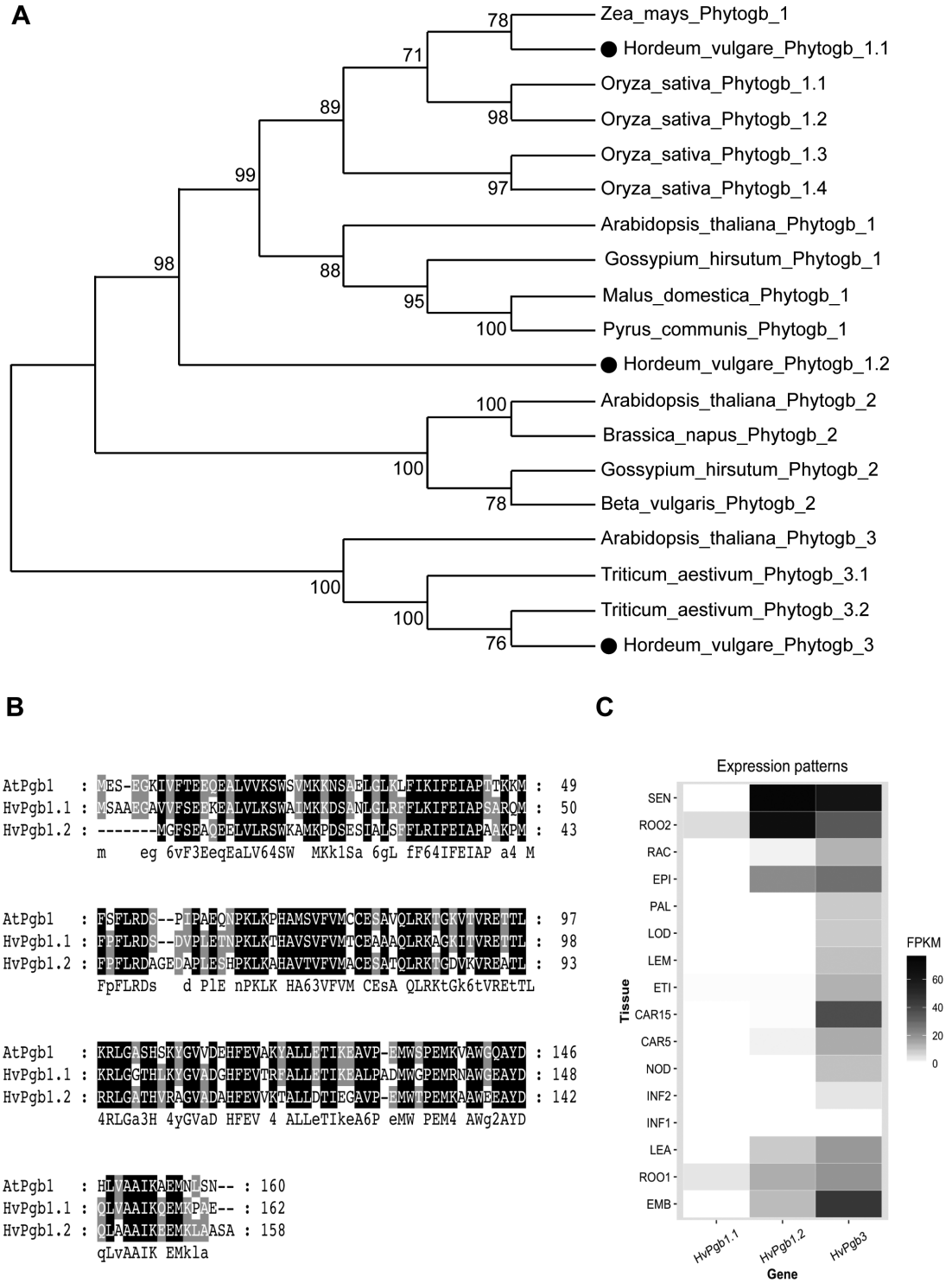

Fig. 1. Plant phytoglobins—relationships and expression pattern. (A) Phylogenetic tree of phytoglobins. The tree was constructed with the neighbor-joining method (1000 replications of bootstrap test, JTT model+Gamma distribution using MEGA 6.06. The NCBI accessions of labelled phytoglobins are listed 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), *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).

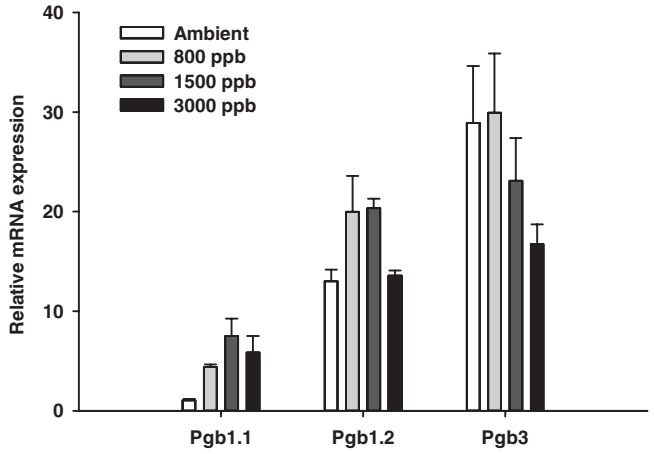

Fig. 2. Transcription levels of of *HvPgb1.1*, *HvPgb1.2*, and *HvPgb3* in wild type barley leaves after NO fumigation. Leaf samples were taken after 20 d of NO fumigation. *HvGADPH* and *Hvactin* were used as housekeeping genes. Each bar represents mean ±SE (n=3). The expression levels of *HvPgb1.2* and *HvPgb3* were normalized to *HvPgb1.1*. Two independent measurements were performed.

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 nitrite and nitrate contents in leaves of WT, HyPgb1.1−, 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 wa lower in older plants in comparison with younger plants, but with increasing NO concentrations the decreased was less pronounced (Fig. 7B).

The effective quantum yield of PSII ($\Delta F/F_m'$) 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 ($\Delta F/F_m'$) 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 $^{15}NO_3^-$ tracer experiment. Barely plants were grown in a soil-less matrix with additional nutrient solution containing $^{15}NO_3^-$. ^{15}N level in barley leaves after 2, 9, and 12 d were compared among the three lines. We observed no significant differences in ^{15}N levels between WT, HvPgb1.1+, and HvPgb1.1− plants (Fig. 8). We also measured the NO_3^- and NH_4^+ content in soil of plants fumigated

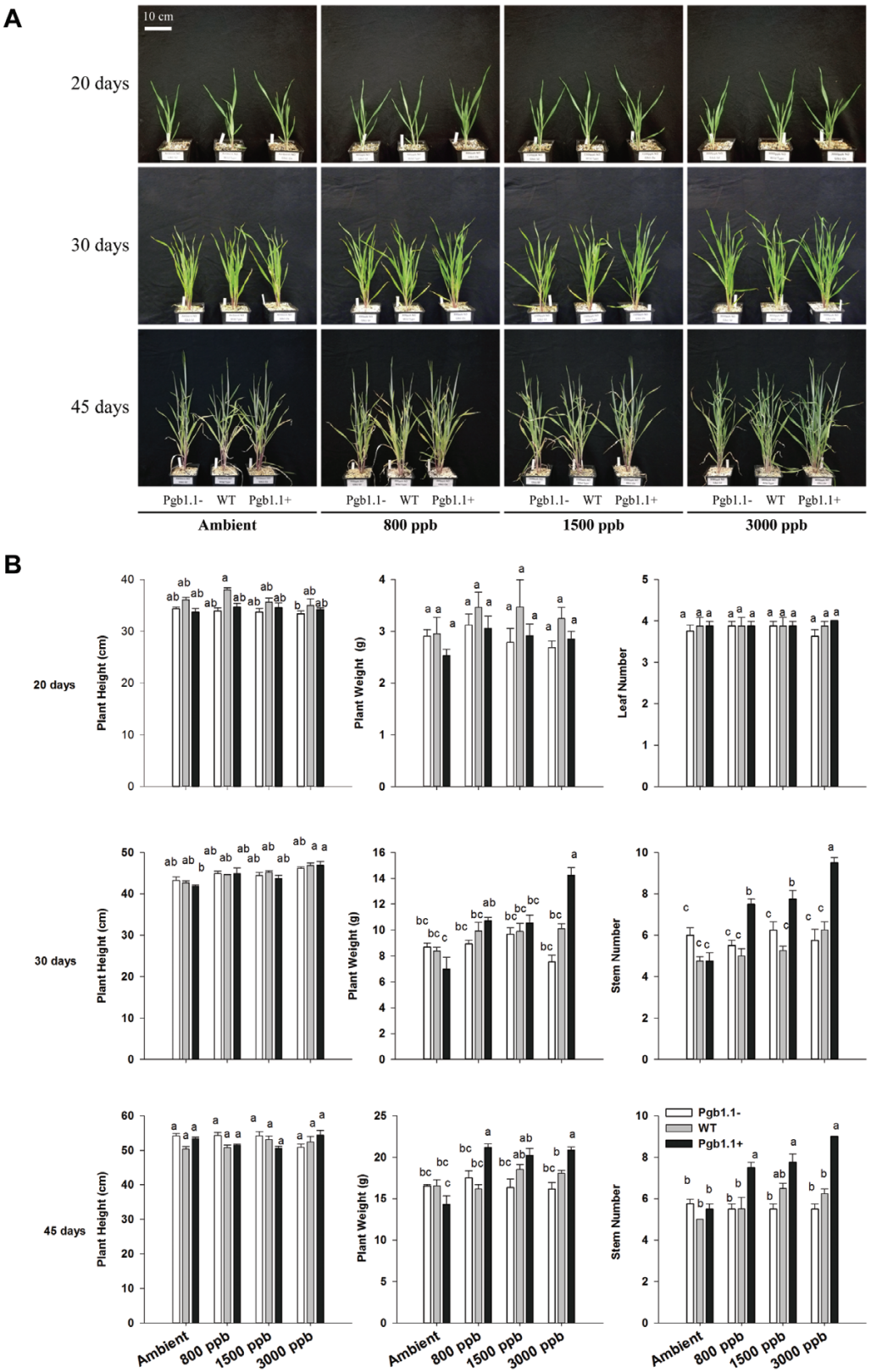

Fig. 3. Phenotypical differences of barley plants fumigated with different concentrations of NO for 20, 30, and 45 d. Barely WT, *HvPgb1.1* overexpressing (HvPgb1.1+), and knockdown (HvPgb1.1–) plants were exposed to ambient, 800, 1500, and 3000 ppb NO and phenotypical parameters were analysed after 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.

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

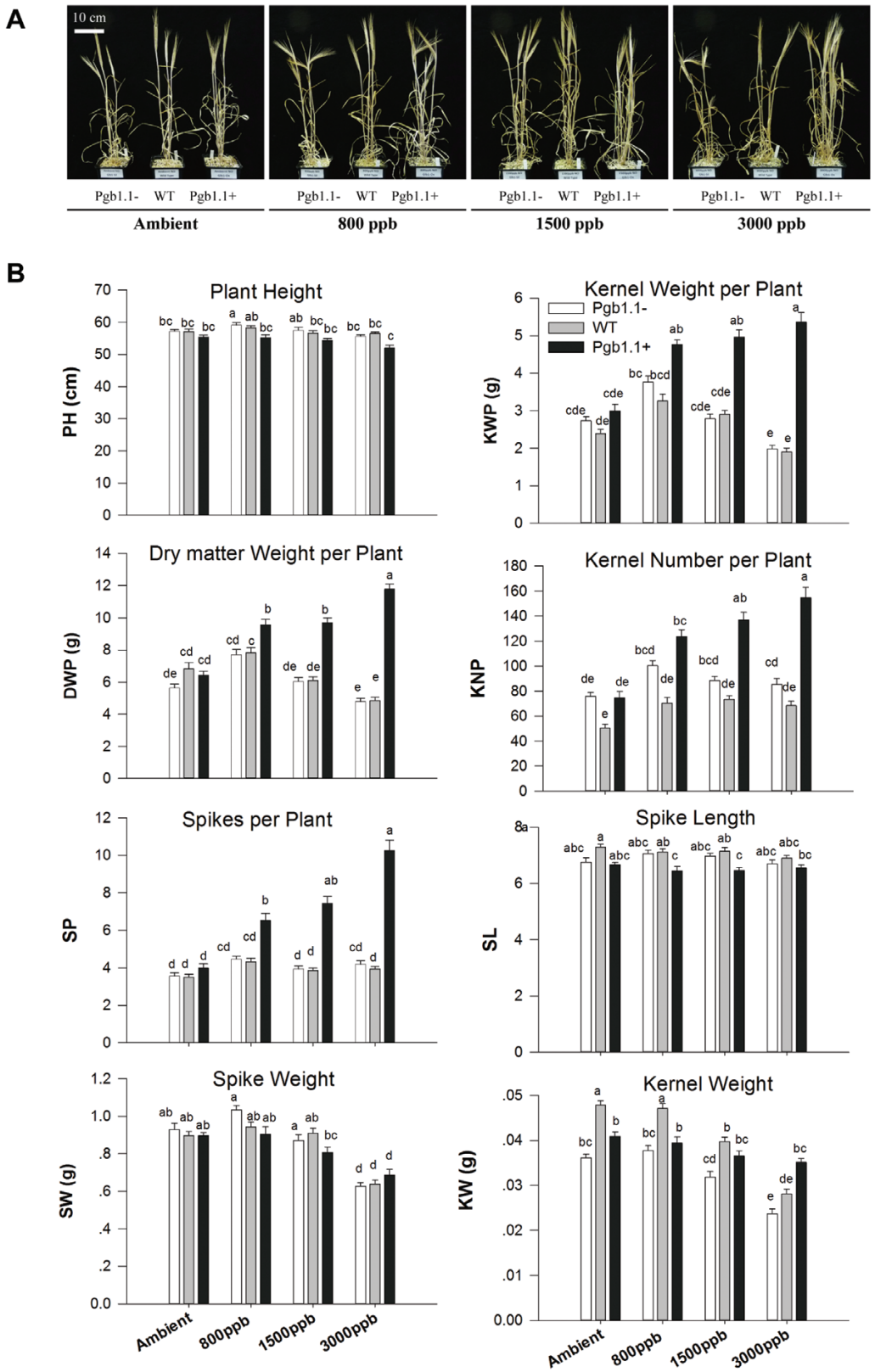

Fig. 4. Yield differences of barley after 80 d NO fumigation. (A) The barley plants fumigated after 80 d. (B) The dry matter weight (DWP), kernel weight (KW), plant height (PH), kernel number per plant (KNP), spikes per plant (SP), spike length (SL), spike weight (SW), and kernel weight (KW). Each bar 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.

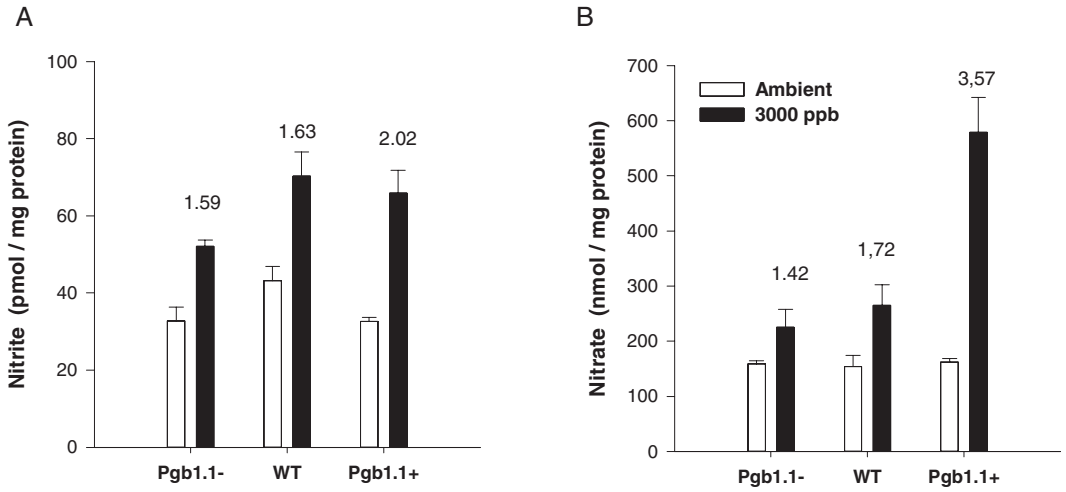

Fig. 5. Nitrite and nitrate content of barley plants after 30 d of NO fumigation. Nitrite and nitrate contents were determined in leaves of barley WT, HvPgb1.1+ and HvPgb1.1– plants fumigated for 30 d with 3000 ppb of NO. Ambient conditions were used as control. The total nitrite and nitrate concentrations 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.

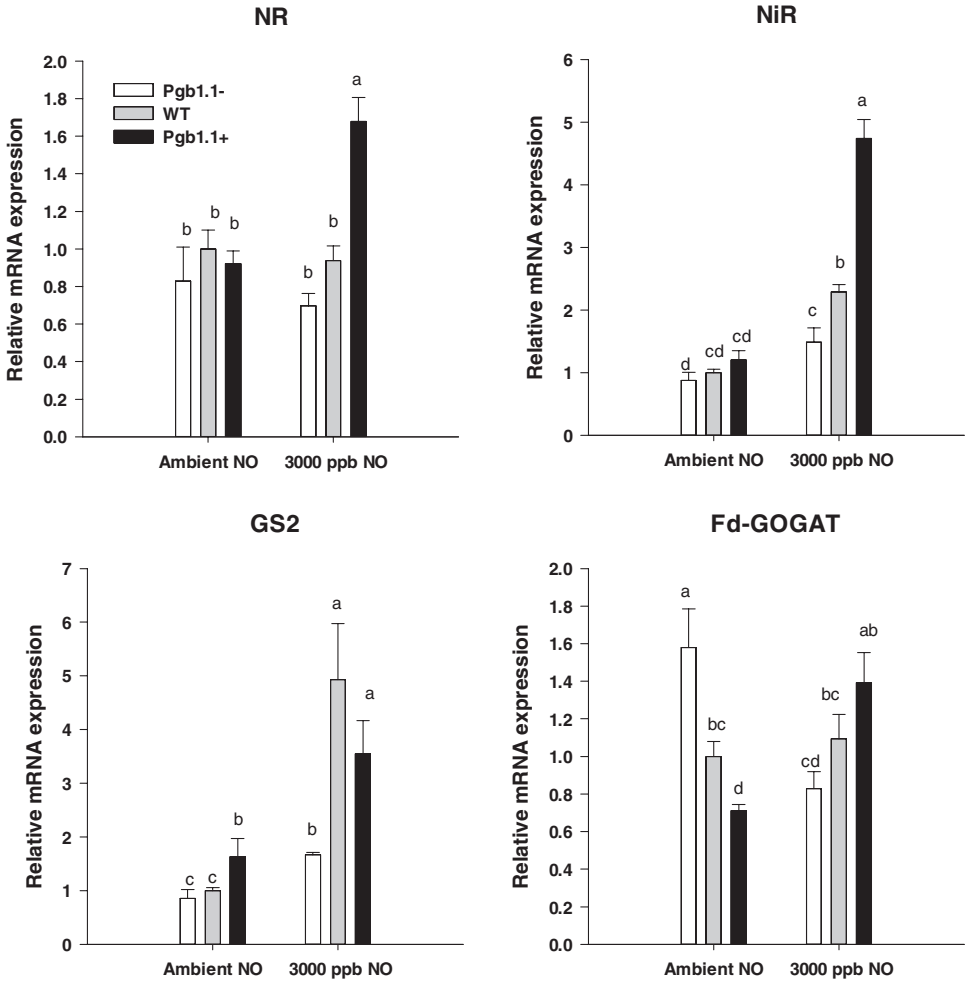

Fig. 6. Transcription levels of of *HvNR*, *HvNiR*, *HvGS2*, and *HvFd-GOGAT* in barley leaves after NO fumigation. Leaf samples were taken after 30 d of NO fumigation. *HvGADPH* and *HvACTIN* were used as housekeeping genes. Each bar represents mean ±SE (n=4). Different letters indicate significant 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.

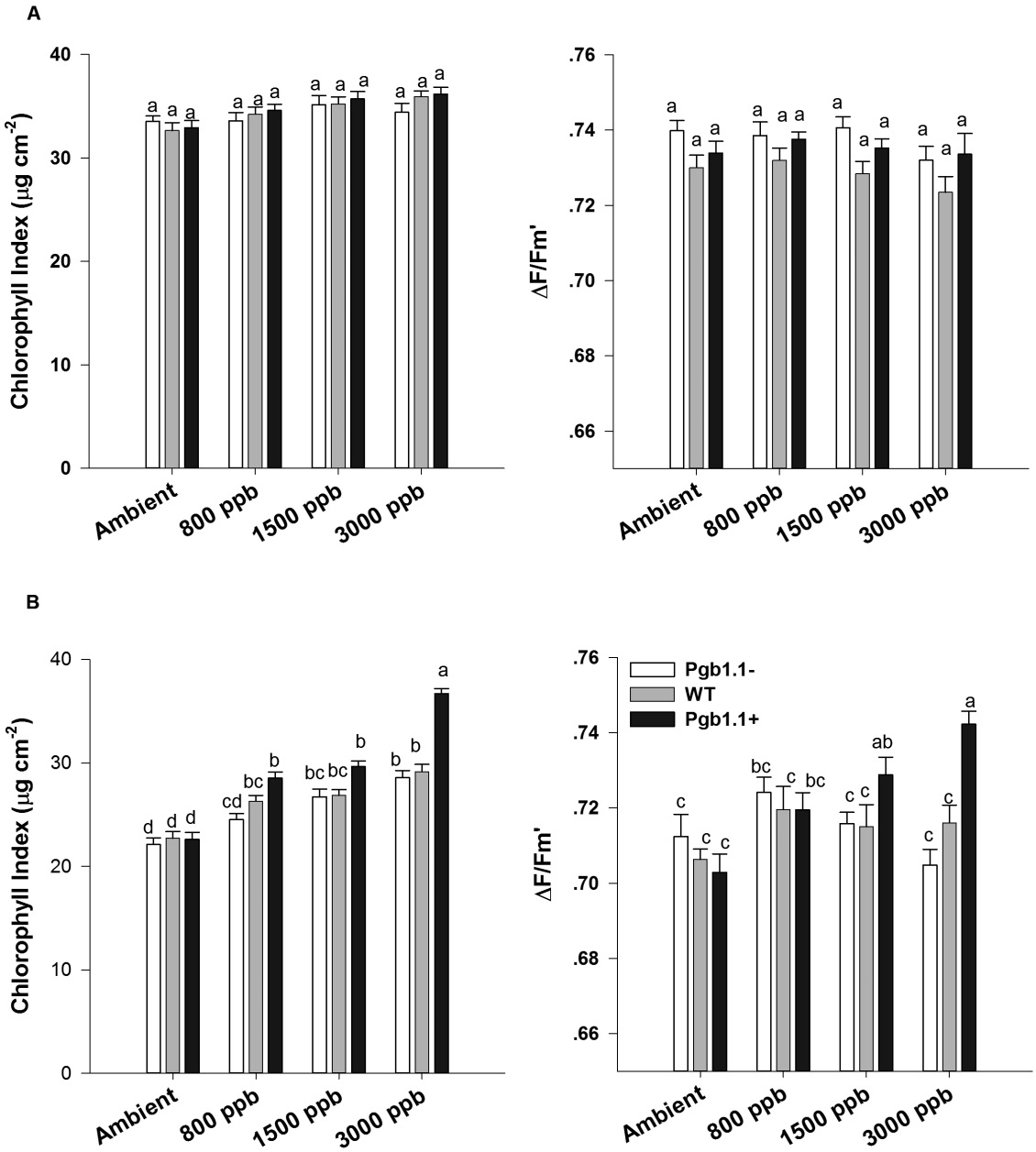

Fig. 7. Chlorophyll index and effective quantum yield of PSII ($\Delta F/F_m'$) of barley leaves after 20 and 35 d of NO fumigation. Chlorophyll index and effective quantum yield of PSII ($\Delta F/F_m'$) were determined in barley WT, HvPgb1.1+, and HvPgb1.1− plants fumigated for 20 d (A) and 30 d (B) with ambient, 800, 1500, and 3000 ppb of NO. Chlorophyll fluorescence was measured using a MINI-PAM-II Photosynthesis Yield Analyzer and the effective quantum yield of PSII ($\Delta F/F_m'=(F_m'-F)/F_m'$) was calculated. 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.

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 ^{15}NO for 7 d and determined the ^{15}N 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 ^{15}NO-fumigated barley leaves, significantly more ^{15}N was detected in all three lines compared with the control fumigation with the natural abundance of ^{15}NO (Fig. 9A). The highest ^{15}N levels were measured in HvPgb1.1+ plants, whereas no difference in ^{15}N 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 ^{15}N has been channeled into the plants' global N metabolism, we measured

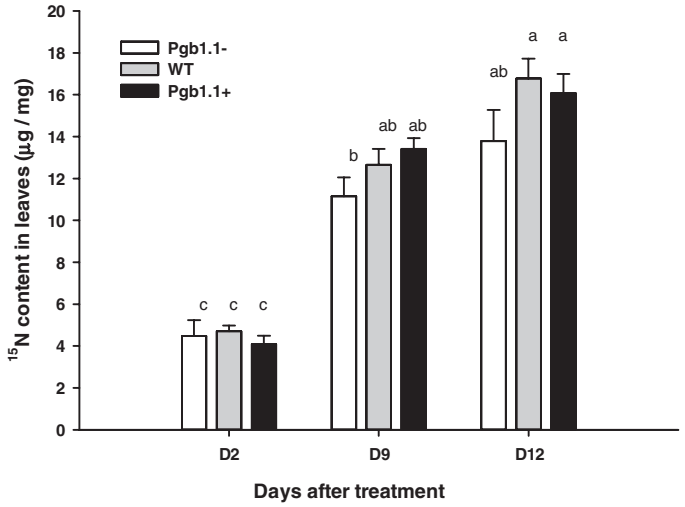

Fig. 8. ^{15}N level in barley leaves after 2, 9, and 12 d under nutrient solutions containing $^{15}NO_3^-$. Plants were grown in soil-less matrix composed of vermiculite and sand. Nutrient solutions with 0.3 mM $^{15}NO_3^-$ were added every day. Leaves were harvested after 2, 9, and 12 d of treatment, dried at 60 °C for 48 h and ground to a homogenous powder. Aliquots of about 2 mg of leaf material were transferred into tin capsules and ^{15}N and ^{14}N content were determined with an isotope ratio mass spectrometer coupled to an elemental analyser. Each bar represents mean ±SE (n=5). Different letters indicate significant differences among treatments at P<0.05, according to Tukey's test.

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 use 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_2 uptake demonstrating that 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− lines, 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

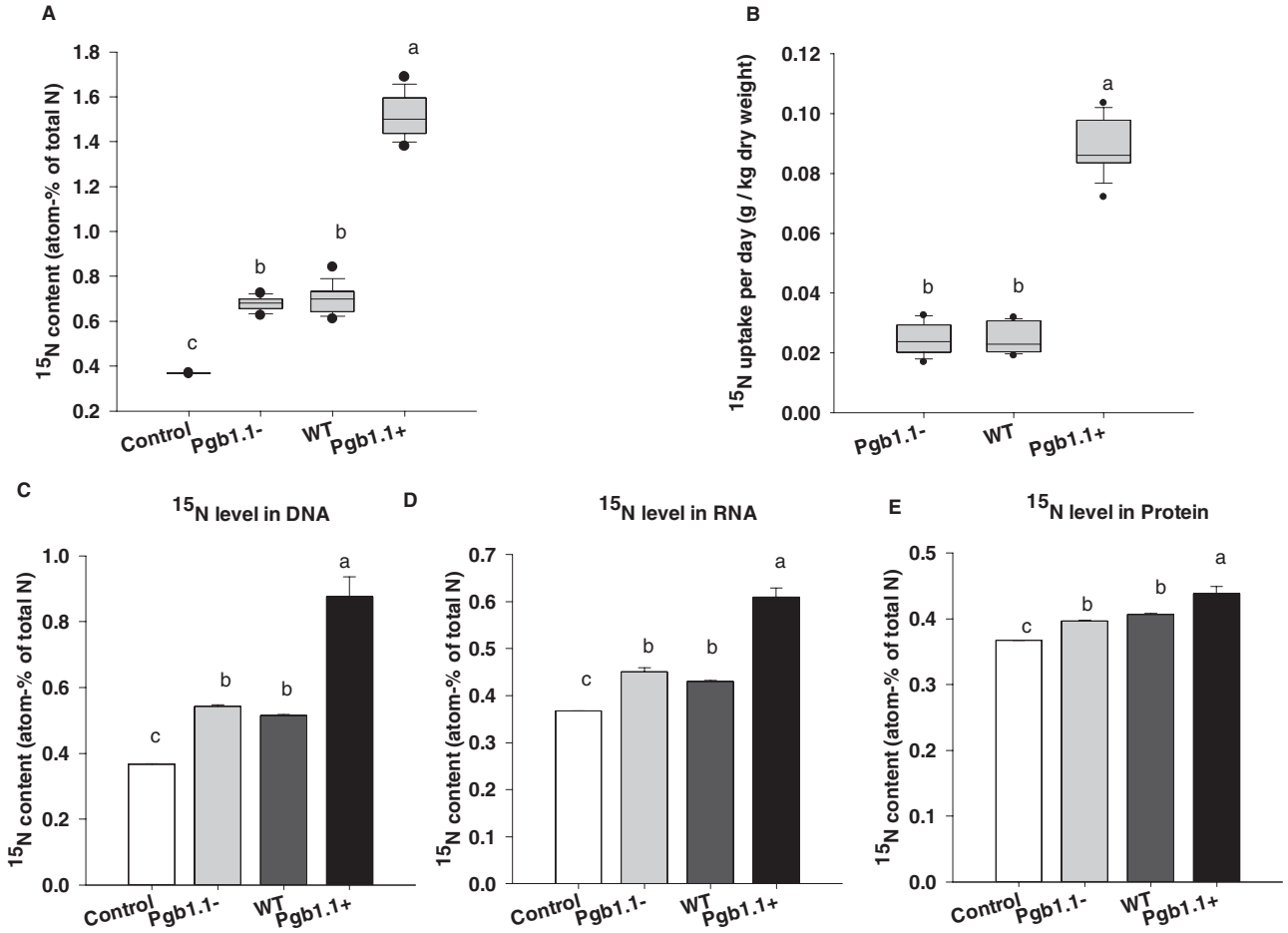

Fig. 9. ^{15}N level in barley leaves, proteins, and nucleic acids after ^{15}NO fumigation. Twenty-day-old barley plants were fumigated with 90 ppb ^{15}NO in the daytime (08.00–20.00 h). (A) ^{15}N content was determined in barley leaves from at least 10 plants after 7 d. (B) The ^{15}N fixed per day was calculated based on the ^{15}N data of (A). (C–E) ^{15}N level in DNA (C), RNA (D) and protein (E) was measured from the extract solutions of barley leaves. Control signifies plants fumigated with 90 ppb NO. For (C–E), each bar represents mean ±SE (n=3). Different letters indicate significant differences among treatments at P<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

nitrate level in NO-fumigated plants might be the result of NO-induced nitrate assimilation. However, no differences in N content were observed in WT plants grown in soil under ambient conditions or fumigated with 3000 ppb of NO (Supplementary Fig. S4) indicating that NO fumigation had no obvious effect on nitrogen uptake from the soil. Moreover, no differences in ^{15}N levels in barley leaves could be detected within the three barley lines grown in the presence of $^{15}NO_3^-$ (Fig. 8). This confirms that phytoglobins did not affect the NO-induced N assimilation. In the presence of 3000 ppb of NO, the higher nitrate level in HvPgb1+ plants (Fig. 5) and the higher N content in HvPgb1+ grown in soil (Supplementary Fig. S4) indicated that the phytoglobin-dependent NO fixation provided significant additional N for plant growth, and the N absorption from soil was reduced. Thus, phytoglobin overexpressing plants benefit from high levels of atmospheric NO, providing significant amounts of N via a NO-fixation to the plants' N assimilation.

But such high NO levels are not expected to occur in the atmosphere. Therefore, a possible practical application of the NO-fixing pathway would require an improvement of the NO-fixation process, e.g. by enhancing the NO binding efficiency and improving the reaction of NO_3^- formation. Moreover, it has to be emphasized that this pathway would be rather of importance under N-limited soil conditions. The N content in plants is closely linked to chlorophyll content and photosynthetic capacity, because N is an essential chemical element of chlorophyll and protein molecules, thereby affecting chloroplast development and chlorophyll accumulation (Bojovic *et al.*, 2005; Bojović and Marković, 2009; Akhter *et al.*, 2016). As a signaling molecule, NO also has a function in plant photosynthesis. As chloroplasts are the main site of C and N metabolism, as well as reactive oxygen species (ROS) production, NO and related species can potentially affect and regulate a wide range of downstream signals through their effects on chloroplasts (Procházková *et al.*, 2013). NO effects are mostly found to impair the photosynthetic apparatus and inhibit photosynthesis (Procházková *et al.*, 2013). Several studies have also demonstrated that NO can prevent chlorophyll losses under stress conditions (Uchida *et al.*, 2002; Shi *et al.*, 2005). In the present work no differences in chlorophyll content and effective quantum yield of PSII ($\Delta F/F_m'$) could be observed after 20 d of exposure to different NO concentrations (Fig. 7A) indicating that NO fumigation has no effect on plant photosynthesis in this early stage of barley development. However, in a later stage under N-limited soil conditions (35 d of fumigation) chlorophyll content and photosynthetic rate is still high in HvPgb1.1+ plants fumigated with NO. This demonstrates the positive effect of N supply due to the enhanced NO-fixing capability of HvPgb1.1+ plants in the presence of high concentrations of atmospheric NO. In WT and HvPgb1.1− plants the chlorophyll content is clearly lower in older plants in comparison with younger plants. However, with increasing NO concentrations the decreased is less pronounced (Fig. 7B). This demonstrates that WT and HvPgb1.1− (knock-down) plants can also fix significant amounts of NO, since both lines contain functional Pgb1.1—of course the levels are lower in comparison with Pgb1.1+. The NO uptake by WT and Pgb1.1− barley plants was also demonstrated by ^{15}NO fumigation experiments (Supplementary Fig. S6). But besides the phytoglobin-dependent NO uptake, it cannot be excluded that traces of NO dissolve in aqueous solutions (e.g. in the apoplast) forming HNO_2, which can also 'enter' N metabolism. In conclusion, the observed higher biomass accumulation in HvPgb1.1+ plants (Fig. 3) is mainly based on the additional N supply through HvPgb1.1-dependent NO-fixation.

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 ^{15}NO labeling experiments. The highest ^{15}N 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 ^{15}N 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 ^{15}NO in and on the plants. In the presence of O_2 and H_2O this deposited ^{15}NO can be converted to nitrite, thus leading to an increased ^{15}N level. Additionally, HvPgb1.2 might also be involved in NO fixation, which could contribute to the increased ^{15}N 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 ^{15}NO fumigation experiment, plants were fumigated with 90 ppb ^{15}NO, a concentration which can be reached in nature (Corradi *et al.*, 1998; Kuruthukulangarakoola *et al.*, 2017). The rate of 0.09 g ^{15}N kg^{-1} leaf dry weight day^{-1} in HvPgb1.1+ barley is 2.6 times higher 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 N_2O. 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 ^{15}N level in RNA, DNA, and protein demonstrated that the uptaken NO was used as an additional N source in plants. The much higher ^{15}N 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 ^{15}N level and the ^{15}N 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 ^{15}N 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

$^{15}N/N_{total}$ ratio in barley protein was much lower compared with $^{15}N/N_{total}$ in leaves, while the $^{15}N/N_{total}$ in Arabidopsis protein was more similar to the leaf values (Supplementary Fig. S6). The differences of ^{15}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}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}C isotope because the key photosynthetic enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase, favors more strongly ^{12}C (Farquhar *et al.*, 1989). Thus, the lower ^{15}N level in barley protein could also be the result of the activity of a few key protein biosynthetic enzymes that might prefer ^{14}N to ^{15}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}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}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}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).

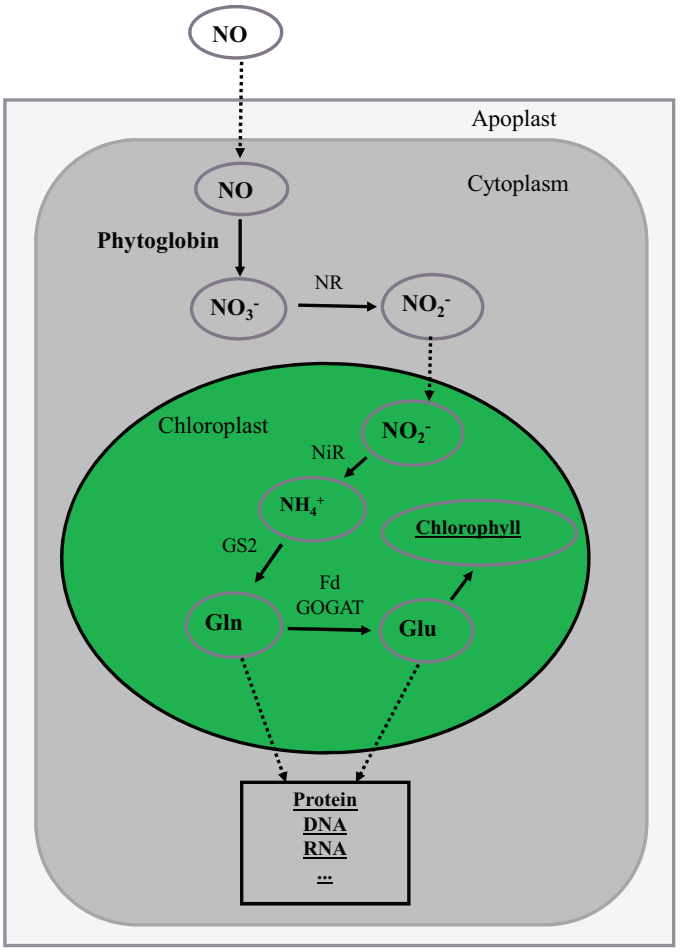

Fig. 10. Proposed model of atmospheric NO entry into plant N metabolism. The model is based on the current work and integrates some information from the literature (Krapp, 2015; Lindermayr and Hebelstrup, 2016). Atmospheric NO can enter the plant cell and first be converted to nitrate by phytoglobins in the cytoplasm. Nitrate is reduced in the cytoplasm to nitrite by nitrate reductase (NR). Nitrite is then transported into the chloroplast and reduced to ammonium by nitrite reductase (NiR). Ammonium is incorporated into glutamine (Gln) and glutamate (Glu) by the GS/GOGAT cycle. Glutamate can be used for chlorophyll synthesis in the chloroplast. Glutamine and glutamate also contribute to synthesis of protein, DNA, RNA, and other N-containing compounds (Krapp, 2015; Lindermayr and Hebelstrup, 2016). Fd-GOGAT, ferredoxin-dependent glutamate–oxoglutarate aminotransferase; GS, glutamine synthetase; NiR, nitrite reductase; NR, nitrate reductase.

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 *HvPgb1.1*, *HvPgb1.2*, and *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 NO_2 measurements from 13 to 19 February 2019 at the Helmholtz Zentrum München.

Fig. S6. ^{15}N level in barley and Arabidopsis leaves after ^{15}NO fumigation.

Fig. S7. Stomatal conductance of barley and Arabidopsis plants.

Fig. S8. ^{15}N level in barley and Arabidopsis leaves after $^{15}NO_2$ 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.

Acknowledgements

We thank Felix Antritter, Elke Mattes, Lucia Gößl and Rosina Ludwig for excellent technical assistance. JZ gratefully acknowledges the financial support from China Scholarship Council (CSC, File No. 201406300083). This work was supported by the Bundesministerium für Bildung und Forschung.

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