TECHNISCHE UNIVERSITÄT MÜNCHEN

Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

Lehrstuhl für Biochemische Pflanzenpathologie

Nitric oxide-fixation by phytoglobin proteins in plants

Jiangli Zhang

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender:	Prof. Dr. Erwin. Grill
Prüfer der Dissertation:	1. Prof. Dr. Jörg Durner
	2. Prof. Dr. Ralph Hückelhoven

Die Dissertation wurde am 16. 04. 2019 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 11. 07. 2019 angenommen.

I. Tables of contents

II. Lists of figures

III. Lists of tables

Lists of figures and tables

IV. Abbreviations

ABA	Abscisic acid
AUX	Auxins
BSA	Bovine serum albumin
cDNA	Complementary DNA
CO	Carbon monoxide
CO_2	Carbon dioxide
CKs	Cytokinins
DNA	Deoxyribonucleic acid
ETs	Ethylene
EDTA	Ethylenediaminetetraacetic acid
Fd-GOGAT	Ferredoxin-dependent glutamate-oxoglutarate-aminotransferase
FPKM	Fragments Per Kilobase Million
GA	Gibberellins
Gln	Glutamine
Glu	Glutamate
GS	Glutamine synthetase
GSH	Glutathione
GSNO	S-Nitrosoglutathione
KO	knock-out
Km	Michaelis constant
N	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NiNOR	Nitrite-NO reductase
NiR	Nitrite reductase

Abbreviation

NO	Nitric oxide
NO_2	Nitrogen dioxide
NO_x	Nitrogen oxides
NOA	Nitric oxide analyzer
NOD	Nitric oxide degrading dioxygenase
NOS	Nitric oxide synthase
NR	Nitrate reductase
O_2	Oxygen
PCR	Polymerase chain reaction
ppb	Parts-per-billion
PPFD	Photosynthetic photon flux density
Pgb	Pgb
PS II	Photosystem II
PTM	Posttranslational modification
qPCR	Real time quantitative PCR
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
RSNO	Nitrosothiol
SE	Standard error of the mean
SNP	Sodium nitroprusside
UV	Ultraviolet
WT	Wild type

V. Summary

Nitric oxide (NO) is naturally present in the atmosphere as part of earth's nitrogen cycle and is regarded as a molecular signal in plant, which plays significant role in the regulation of several biological processes. Phytoglobins are ubiquitously distributed in plants and can metabolize NO into nitrate during hypoxic stress.

In this research, we demonstrated that phytoglobin-dependent NO-fixation results in enhanced nitrogen meboblism and better growth for hydroponic *Arabidopsis* under high concentrations (3000 ppb) of NO fumigation. Such NO-fixation allows a channeling of atmospheric NO into the plant N metabolism and results in a decreased atmospheric NO level.

The NO-fixation were also studied in the crop plant barley. We performed a long-term study with barley "Goden Promise" wild type, class 1 phytoglobin knockdown (Pgb1.1-) and overexpression (Pgb1.1+) lines fumigated with different NO concentration during the whole growth period. Analysis of fresh weight, stem number, chlorophyll content, and the effective quantum yield of PSII showed that NO fumigation promoted plant growth and tillering significantly in the HvPgb1.1+ line. After 80 days 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 levels. Application of atmospheric ^{15}NO and $^{15}NO_2$ demonstrated NO-specificity of phytoglobins. ^{15}N of ^{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 to HvPgb1.1- and WT plants. These results demonstrate that overexpression of phytoglobins allows the plants more efficiently using atmospheric NO as N source.

Summary

The plant-based NO uptake could lower the concentration of atmospheric NOx, which has a beneficial effect on air quality and human health. Thus, the uptake capacity of NO and NO_2 were analyzed in different species of city trees. We found that the NO uptake capacity in different plant species has a positive correlation with leaf moisture content. Besides, overexpression of phytoglobins significantly enhanced the NO uptake capacity in *Arabidopsis*, barley and poplar, which provides a potential biotechnological application to improve the NO uptake capacity in city trees.

1 Introduction

1.1 NO signaling in plants

NO is an important signalling molecule with diverse physiological functions in plants. Since NO was identified as mediator of plant defense responses in plants (Durner *et al*., 1998; Delledonne *et al*., 1998), the functions of NO in plants have been widely studied over the past decades and a significant amount of evidence demonstrated the involvement of NO in the regulation of several biological processes (Neill *et al*., 2002; Garcia-Mata *et al*., 2003; He *et al*., 2004; Huang *et al*., 2004; Bethke *et al*., 2006, 2007; Grün *et al*., 2006; Corpas *et al*., 2011). In this part, we introduce the biosynthesis and homeostasis of NO in plant and summarize the function of NO in plant growth and development, biotic and abiotic stress, and hormonal signalling.

1.1.1 Biosynthesis and homeostasis of NO in plants

Plants have various pathways for NO synthesis, which can be can be classified as either reductive pathway or oxidative pathway (Figure 1). The reductive pathways dependent on nitrite as a primary substrate, while the oxidative pathways depend on L-Arginine, hydroxylamine or polyamines as substrates (Gupta *et al*., 2011a, Figure 1).

The best-characterized production pathway for NO in plants is the NAD(P)H-dependent nitrate reductase (NR) pathway. NR is localized in cytosol and catalyzes the reduction of nitrate to nitrite. This enzyme is encoded by two homologous genes NIA1 and NIA2 (Wilkinson and Crawford, 1993) in *Arabidopsis*, and can also catalyze the reduction of nitrite to NO via the reaction: $NAD(P)H + 3H_3O^+ + 2NO_2^- \rightarrow NAD^+ + 2NO + 5H_2O$. Since the discovery that plant NR could produce NO both under in vitro and in vivo conditions (Harper, 1981), a great deal of

evidences have indicated this enzyme as one of the major plant biosynthetic sources of NO (Rockel *et al*., 2002; Meyer *et al*., 2005). NR-mediated NO production is involved in response to various abiotic and biotic factors, such as fungal plant pathogens (Yamamoto-Katou *et al*., 2006; Shi and Li, 2008; Srivastava *et al*., 2009), osmotic stress (Kolbert *et al*., 2010) water stress (Sang *et al*., 2008), and hypoxia (Benamar *et al*., 2008 Blokhinaand and Fagerstedt, 2010). Nitrite-derived NO production was also determined in membrane fractions isolated from tobacco (Nicotiana tabacum) roots (Stöhr *et al*., 2001) Nitrite, produced by apoplasmic plasma membrane-bound NR, is substrate for NiNOR. NiNOR is bound to the plasma membrane of roots and lead to the NO release at the apoplasmic side of the membrane. The root specific plasma membrane-bound NR:NiNOR system has been suggested to be involved in the sensing of nitrate availability in the soil (Meyer and Stöhr, 2002). Furthermore, NiNOR mediated NO production also plays a role in the regulation of root infection by mycorrhizal fungi (Moche *et al*., 2010)

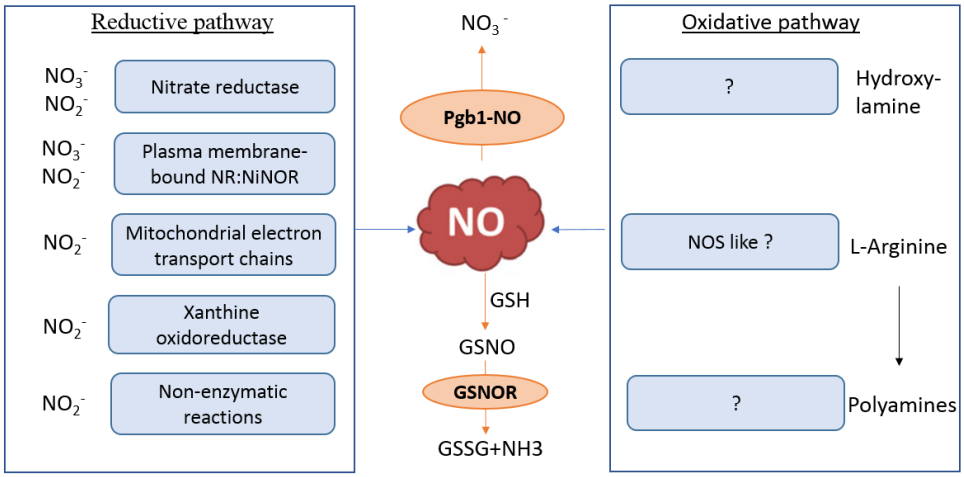


Figure 1 Overview of NO biosynthesis and homeostasis in plant cells.

The oxidative pathway involves a NO synthase (NOS)-like enzyme and two other ways of NO production using polyamines and hydroxylamines as substrates. The reductive pathway of NO synthesis includes: nitrate reductase pathway, plasma membrane nitrate reductase (NR)/ nitrite-NO reductase (NiNOR) system, mitochondrial electron transport chain, xanthine oxidoreductase and a non-enzymatic way of NO formation under acidic pH or through the reduction of NO2- by carotenoids. NO can react with reduced glutathione (GSH) to form S-nitrosoglutathione (GSNO),

which, in turn, can be converted into oxidized GSSG and ammonia by the action of GSNO reductase (GSNOR). Phytoglobins (Pgbs), can scavenge NO in presence of oxygen to produce nitrate. Modified from Arc *et al*., 2013.

Another nitrite-derived reduction occurs in the mitochondrial inner membrane, probably via cytochrome c oxidase and/or reductase. Nitrite is the substrate and NAD(P)H provides electrons via ubiquinone and the mitochondrial electron transport chain. Mitochondrial nitrite reduction produces small amounts of ATP during anoxia (Stoimenova *et al*., 2007).

Nitrite reduction to NO can also be catalyzed by the peroxisomal enzyme xanthine oxidoreductase (XOR). Under anaerobic conditions, XOR can reduce nitrite to NO, using NADH or xanthine as reducing substrate. The XOR mediated NO production has been demonstrated to be involved in phosphate deficiency stress (Wang *et al*., 2010).

In addition, nitrite-derived NO production can be produced through non-enzymatic reactions. For instance, an increase in cellular NO levels was demonstrated under acidic conditions (Bethke *et al*., 2007; Freschi *et al*., 2010), and the light-mediated reduction of nitrite to NO by carotenoids has been reported (Neill *et al*., 2008).

Production of NO via the oxidative pathway is based on the existence of NOS-like activity in plants, which was first found in animals. In animals, NO synthase (NOS) is the enzyme that generates NO in an oxidative pathway using arginine as substrate and producing NO and citrulline in the presence of O_2, whereas NAD(P)H acts as an electron donor. NOS-like activity has been found in plant chloroplasts (Jasid *et al*., 2006) and peroxisomes (Ribeiro *et al*., 1999; Barroso *et al*., 1999; Corpas *et al*., 2009). (NOS)-like activity is involved in the induction of cadmium accumulation and cadmium-induced programmed cell death (Besson-Bard *et al*., 2009; De Michele *et al*., 2009, Ma *et al*., 2010), pathogen signaling induced by specific elicitors (Delledonne *et al*., 1998; Asai and Yoshioka 2009; Besson-Bard *et al*., 2008), mediation of protective responses against UV-B

radiation (Tossi *et al*. 2009), ABA-induced stomatal closure (Guo *et al*., 2003; Bright *et al*., 2006) and root development (Wang *et al*., 2010). However, the existence of NOS-like activity in plants is exclusively supported by biochemical and pharmacological evidence since a canonical NOS gene or a mutant deficient in NOS-like-dependent NO production has not been identified in higher plants yet. Recently, the 1000 plant genome project 1KP international consortium, including the expression analysis in plants and algae, have depicted an embryonic picture of the NOS presence in photosynthetic organisms, concluding that no NOS gene is present in land plant genomes (Jeandroz *et al*., 2016). Besides the NOS-like activity, it was also suggested that polyamine oxidases and copper containing amine oxidases participate in oxidative NO production (Tun *et al*., 2006; Wimalasekera *et al*., 2011). However, the biochemical mechanisms are still not clear.

NO homeostasis is relying on the biosynthesis, but also the buffering and scavenging of NO. NO can react with reduced glutathione (GSH) to form S-nitrosoglutathione (GSNO), which, in turn, can be converted into oxidized GSSG and ammonia by the action of GSNO reductase (GSNOR) (Liu *et al*., 2001). GSNO is considered a cellular reservoir of NO and its abundance influences the activity of enzymes and transcription factors via nitrosylation. Besides, phytoglobins (Pgbs), a kind of plant globular proteins that can scavenge NO in presence of oxygen to produce nitrate and play a significant role in the NO homeostasis.

1.1.2 NO function in plant development

As a signaling molecular, NO is thought to modulate a variety of developmental processes. In this part, we discuss the role of NO plays in dormancy and germination, root growth and formation, leaf senescence, flowering and fruit ripening.

NO can efficiently break the dormancy, promote seeds germination, and play a pivotal role in sensing environmental conditions appropriate for seed germination (Kopyra and Gwóźdź, 2003; Krasuska *et al*., 2015, Bethke *et al*., 2004, 2006, 2007; Beligni and Lamattina, 2000). NO is produced rapidly after seed imbibition and promotes germination by inducing the expression of the abscisic acid 8-hydroxylase gene, CYP707A2, and stimulating ethylene (ET) production (Bethke *et al*., 2007; Yang *et al*., 2006). Moreover, enhanced expression of gibberellic acid 3 oxidase genes by NO was observed in dormant seeds whereby these genes play an important role in breaking dormancy (Liu *et al*., 2010).

NO has been reported to regulate lateral root formation (Correa-Aragunde *et al*., 2004, 2008), primary root growth (Fernández-Marcos *et al*., 2011), adventitious roots formation (Pagnussat *et al*., 2002, 2004) and root hair development (Lombardo *et al*., 2006). Auxin plays central role in modulating root architecture. NO is thought to act as a downstream messenger in auxin signaling (Chen *et al*., 2010). Further, NO can indirectly increase auxin levels by reduceing auxin degradation by inhibiting IAA oxidase activity and acts positively on auxin signalling through S-nitrosylation of the auxin receptor F-box protein TIR1 (Terrile *et al*., 2012). Moreover, NO is able to induce lateral root formation even in the absence of auxin treatment (Correa-Aragunde *et al*., 2004).

The body of evidences reveal that NO acts as a negative regulator of leaf senescence in several plant species. In pea leaves, it was found that NO donor inhibited ET biosynthesis and thus decreased ET level and ultimately inhibited senescence in pea leaves (Leshem and Haramaty, 1996). In rice leaves, NO mediates inhibition of senescence by increasing superoxide dismutase activity and plays a protective role in methyl jasmonate-induced senescence (Hung and Kao, 2003, 2004). In *Arabidopsis*, NO-deficient mutants were more prone to senescence as compared to wild type plants and massive upregulation of senescence-

associated genes resulted in early senescence (Du *et al*., 2014). These studies demonstrated that NO possibly acts as an anti-senescence agent.

NO is also involved in plant flowering. NO was found to delay floral transition in *Arabidopsis* (He *et al*., 2004). The expression of MADS box transcription factor, FLOWERING LOCUS C (FLC), a key repressor of flowering is enhanced by NO (Kolbert *et al*., 2011; Astie *et al*., 2011). In contrast, AtNOA1 mutants (loss of function of NOS-like activity) shows reduced expression of FLC and enhanced expression of floral promoter CONSTANS results in early flowering (He *et al*., 2004; Crawford *et al*., 2006)

NO participates in the fruit ripening. NO fumigation suppressed respiration and ET production and thus leading to a delay in ripening of commercial fruits (Leshem and Pinchasov, 2000; Singh *et al*., 2009; Manjunatha *et al*., 2010, 2012). In sweet pepper, it has been demonstrated that NO content diminishes during ripening, whereas other elements of the RNS metabolism change following patterns, such as an increase of protein nitration and SNO content accompanied by a decreased S-nitrosoglutahione reductase activity (Chaki *et al*., 2015; Rodríguez-Ruiz *et al*., 2017).

1.1.3 Crosstalk between NO and hormones

NO is one of the major players in plant signaling networks. Emerging evidences support that NO interplays with signaling pathways of auxins (AUX), cytokinins (CK), abscisic acid (ABA), gibberellins (GA), ET and other plant hormones to regulate plant metabolism, growth, and development (Freschi, 2013; Sanz *et at*., 2015; Nawaz *et al*., 2017; Sami *et al*., 2018). Generally, NO interplay with other hormonal signals through three ways: i. NO act as upstream signal of hormonal; ii. NO act as downstream signal of hormones; iii. NO-dependent post-translational modifications (PTMs) in biosynthesis, distribution, degradation, and conjugation of elements involved in hormone transport and signaling (Freschi, 2013).

Synergistic effects of NO and auxin have been observed during the regulation of a series of plant development and stress responses, including root organogenesis (Pagnussat *et al*., 2002, 2003, 2004; Lanteri *et al*., 2006), gravitropic responses (Hu *et al*., 2005), root nodule formation (Pii *et al*., 2007), root responses to iron deficiency (Chen *et al*., 2010), cell division and formation (Ötvös *et al*., 2005). In most cases, NO was shown to function as downstream of auxins, apparently through linear signaling pathways. NO production was increased after exogenous auxin application (Pagnussat *et al*., 2002; Correa-Aragunde *et al*., 2004; Hu *et al*., 2005; Lombardo *et al*., 2006) or in auxin overproducer mutants (Chen *et al*., 2010). However, no stimulation or weak stimulation in NO production by auxins was also reported in some particular experimental conditions or cell types (Tun *et al*., 2001; Guo *et al*., 2003), suggesting that the auxin-dependent NO production may occur exclusively under specific temporal and spatial contexts (Hu *et al*., 2005).

It has been shown that NO and CKs are intricately interconnected to regulate leaf senescence, photosynthesis, cell division and differentiation, and drought stress (Shen *et al*., 2013; Simontacchi *et al*., 2015). CKs can increase NO production. Several studies reported about rapid and dose-dependent increases in NO production triggered by CKs in both plant cell cultures (Tun *et al*., 2001; Carimi *et al*., 2005) and intact seedlings (Tun *et al*., 2008; Shen *et al*., 2013). Besides, potential action of CKs in scavenging NO produced under dark conditions was also found (Xiao-Ping and Xi-Gui, 2006).

As important “stress-related” molecules, NO and ABA intensively crosstalk during certain signaling cascades triggered by environmental stresses, such as water limitation and UV-B radiation, which ultimately leads to the induction of plant adaptive responses, such as stomatal closure and antioxidant defenses (Neill *et al*., 2008; Tossi *et al*., 2009; Hancock *et al*., 2011). During the regulation of stomatal movements, NO apparently acts downstream of ABA and upstream of cytosolic calcium in the ABA-dependent signaling cascade leading to the up-

regulation of the crassulacean acid metabolism and does not participate in the ABA-independent pathway (Freschi *et al*., 2010). Besides, it was also demonstrated that NO can regulate ABA level via enhancing the transcript and protein levels of the ABA 8'-hydroxylase gene CYP707A2, a key enzyme in ABA catabolism (Liu *et al*., 2009; Arc et *al*., 2013).

NO influences several plant developmental events in which GA play crucial roles, such as seeds germination, hypocotyl elongation, photomorphogenesis, primary root growth, reorientation, and growth of pollen tubes (Beligni and Lamattina, 2000; Prado *et al*., 2008; Tonón *et al*., 2010; Lozano-Juste and Leon, 2011). During these responses, NO has been described to act upstream of GA (Bethke *et al*., 2007), regulating both GA biosynthesis and transduction (Lozano-Juste and Leon, 2011).

As important gas molecules, NO and ET play significant role in fruit ripening and leaf/flower senescence. A large number of reports on the interaction between NO and ET suggest an antagonistic relationship between these two gaseous molecules (Leshem *et al*., 1998; Lamattina *et al*., 2003; Manjunatha *et al*., 2010). NO was demonstrated to inhibit ET production and action in fruit ripening and leaf/flower senescence (Leshem *et al*., 1998; Manjunatha *et al*., 2010). Additional studies revealed that exogenous application of NO, either by direct fumigation or by means of NO donors, delays senescence of both vegetative and reproductive organs by negatively regulating a number of elements involved in ET production (Leshem and Haramaty, 1996; Leshem *et al*., 1998; Wills *et al*., 2000; Zhu *et al*., 2006; Liu *et al*., 2007; Manjunatha *et al*., 2010, 2012). Recent studies have revealed that the inhibition of fruit ET production by NO may be attributed to a reduction in the transcript level and activity of key ET biosynthetic enzymes (Manjunatha *et al*., 2010).

1.1.4 NO function in biotic and abiotic stress

Plants are continuously exposed to a wide range of adverse environmental conditions, including drought, salinity, heavy metals, nutrient deficiencies, and pathogens, among other factors, which usually limit agricultural production considerably. NO has been regarded as an important endogenous signaling molecule in the adaptation of plants to various biotic and abiotic stresses (Durner *et al*., 1998; Delledonne *et al*., 1998; Arasimowicz and Floryszak-Wieczorek, 2007; Lindermayr *et al*., 2010; Fancy *et al*., 2017).

NO acts as a stress-coping factor in plants. Similar as ROS, the production of NO was induced after both abiotic stress and biotic stress stimulation. In soybean and tobacco cell, a rapid NO burst was found after 1h treatment with incompatible P. syringae (Delledonne *et al*., 1998). In pelargonium leaves, a transient NO burst is also observed among the earliest responses to wounding (Arasimowicz *et al*., 2009). In *Arabidopsis*, drought and salt stresses are also suggested to induce NO production, which activates cellular processes that afford some protection against the oxidative stress under these conditions (Neill *et al*., 2008). In wheat, the NR-mediated NO burst was found to maintain root function and enhance antioxidant enzyme activities under Al toxicity (Sun *et al*., 2014)

NO can interact with plant hormonals to help plants to adapt stress, such as the crosstalk of with ABA and CKs in drought stress, which has already been discussed in 1.2.3. Besides, the NO-meditated PTMs were also shown to regulate plant stress responses (Lindermayr *et al*., 2010; Begara-Morales *et al*., 2016).

1.2 Effect of NO and NO_2 on plant growth

In atmospheric chemistry, NOx is a generic term for the nitrogen oxides that are most relevant for air pollution, namely NO and NO_2. NO and its oxidation product NO_2 are involved in many environmental effects, including global warming, formation of smog, acid rain, and depletion of the ozone layer (Figure 2, Singh

and Agrawal, 2008; Thomson *et al*. 2012; Kanter *et al*. 2013). In plants, not only NO, but also NO_2 has been widely regarded as signal molecules, which play significant role in plant growth and development (Simontacchi *et al*., 2015; Takahashi *et al*., 2005, 2014). Plants themselves can produce and emit NO and NO_2 (Chen *et al*. 2012; Klepper, 1979, 1990; Dean and Harper, 1986). Besides, atmospheric NO and NO_2 can be taken up by plants. Moreover, there is also NO and NO_2 exchange from soils, which involves both microbial activity and chemical reactions (Pilegaard 2013; Vinken *et al*. 2014) where nitrous oxide (N_2O) chemistry also contributes (Figure 2, Hu *et al*. 2015).

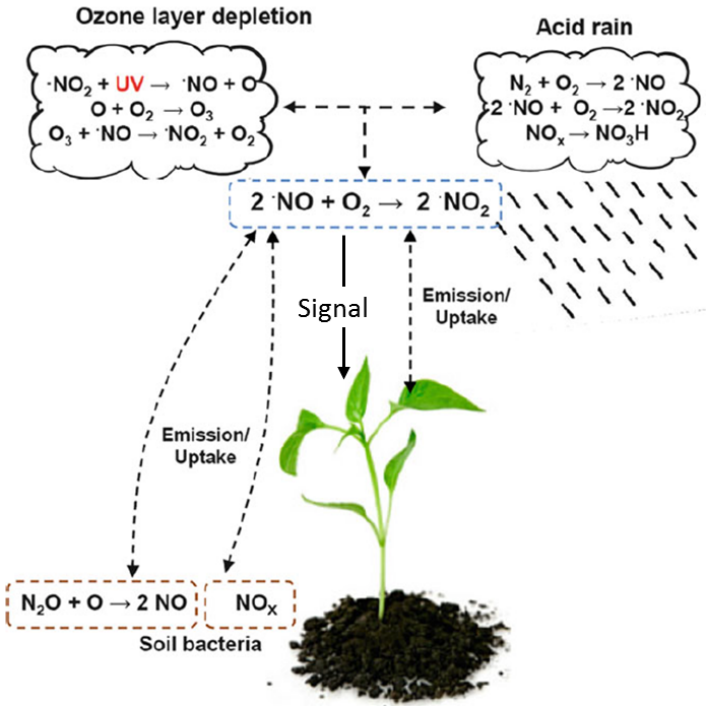


Figure 2 Simple model of NO/NO_2 emission/uptake among plants, atmosphere, and soil bacteria.

In the atmosphere several chemical reactions take place contributing to the nitric acid rain and ozone (O_3) layer depletion through the photolytic nitrogen dioxide (•NO_2) cycle. Acid rain takes place as a consequence of the formation of nitric acid through a series of reactions which involve nitrogen oxides (•NO and •NO_2). Both plants and soil bacteria can contribute by emission/uptake to the NO/NO_2 atmospheric pool of nitrogen oxides (NOx). As molecular signal, NO and NO_2 also play significant role in plant growth and development. Bacterial action in the soil can release nitrous oxide (N_2O) to the atmosphere where it can react with atomic oxygen to form •NO. Modified from Corpas *et al*., 2016.

1.2.1 Effects of atmospheric NO and NO_2 on plant growth and development

Atmospheric NOx has long been discussed as either detrimental or beneficial for plant growth and development (Capron and Mansfield, 1976; Sandhu and Gupta, 1989; Wellburn, 1990; Saxe, 1994). High concentrations of NOx were found to impair plant growth in several species. In tomato, more than 400 ppb NO treatment caused an inhibition in photosynthesis and a reduction in plant biomass (Capron and Mansfield, 1976; Anderson and Mansfield, 1979; Bruggink *et al*., 1988). In *Lolium perenne*, long-term exposure to 400 ppb NO leads to 32–39% reduction in shoot growth (Lane and Bell, 1984). In *Arabidopsis*, more than 500 ppb NO_2 fumigation leading to a decrease in chlorophyll content and photosynthetic rate and caused injury (Xu *et al*., 2010).

Low concentrations of NOx, however, can stimulate plant growth by affecting plant biochemical, physiological and growth aspects. 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 found in pea leaf discs and spinach (Leshem and Haramaty, 1996; Jin *et al*., 2009). In spinach, the shoot biomass of soil cultivated spinach plants became significantly increased after treatment with additional low concentrations (200 ppb) of NO. Moreover, the photosynthetic rate of leaves is increased in NO-treated plants, indicating that enhanced biomass accumulation is based on NO-induced increase of photosynthetic activity.

Exogenous NO_2 fumigation at ambient concentrations can nearly double the total leaf area, nutrient uptake and shoot biomass in plants fed root N (Takahashi *et al*., 2005). Similar results have been reported in various plant species, including *Arabidopsis* and various horticultural species (Ma *et al*., 2007; Adam *et al*., 2008; Takahashi *et al*., 2008, 2013; Xu *et al*., 2010). Except the atmospheric concentration, the biological effect of NOx on plants also depends on exposure time, plant species, and soil fertility (Anderson and Mansfield, 1979; Wellburn *et al*., 1980).

All these studies in different plant species demonstrate the positive effect of NO/NO_2 on plant growth and development under available concentrations. However, the molecular mode of action underlying these effects has often remained elusive. Lindermayr and Hebelstrup (2016) suggested the possible function of these molecules either as signaling which affect N uptake through root system and promote activity of plant hormone, or NOx can be directly used as N source for plant growth (Figure 3). There is no doubt that both NO and NO_2 can act as a molecular signal and regulate plant growth and development. However, different to NO_2, which can directly react with H_2O in plant cell forming nitrate and nitrite, NO is converted to nitrate in the presence of Pgbs (Figure 3).

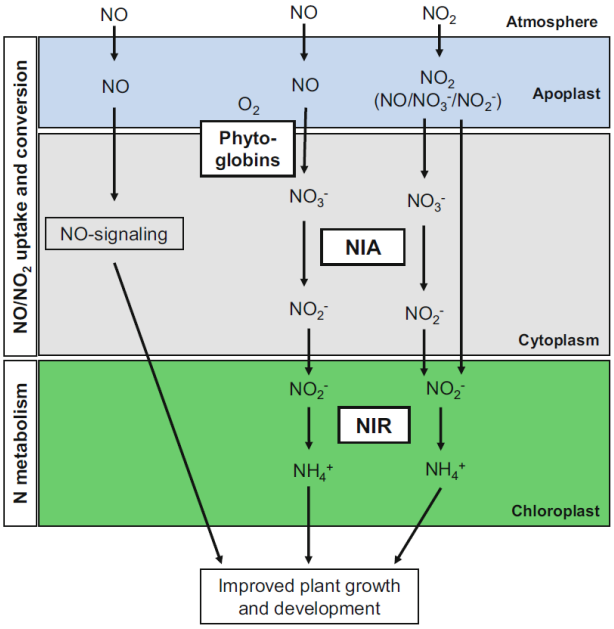


Figure 3 Pathways by which atmospheric NO and NO_2 could enter in plant N metabolism and affect plant growth and development.

Pathways by which atmospheric NO and NO_2 could enter in plant N metabolism and affect plant growth and development. As lipophilic molecule NO can enter the plant cell and act as signaling molecule. In the presence of phytoglobins, NO can be converted to NO_3^-, which can be reduced in a two-step reaction (catalyzed by nitrate reductase [NIA] and nitrite reductase [NIR]) to ammonium (NH_4^+). NO_2 can react in water, depending on the chemical conditions, to NO, NO_2, and/or NO_3^-. All these molecules can be converted to NH_4^+ in the same way as described above and finally can result in improved plant growth and development. From Lindermayr and Hebelstrup, 2016.

1.2.2 NO and NO_2 uptake of plants

It is well known that plants act as a major 'sink' for atmospheric pollutants in terrestrial ecosystems (Hill, 1971). Trees and other plant species offer the ability to remove significant amounts of air pollutants and consequently improve environmental quality and human healthy. Plants remove gaseous air pollution primarily by uptake via leaf stomata, though some gases are deposited on plant surface (Nowak *et al*., 2006).

The NO_2 uptake by plants has been reported in a large numbers of plant species. Uptake of NO_2 by different species including corn (*Zea mays*), soybean (*Glycine max*), loblolly pine (*Pinus taeda*), and white oak (*Quercus alba*) was found increased with the level of photosynthetic radiation (Rogers *et al*., 1979). Besides, similar uptake level of NO_2 among the different species suggested that this process could be mediated by a physical exchange but not by a metabolic process. Since guard cells regulate plant gas exchange and transpiration by modulation of stomatal aperture, it was suggested that the NOx uptake capacity should be related to the stomatal behavior. Indeed, several studies found that the NO_2 uptake capacity depends on stomatal opening (Chaparro-Suarez *et al*., 2011). Besides, other researches also indicated that the NO_2 uptake could be affected by nitrogen status, leaf growth state, rate of photosynthesis, and height within the canopy (Sparks *et al*., 2001; Hu and Sun, 2010, Morikawa *et al*., 1998). Unlike NO_2, researches about the NO uptake by plants are relatively scarce. Nevertheless, the analysis of NO concentrations in the atmosphere in the presence of horticultural crops, including lettuce, strawberry, apple, and banana, demonstrated a significant reduction of NO in the atmosphere, indicating the capacity of these plants to uptake NO (Soegiarto *et al*. 2003).

1.3 Phytoglobins

Hemoglobins (Hbs) are heme proteins that reversibly bind to oxygen and are known to exist ubiquitously across unicellular (archaea, bacteria, and protozoans) and multicellular organisms (fungi, plants, and animals) (Vinogradov *et al*., 2006, 2011). Besides oxygen, Hbs also bind to other gaseous ligands such as NO, carbon monoxide (CO), hydrogen sulphide (H_2S), and with some organic molecules (Frey and Kallio, 2005; D'Angelo *et al*., 2004; Rinaldi *et al*., 2006), which suggests that they are multifunctional proteins in living organisms (Garrocho-Villegas *et al*., 2007).

In plants, Hbs are termed as phytoglobins (Pgbs). Pgbs were discovered in 1939 by Kubo after spectroscopic and chemical analysis of the red pigment of soybean root nodules (Kubo, 1939). The isolation of Pgb gene from Trema tomentosa, was the first demonstration of the presence of Hb in a non-nodulating plant (Bogusz *et al*., 1988). After that, Pgbs 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 and evolved angiosperms (Garrocho-Villegas andArredondo-Peter, 2008; Vázquez-Limón *et al*., 2012). The presence of Pgbs is widespread in the plant kingdom suggests that Pgbs are likely to have an important role in the metabolism of plants.

1.3.1 Classification and characteristics of phytoglobins

The different types of Pgbs consist of Pgb class 0 (Pgb0), Pgb class 1 (Pgb1), Pgb class 2 (Pgb2), symbiotic Pgb (sPgb), legPgb (Lb) and Pgb class 3 (Pgb3) (Hill *et al*., 2016). The Pgb0 is primitive Pgbs found in algae, bryophytes and gymnosperms. sPgb is specifically localized in N_2-fixing nodules of nonlegume land plants, whereas Lbs are found in nodules of N_2-fixing legumes (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 (Km in the order of 2 nM, Smagghe *et al*., 2009; Hargrove *et al*., 2000) while Pgb2 varies between a moderate to high affinity (Km 100–200 nM, Dordas, 2009; Vigeolas *et al*., 2011). The Phytogbs 3 are structurally similar to the bacterial truncated globins and are found in algae and land plants, with a very low similarity to Pgb1 and Pgb2, and having low affinity to O_2. (Km 1500 nM, Watts *et al*., 2001). The evolution of different types of Pgbs and new functions has shown to parallel major transitions in plant evolution (Vázquez-Limón *et al*., 2012).

Biochemically, Pgbs share structural similarity with animal Hbs as they contain a globular structure that is further attached to prosthetic groups facilitating the binding of ligands such as O_2, NO, CO H_2S and certain membrane lipids (Kundu *et al*. 2003, Figure 4). The heme prosthetic group contains an iron atom with four of the six coordination sites occupied by the heme pyrrole nitrogens. It is further attached to histidines of the globin moiety through coordination of either one or two histidine side chains. Based on coordination of heme iron, Pgbs can be hexa-coordinated and penta-coordinated (Gupta *et al*., 2011b). The Pgb2, sPgb and Lbs are predominantly penta-coordinate whereas Pgb1 are predominantly hexa-coordinate and Pgb0 and Pgb3 are a combination of penta- and hexa-coordinate. In the penta-coordinated structure, only the proximal histidine coordinates with the fifth site of the heme iron, leaving the sixth site open for reversible binding of ligands such as O_2 and NO (Figure 4). However, in the hexa-coordinated structure, both the proximal and distal histidine coordinate with the heme iron, facilitating tight binding of O_2 that can further accept an electron from iron and oxygenate NO to form nitrate (Gupta *et al*., 2011b, Figure 4).

Pgbs are expressed in callus, cell suspension, seed, root and stem tissue of both dicot and monocot plants (Hill, 1998). However, Pgbs are generally found at low concentrations (1-20 μM) in plant organs except the legPgb, which can reach 0.7

mM in nodules rendering them with their characteristic red color (Gupta *et al*., 2011b). The number of Pgbs varies amongst plant species. It has been suggested that it is likely that all dicots have both class 1 and class 2 Pgbs (Trevaskis *et al*., 1997), whereas in monocots only class 1 genes have been detected (Hunt *et al*., 2001).

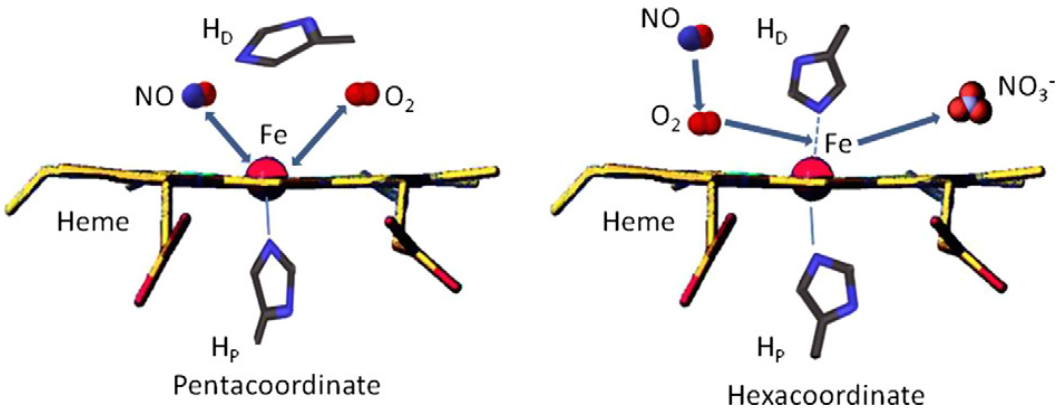


Figure 4 Chemical structures of phytoglobins showing Penta coordination and Hexa coordination.

Coordination of proximal (H_P) and distal (H_D) histidines in pentacoordinate and hexacoordinate heme. The pentacoordinate structure is open for reversible binding of ligands such as O_2 and NO, while the hexacoordinate structure facilitates tight binding of oxygen that can further accept an electron from iron and oxygenate NO resulting in formation of nitrate. From Gupta *et al*., 2011.

1.3.2 Phytoglobins and NO

Like other globins, penta-coordinate Pgbs reversibly bind and transport O_2. Wittenberg's group (Wittenberg *et al*., 1974) elucidated the function of Lbs in nodules. The apparent function of Lbs in nodules is to facilitate the diffusion of O_2 to the respiring bacteroids for nitrogen-fixation. At the same time, Lb contributes to maintain low O_2 levels (10 nM) to avoid inactivation of the O_2-sensitive nitrogenase that fixes the atmospheric nitrogen (Appleby, 1984). Furthermore, Pgbs bind other gaseous ligands, most notably NO, and exhibit a NO dioxygenase activity (Smagghe *et al*., 2008). Work by Hill and collaborators during the last ~15 years has shown that levels of endogenous NO vary with the concentration of Pgb1 in transgenic maize and alfalfa (Hill, 2012). Based on these observations, they

have proposed that a function of oxygenated Pgbs is to modulate levels of NO via a NO dioxygenase activity and to indirectly regulate a wide variety of cell functions that are modulated by levels of NO. The NO dioxygenase activity was mainly researched in Pgb1. The structural properties of Pgb1 allow them to serve as soluble electron transport proteins in the enzymatic system scavenging NO produced in low oxygen conditions primarily via reduction of nitrite in plants, which is called the Pgb/NO cycle (Perazzolli *et al*., 2004; Berger *et al*., 2018).

Class 1 Pgbs possess weak penta-coordination characteristic and are expressed in cells under low oxygen tension (Hargrove *et al*., 2000). Upon binding of a ligand, such as oxygen, the distal histidine moves away from the iron atom and the protein attains in a more stable conformation (Hoy *et al*., 2008) which allows a very tight but slow oxygen binding during the scavenging of NO under near anaerobic conditions (Perazzolli *et al*. 2004). During this interaction, Pgbs and oxygen interacts to form oxyPgb that participates in oxygen dependent NO binding and/or scavenging under oxygen deficit conditions and produces nitrate and metPgb (Igamberdiev and Hill, 2004; Nienhaus *et al*., 2010). At the same time, reductase activity is needed to convert the ferric state in metPgb to the ferrous state (Igamberdiev *et al*., 2006, Figure 5).

Class 2 Pgb (Pgb2), on the other hand, has very low affinity for O_2 because it is completely penta-coordinated in the physiological conditions. This makes them less efficient in NO scavenging but increases the possibility of functions related to sensing low levels of oxygen and to oxygen storage and diffusion by Vigeolas *et al*., 2011. However, an optimum for stimulation of growth at 25 µM SNP for wild type seedlings, whereas seedlings with overexpression of Pgb2 had an optimum shifted towards a higher concentration, indicating that class 2 Pgb is also part of a NO dioxygenase activity (Hebelstrup and Jensen, 2008).

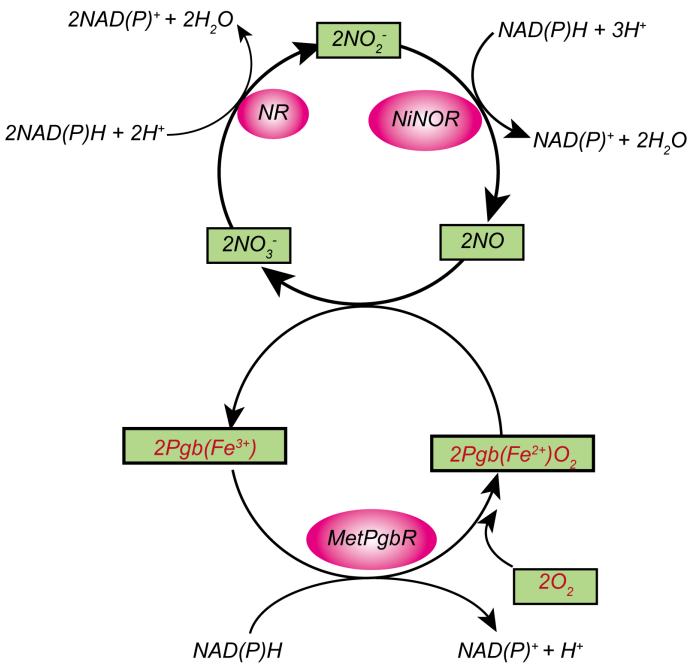


Figure 5 Scavenging of NO by class 1 phytoglobins.

NO is converted to NO_3^- by the oxygenated ferrous (Fe^{2+}) phytoglobin (Pgb), which turns to the MetPgb (ferric, Fe^{3+}) form. The latter can be reduced by a corresponding reductase (MetPgbR) and oxygenated again. NO_3^- is converted to NO_2^- by NR, while NO_2^- can form NO in reactions of hemeproteins and other redox systems possessing nitrite: NO reductase (NiNOR) activity. Modified from Gupta *et al.*, 2011.

Unlike the well-documented role of Pgb1 and Pgb2 in plants metabolism, development and various abiotic and biotic stresses, there is lack of evidence pertaining to the physiological significance of Pgb3 in plants. However, NO dioxygenase activity of *Arabidopsis* Pgb3 was suggested by crystallographic studies in vitro (Mukhi *et al*., 2016), and was then confirmed in vivo (Mukhi *et al.*, 2017).

1.3.3 Physiological functions of phytoglobins

Pgb has been found to play a significant role in plant growth and development**.** The reverse genetic approaches on Pgbs in *Arabidopsis* thaliana have emphasized their paramount role during plant growth and development by demonstrating that at least one functional Pgb gene is necessary for survival of young seedlings (Hebelstrup *et al*., 2013; Hill, 2012). Silencing of Pgb1 results in abnormal

development of leaf hydathodes, flowers and floral buds (Hebelstrup *et al*., 2006), Pgb2 knockout and overexpression lines show normal growth and development. Overexpression of Pgb1 in *Arabidopsis* 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, in barley, the ectopic overexpression delayed growth and development, and seed specific overexpression reduced seed yield, which was different from that in *Arabidopsis* (Hebelstrup *et al*., 2014).

Pgbs are essential to plant survival in response to both biotic and abiotic stress. Pgbs has been demonstrated play a role in various stresses, including hypoxia, nutrient deprivation, osmotic, cold, nutrition deficiency, oxidative, drought and nitrative stress (Arredondo-Peter *et al*., 2014; Mira *et al*., 2016; Mira *et al*., 2017; Montilla-Bascón *et al*., 2016; Shankar *et al*., 2018). Pgb also plays a very important role during plant pathogen interaction. Production of transgenic tobacco plants overexpressing the alfalfa Pgb showed altered necrotic symptoms after treatment with NO generating compounds or infection by necrotic pathogens (Seregélyes *et al*., 2004). Overexpression of the *Gossypium sp*. Pgb1 in *Arabidopsis* increased pathogen resistance as well as enhanced tolerance to NO (Qu *et al*., 2006). Overexpression of Pgb in barley influenced the status of plants infected with *B. graminis*, expressed as a shift in the defence response against avirulent genotypes and resulting in higher tolerance response to virulent pathogen genotypes (Sørensen *et al*., 2018).

Pgbs can modulate many hormonal signal transduction pathways through their metabolism of NO (Hill, 2012). In dicot somatic embryogenesis, Pgbs affect the expression of auxin and jasmonic acid genes through modulation of cellular NO (Elhiti *et al*., 2013; Mira *et al*., 2016). Pgbs also induce program cell death by altering the expression of genes encoding ABA and ET signaling in developing somatic embryos (Stasolla and Hill, 2017; Kapoor *et al*., 2018).

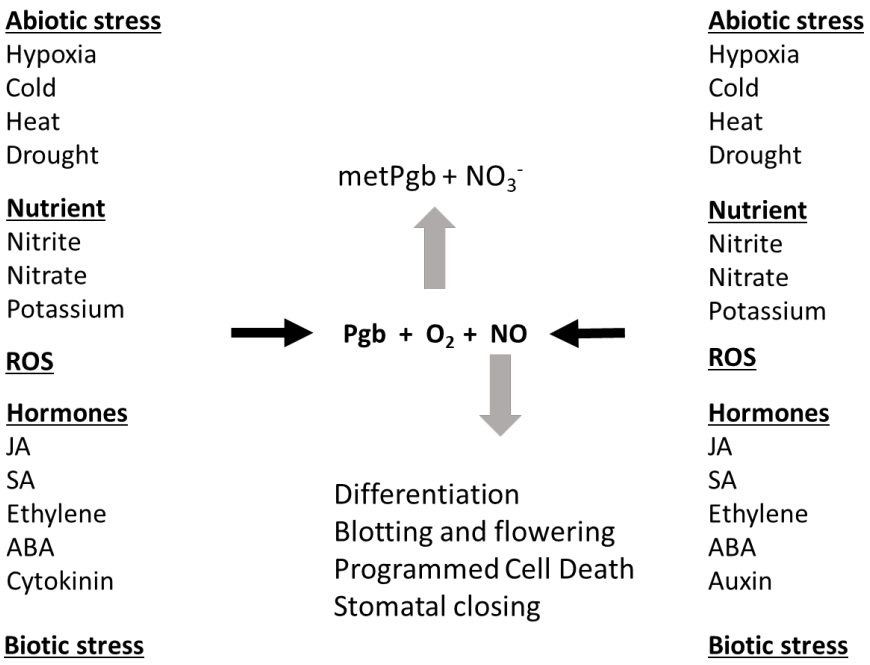


Figure 6 Schematic function of phytoglobins and NO in plants.

Phytoglobins and NO play significant role in biotic and abiotic stress, hormones regulation, nutrient, and plant development. Modified from Hill, 2012.

As shown in Figure 6, Pgbs have extensive functions, including but not limited to, plant growth and development, abiotic and biotic stress responses, interaction with hormonal signal. NO acts as a signalling molecule in the appropriate signal transduction pathway, resulting in a specific biological outcome. If Pgb is induced as a result of the induction process, it can interact with NO to produce metHb (Fe_3^+) and nitrate, reducing the levels of NO and modulating the biological response.

1.4 Aim of the study

NO is an air pollutant, which contributes to the formation of smog and acid rain together with its oxidation product NO_2. NO is also regarded as a ubiquitous signaling molecular which mediates many developmental and physiological processes. In our previous studies, we observed a positive effect of NO fumigation on plant growth in soil grown *Arabidopsis* and identified the phytoglobin

dependent NO-fixation pathway. However, until now, little is known about the effect of atmospheric NO on important crops, e.g. barley.

The plant-based NO-fixation lowers the concentration of atmospheric NOx and in this context, plants have a beneficial effect on air quality and human health. With regard to the air quality in cities with high concentrations of nitrogen oxides, the NO fixing capability of city trees could contribute significantly to the reduction of NOx and thus improve air quality.

Therefore, the aims of the study are:

i. Analyzing NO-fixation under N-limited conditions in *Arabidopsis*;
ii. Investigating the effect of atmospheric NO on the crop plants barley and the role of Pgbs under these conditions;
iii. Determining the NO-uptake using ^{15}NO;
iv. Analyzing the NO-N metabolism in transgenic plants to obtain insights into NO-fixing pathway;
v. Analyzing the NOx uptake capacity of different city trees and verifying the enhanced NOx uptake capacity in Pgb transgenic trees.

2 Materials and methods

2.1 Plant material

The plants used in this study and their sources have been summarized in Table 1. *Arabidopsis* with overexpressing class 1 Pgb (AtPgb1+) or class 2 Pgb (AtPgb2+), as well as plants with reduced (AtPgb1-) or knocked out (AtPgb2-) Pgb expression were obtained in *Aarhus* University as described (Hebelstrup *et al*., 2006). Barley plants overexpressing class 1 Pgb (HvPgb1.1+) and silenced (HvPgb1.1-) lines were described Hebelstrup *et al.* (2014). Transgenic poplar PcPgb1+ line is overexpressing *Arabidopsis* class 1 Pgb gene (AtPgb1); transgenic poplar PcPgb2+ is overexpressing *Arabidopsis* class 2-Pgb gene (AtPgb2).

Table 1 Plant species used in this study.

Species	Ecotype	Plant line	Source of the Plant/Seed
Arabidopsis thaliana	Columbia-0	Wild-type	Lindermayr C, HMGU, BIOP
Arabidopsis thaliana	Columbia-0	*AtPgb1-(glb1-RNAi)*	Hebelstrup K, MBG, Aarhus Univ.
Arabidopsis thaliana	Columbia-0	*AtPgb1+ (GLB1-Ox)*	Hebelstrup K, MBG, Aarhus Univ.
Arabidopsis thaliana	Columbia-0	*AtPgb2-(glb2*-KO)	Hebelstrup K, MBG, Aarhus Univ.
Arabidopsis thaliana	Columbia-0	*AtPgb2+ (GLB2*-Ox)	Hebelstrup K, MBG, Aarhus Univ.
Hordeum vulgare	Golden Promise	Wild-type	Hebelstrup K, MBG, Aarhus Univ.
Hordeum vulgare	Golden Promise	*HvPgb1.1-*	Hebelstrup K, MBG, Aarhus Univ.
Hordeum vulgare	Golden Promise	*HvPgb1.1+*	Hebelstrup K, MBG, Aarhus Univ
Poplulus canescens	syn. *P. tremula* × *P. alba*	Wild-type	Lindermayr C, HMGU, BIOP
Poplulus canescens	syn. *P. tremula* × *P. alba*	PcPgb1+	Lindermayr C, HMGU, BIOP

Poplulus canescens	syn. *P. tremula* × *P. alba*	PcPgb2+	Lindermayr C, HMGU, BIOP
Carpinus betulus	Frans Fontaine	Wild-type	Wilhelm Ley Baumschulen
Fraxinus ornus	Loisa Lady	Wild-type	Wilhelm Ley Baumschulen
Fraxinus pennsylvanica	Summit	Wild-type	Wilhelm Ley Baumschulen
Ostrya carpinifolia		Wild-type	Wilhelm Ley Baumschulen
Celtis australis		Wild-type	Wilhelm Ley Baumschulen
Alnus spaethii		Wild-type	Wilhelm Ley Baumschulen
Alnus glutinosa	Imperialias	Wild-type	Wilhelm Ley Baumschulen
Tilia henryana		Wild-type	Wilhelm Ley Baumschulen

2.2 Hydroponic culture system for *Arabidopsis*

Nutrient solutions for hydroponic cultures were prepared according to Table 2. Tips, 1.5 ml eppendorf tubes in the ranks with the lids, tooth sticks and ddH2O were autoclaved for later use. Prepared the 0.65% (0.65 g/100 mL) bacto agar (dissolved the agar with ddH_2O) and then heated it in the micro oven until transparent. After the agar cooling down enough, filled 1.7 ml in the sterilized eppendorf tubes and put them at 4 centi-degrees at least overnight or 2 days with the lids covered after about 10 minutes.

Sterilized the seeds (2 times with 90%EtOH – pipette 2 × 1 ml to the seeds on a filter paper under the sterile bench). Cut the bottom by the machine at the position about 0.5 mL and planted the seeds on the agar (2 seeds in each tube). Added available prepared nutrient solutions to plant growth box and put the tubes in the hole of the box plate and covered them with the preservative film (cling film) before putting them in the chamber at 11.5h/12.5h light/dark cycle to ensure the roots growth. One week later, open the cling film a little bit to avoid the fungal growth and supply enough fresh air for the plants. At the same time, checked the root of the plants can straightly grow into the agar and removed the other one whose root cannot grow straightly and have too much lateral roots.

Table 2 Hydroponic nutrient solutions for *Arabidopsis*.

With Nitrogen		**Without Nitrogen**	
Reagents	**Concentration**	**Reagents**	**Concentration**
KNO_3	1.25 mM		
$Ca(NO_3)_2$	1.5 mM	$CaCl_2$	1.5 mM
$MgSO_4$	0.75 mM	$MgSO_4$	0.75 mM
KH_2PO_4	0.5 mM	KH_2PO_4	0.5 mM
KCl	50 uM	KCl	1 mM
H_3BO_3	50 uM	H_3BO_3	50 uM
$CuSO_4$	1.5 uM	$CuSO_4$	1.5 uM
$MnSO_4$	10 uM	$MnSO_4$	10 uM
$ZnSO_4$	2.0 uM	$ZnSO_4$	2.0 uM
$(NH_4)_6MoO_{24}$	0.075 uM	Na_2MoO_4	0.075 uM
Na_2SiO_3	0.1 mM	Na_2SiO_3	0.1 mM
Fe-EDTA	72 uM	Fe-EDTA	72 uM

Note: Add 0.5g/L MES and adjust the PH to 5.7 with KOH.

2.3 NO and NO_2 fumigation treatment

All experiments were performed in climate chambers under controled. The chambers and NO treatment facilities were provided by the Research unit of Environmental Simulation in the Department of Biochemical Plant Pathology (BIOP) at Helmholtz Zentrum Munich, Germany. In all experiments, the NO and NO_2 levels in the chambers were monitored with an AC32M (Ansyco, Karlsruhe, Germany) chemiluminescent NOx analyser. NO was obtained from Air Liquide (Düsseldorf, Germany) in cylinders containing 2 or 15% NO in N_2.

2.3.1 NO fumigation of hydroponic *Arabidopsis*

Arabidopsis grown in the hydroponic culture system were used (Gilbert *et al.*, 1997) in three NO fumigation experiments.

1) Hydroponically grown plants germinated and grew in N-containing medium for 12 days. Then, they were transferred to medium without any

N-source (1.25 mM KNO_3, $Ca(NO_3)_2$ and 0.075 µM $(NH_4)MoO_{24}$ were replaced by 1.5 mM $CaCl_2$, 1mM KCl and 0.075 µM Na_2MoO_4, respectively, table 1) and fumigated with 3000 ppb NO for 30 days (day/night 24 h), purified air with ambient NO was used as control;

2) 30-days-old hydroponic *Arabidopsis* were transferred to medium without any N-source and fumigated with 250 ppb ^{15}NO for 11 days (day/night 24 h);

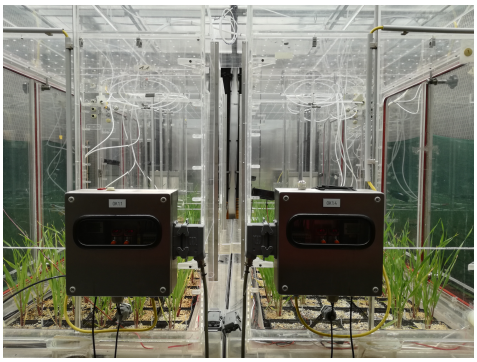
3) 30-days-old hydroponic *Arabidopsis* were transferred to medium with or without N-source and fumigated with 200 ppb ^{15}NO for 5 days (day/night 24 h).

Growth conditions: light – 300 µmolm^{-2} s^{-1}, photosynthetically active radiation (PAR: 400–700 nm); temperature – day: 20 °C (14 h) and night: 16 °C (10 h); and relative humidity – 80%.

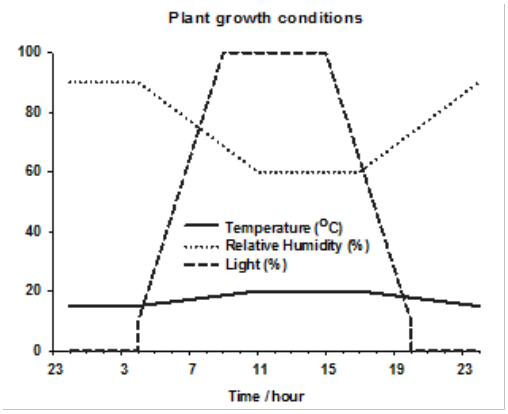
2.3.2 NO fumigation of barley plants

Transgenic barley (Hordeum vulgare L. var. Golden Promise) plants overexpressing Pgb (Pgb1.1+) and Pgb knockdown (Pgb1.1-) lines were obtained from Kim Hebelstrup (Aarhus University). Plants (1 plant/pot, Square Pot 10 × 10 × 11cm) were grown in the matrix with mixed Floragard B, meteorite and sand (floragard B: vermiculite: sand=2:2:1, Floragard B containing 140 mg/L N, 80 mg/L P_2O_5 and 190 mg/L K_2O). The NO fumigation of the soil grown plants was continuous day/night 24h, starting on the 4^{th} day after germination, and was performed in climatic fumigation chambers whose internal NO levels were constantly monitored (Figure 7).

A



B



C

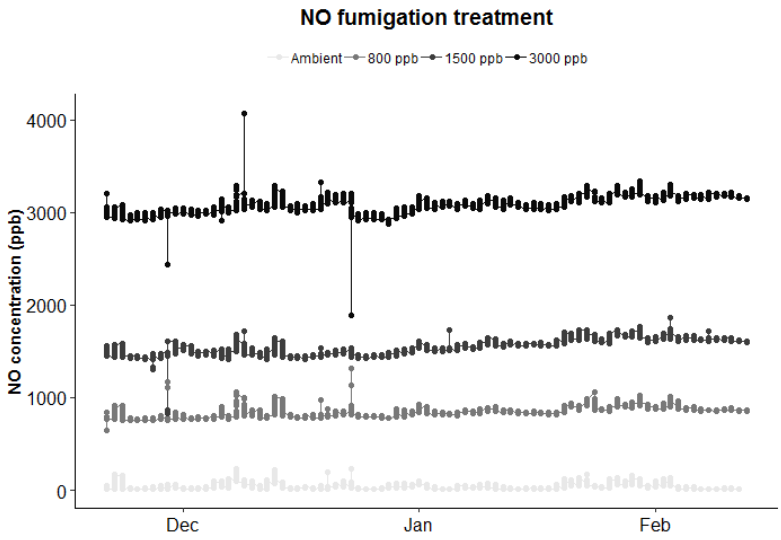


Figure 7 Growth conditions for barley plants during long term NO fumigation treatment.

Barley plants were treated with various concentrations of NO in specially designed exposure chambers (A). The NO levels inside these chambers were continuously monitored using chemiluminescence detection method sensitive to as low as 1 ppb of NO. The plant growth conditions are showed in graph B, the photosynthetic photon flux density (PPFD) of light at 100% from 9:00-15:00 is 300 µmol m^{-2} s^{-1}, the photosynthetically active radiation (PAR) is 400 – 700 nm. The concentration monitored during the experiment is showed in graph C. All the chambers were supplied with ambient air that was directly drawn from the campus of Helmholtz Zentrum München, Germany.

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, Figure 7). During the experiment, 100 ml modified Hoagland nutrient solution without any N-source (KNO_3, Ca $(NO_3)_2$ were replaced by $CaCl_2$, KCl,

respectively) was added every two weeks. Growth conditions: light – <300 μmol m^{-2} s^{-1}; photosynthetically active radiation (PAR: 400–700 nm);
Temperature – night: 15°C (8 h), daytime: 15-20°C (16 h); humidity – (60%-90%). Detailed growth conditions are shown in Figure 7.

2.3.3 $^{15}NO/^{15}NO_2$ fumigation of *Arabidopsis* and barley

20 day-old barley (4 plants/pot, square pot 10 × 10 × 11cm) and 28 day-old *Arabidopsis* (5 plants/pot, square pot 5 × 5 × 5 cm) grown in the substrate (Floragard B: Meteorite: Sand=3:1:1) were used in $^{15}NO/^{15}NO_2$ fumigation experiment. ^{15}N-NO/ ^{15}N-NO_2 (99 % atom isotopic enrichment) was obtained from Linde (Pullach, Germany) and diluted to 2% with nitrogen by Westfalen AG (Münster, Germany). $^{15}NO/^{15}NO_2$ (90 ppb) fumigation treatment was performed 12 hours (8:00-20:00) of daytime for 7 days. 90 ppb NO/NO_2 fumigation was used as control. Growth conditions: light - 300 μmol m^{-2} s^{-1}; photosynthetically active radiation (PAR: 400–700 nm); temperature – day: 20°C (14 h) and night: 16°C (10 h); relative humidity – 80%.

2.3.4 NO and NO_2 fumigation of trees

Four different kinds of trees (*Carpinus betulus*, *Fraxinus ornus*, *Fraxinus pennsylvanica* and *Ostrya carpinifolia*; Figure 8) were used for the NO/NO_2 fumigation. Plants were moved to climate chamber two days before treatment to adapt the environment. Mature and healthy shoots were choosed and drew a blade shape on the paper for measuring leaf area. Choosed shoots were tighten together with a gas tube as air inlet in one side of the open plastic bag, the second gas tube were tightened at another side of plastic bag as air outlet (Figure 8).

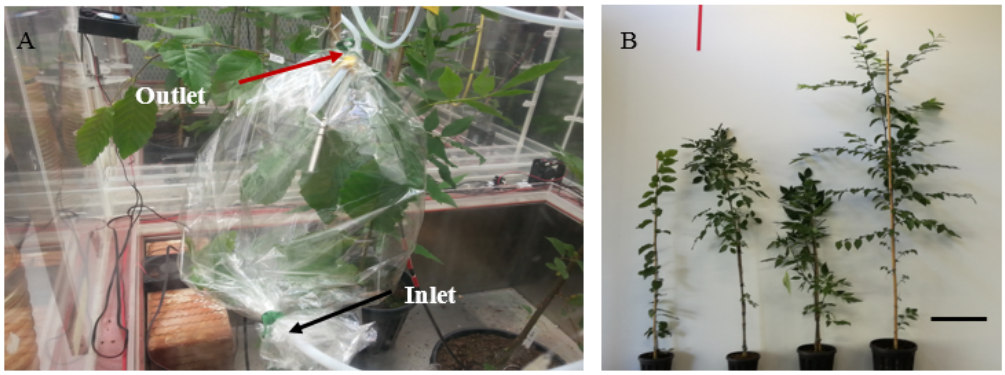


Figure 8 NO and NO_2 fumigation system for trees.

A. Experement was set up in climate chamber. The black arrow represents the air inlet and red arrow represents the outlet. B. Phenotype of four different trees (from left to right, *Carpinus betulus*, *Fraxinus ornus*, *Fraxinus pennsylvanica* and *Ostrya carpinifolia*) used in this experiment.

During the fumigation experiment, the airflow was around 1000 ml/min. Fixed concentrations of NO and NO_2 were controlled and monitored for fumigation. Growth conditions: light - 300 µmol m^{-2} s^{-1}; photosynthetically active radiation (PAR: 400–700 nm); temperature – day: 20°C (14 h) and night: 16°C (10 h); relative humidity – 80%.

2.3.5 ^{15}NO fumigation with trees, *Arabidopsis*, and barley

15 day-old barley, 30 day-old *Arabidopsis*, 15 day-old poplar (the height was around 15 cm) and 40 day-old poplar (the height was around 50 cm), and 8 different trees (*Carpinus betulus*, *Fraxinus ornus*, *Fraxinus pennsylvanica*, *Ostrya carpinifolia*, *Celtis australis*, *Alnus spaethii*, *Alnus glutinosa*, and *Tilia henryana*) were used in this fumigation experiment. All plants were transferred to climate chamber 2 days before. ^{15}N-NO (99 % atom isotopic enrichment) was obtained from Linde (Pullach, Germany) and diluted to 2% with nitrogen by Westfalen AG (Münster, Germany). ^{15}NO (50 ppb) fumigation treatment was performed for 5 days. 50 ppb NO fumigation was used as control. Growth conditions: Growth conditions: light - 300 µmol m^{-2} s^{-1}; photosynthetically active radiation (PAR:

400–700 nm); temperature – night: 16°C (8 h), daytime: 20°C (14 h); humidity – 80%.

2.4 $^{15}NO_3^-$ tracer application

30 day-old *Arabidopsis* plants were germinated and grown under the hydroponic culture system and moved to climate chamber for NO fumigation 2 days before treatment. ^{15}N tacer nutrient solutions (Table 1, 50% of KNO_3 was replaced with $^{15}KNO_3$ (60-atom % ^{15}N, from Sigma-Aldrich, Deisenhofen, Germany) were added to replace the normal nutrient solutions before NO fumigation. Plants were fumigated with 3000 ppb NO, the ambient NO was used as control.

Barley plants were germinated and grown in the matrix without soil (Meteorite: Sand=4:1, 4 plants/pot, square pot 10 ×10 ×11cm). 7 days after sowing, 50 ml nutrient solutions with 0.3 mM $^{15}NO_3^-$ were added for each pot every day. The nutrient solution contains 1 mM KH_2PO_4, 0.5 mM $^{15}KNO_3$ (60 atom % ^{15}N, from Sigma-Aldrich, Deisenhofen, Germany), 0.5 mM Ca $(NO3)_2$, 0.9 mM $MgSO_4$, 50 uM Fe-EDTA, 16 uM H_3BO_3, 0.3 uM $ZnSO_4$, 0.3 uM $CuSO_4$, 0.4 uM Na_2MoO_4. Leaf samples were taken after 2 days, 9 days and 12 days for ^{15}N measurement.

2.5 Growth and yield parameters

For *Arabidopsis*, the rosette size, shoot length and yield were measured. Rosette size was measured by measuring the diameter (in cm) of the biggest circle that was occupied in at least two opposite directions. Shoot length (in cm) was measured by scaling the distance between bottom-most part of the vegetative shoot to its top most part. Yield was measured by measuring the siliques number and seed weight. All measurements were performed with 15-20 plants.

For barley, after 20, 30 and 45 days NO fumigation, at least 4 plants were taken for the measurement of the plant height, leaf number, stem number, and plant weight. After 80 days treatment, 15 plants were taken for the measurements of 8 traits: dry matter weight per plant (DWP), plant height (PH), spike length

excluding awns (SL), spikes per plant (SP), spike weight (SW), kernel numbers per plant (KNP), kernel weight (KW), kernel weight per plant (KWP).

For all plants, fresh weight (in g) was measured by weighing the freshly processed plant leaves after NO fumigation. These leaves were then dried in the hot air oven for more than 48 hours at 60 °C to measure the dry weight (in g). The moisture content of leaves was calculated as: (fresh weight - dry weight) / fresh weight.

2.6 Measuring NO levels in closed reaction chamber

Pots with plants were placed in a closed system/cuvette and levels of accumulated NO were measured after 30 min. Afterward, the plants were cut, and the levels of accumulated NO was determined again after 30min. The difference between the levels with and without plants reflects the amount of NO taken up in 30min by the different genotypes. Gaseous NO was measured using a CLD88 CY p analyzer (ECOPHYSICS, Germany).

2.7 Chlorophyll ratio and chlorophyll fluorescence measurement

Dualex Scientific+™ (FORCE-A, 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 MINI-PAM-II Photosynthesis Yield Analyzer WALZ, Germany. The effective quantum yield of PSII (ΔF/Fm') was determined according to Genty *et al*. (1989). ΔF/ Fm' 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 the measurements were performed between 13:00 - 15:00.

2.8 Nitrate and nitrite measurement in leaves

The total nitrite and nitrate concentration were estimated using a Sievers280i nitric oxide analyser (GE Analytical Instruments, Boulder CO, USA). Rosette proteins

were extracted with extraction buffer (137 mM NaCl, 0.027 mM KCl, 0.081 mM $Na_2HPO_4.2H_2O$ and 0.018 mM NaH_2PO_4) from 300 – 500 mg of plant tissue. 100 µL of leaf protein extraction was injected into the purging vessel of NOA containing 3.5 mL of acidified KI/I_3 solution (reducing agent) at 30°C. The recorded mV signals were plotted against a 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

2.9 cDNA synthesis and polymerase chain reaction (PCR)

100 mg of plant material was ground to powder, followed by RNA extraction using the RNeasy Plant Mini Kit (Qiagen, Cat No. 74904) according to the manufacturer's instruction. RNA concentration and quality were determined spectrophotmetrically (NanoDrop 1000). 1 µg of total RNA were used for cDNA synthesis with the QuantiTect Rev. Transcription Kit (Qiagen, Cat No. 205311).

A real time PCR reaction was composed of 10 µl of Sybr green (Bioline, 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 ^{0}C for 10 minutes followed by 45 cycles of 95 ^{0}C for 15 s, 55 ^{0}C for 15 s, and 72 ^{0}C for 45 s. Each sample was run in triplicates. *HvGADPH* and *Hvactin* were used as housekeeping genes. Primers used are listed in Table 3.

A semi-quantitative reverse transcriptase PCR analysis was composed of 2 µl 20 mM dNTPs, 0.5 µl of 10 µM specific primers, 0.2 µl iProof High-fidelity™ Phusion Polymerase (Biorad, Cat No. 1725300, 2 U/µl), and 4 ul of 1:20 diluted cDNA template. Cycling conditions were 98 ^{0}C for 5 minutes followed by 35 cycles of 98 ^{0}C for 10 s, 55 ^{0}C for 30 s and 72 ^{0}C for 45 s, and then 72 ^{0}C for 10 minutes. The separation of amplified DNA fragments after PCR was done in agarose gels using TAE buffer. 1% agarose gel (1 g agarose ultra-pure solved in

100 ml of TAE buffer) supplemented with ethidium bromide (Carl Roth GmbH, Karlsruhe, Germany) was used. Samples were mixed with 6 × loading dye (MBI Fermentas, St Leon-Rot, Germany) solution before loading and the gels were run at voltage of 120 volts for 30 minutes and was visulalized on UV transilluminator (UVP, Inc, Jena, Germany). Primers used are listed in Table 3.

Table 3 Primers used for Real-Time PCR and semi-quantitative reverse transcriptase PCR analysis.

Gene identifier	Name	Forward primer	Reverse primer
AY145451.1	*HvActin*	GCCGTGCTTTCCCTCTATGC	GCTTCTCCTTGATGTCCCTTAC
X60343.1	*HvGADPH*	GCTCAAGGGTATCATGGGTTACG	GCAATTCCACCCTTAGCATCAAAG
U94968.1	*HvPgb1.1*	TCGTCTTCAGCGAGGAGAAG	GATCTCGAAGATCTTGAGGAAG
AK376331.1	*HvPgb1.2*	ATGTGGACGCCGGAGATGAA	GCAGAGGCAGCGAGCTTCAT
AF376063.1	*HvPgb3*	CCTCTCCACCAACTTCTACACCA	TGGCCGATGTCGTCCTATCAAG
X57844.1	*HvNR*	GTCGACGCCGAGCTCGCCAA	GCGCACCTCGGACATGGT
LC097012.1	*HvNiR*	TCAAGTGGCTCGGCCTCTT	ACGCACACGTTCCACTTCCT
X53580.1	*HvGS2*	TGCTCGACATGGACACCA	CGTTTGTTAGTAGGGATGGGT
S58774.1	*HvFd-GOGAT*	TGCATGGAGCACCGTGGT	CCATCTAGGGCTTGTATTGGTACT
XM002298946	*PtActin*	CGGAGAGAGGTTACACATTCAC	CGTTTCAAGCTCCTGCTCATA
U94998.1	*AtPgb1*	TCCAAAGCTCAAGCCTCACGCA	AGCCTGACCCCAAGCCACCT
U94999.1	*AtPgb2*	GAGATGGGAGAGATTGGGTTTAC	GTGAGAAGAAGTGAAGGCTGTAT

2.10 Phytoglobins phylogenetic and expression pattern analysis

All Pgb protein sequences from different plant species were aligned using Clustal W. The phylogenetic analysis was carried out by the Neighbor–Joining method with JTT+G model using the MEGA 6.06 program. Amico acid sequences

alignment were analyzed by online software - Pairwise Sequence Alignment (https://www.ebi.ac.uk/Tools/psa/). Accession numbers of Pgb sequences employed in the multiple alignments and used to generate the phylogenetic tree are listed in supplementary Table 1. The expression pattern analysis was performed based on the collected data from morexGenes-Barley RNA-seq Database.

2.11 Nitrate and ammonium measurement in soil samples

After totally removing roots, a mixture of 5 g of soil was shaken with 20 ml of 0.1% $CaCl_2$ for two hours. After centrifugation (Rotanta 460R, Hettich AG, Bäch, Schweiz) for 20 minutes at 4000 rpm, the supernatant was filtered using black ribbon filter paper. The concentrations of ammonium and nitrate were determined simultaneously with an N-autoanalyzer (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.

2.12 DNA, RNA and protein extraction for ^{15}N measurement

Genomic DNA was extracted with a modified CTAB method according to Krizman *et al*., 2016. TRIzol reagent (Ambion, Life technologies, Austin, USA) was used to extract RNA from the leaves, following the manufacturer's instruction. Purified total DNA and RNA were quantified using the Nanodrop ND -1000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA).

For protein extraction, homogenised frozen rosette material (400 mg) was vortexed using 1 mL extraction buffer (100 mM Tris/HCl-pH 8.0, 10 mM EDTA, 1 mM $MgCl_2.H_2O$). Homogenate was centrifuged (12000 g for 20 min at 4°C) and supernatant was filtered using 70 µm nylon membrane. Protein extraction was then

desalted using PD-10 desalting columns (GE Healthcare, Freiburg, Germany) according to the manufacture's instruction. 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 595nm, a series of dilutions of bovine serum albumin (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.

2.13 Determination of ^{15}N content in leaves and N content in soil

Plant and soil materials were dried at 60 °C for 48 h and ground to a homogenous 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 into tin capsules (IVA Analysentechnik, Meerbusch, Germany). ^{15}N abundance and N content were determined with an Isotope Ratio Mass Spectrometer (IRMS, delta V Advantage, Thermo Fisher, Dreieich, Germany) coupled to an Elemental Analyzer (Euro EA, Eurovector, Milano, Italy).

As carrier gas, Helium 5.0 was used with a flow of approx. 80 ml/min. The Autosampler introduced the samples into a combustion column, which was heated up to 1000 °C and filled with tungsten oxide and silver coated cobalt oxide to improve the oxidation and adsorb halogens. Oxidation took place in an excess of oxygen. The exothermic oxidation of tin leaded to a local temperature of about 1700 °C, which ensured a quantitative oxidation of the samples. The combustion products like CO_2, NO_x and water were passed into a reduction reactor filled with metallic copper at 650 °C, where nitrous gases were reduced to molecular nitrogen and oxygen was removed by reacting with copper. After elimination of water with magnesium perchlorate, only CO_2 and N_2 were left for separation on a packed column.

Only a small part of both gases was introduced to the ion source of the IRMS using a so-called ConFlow Interface. The flow into the ion source was about 0.3 ml/min. In the ion source, ions (N_2^+, CO_2^+) were generated by colliding with electrons. The ions were accelerated in an electric and separated in a magnetic field depending on their masses. Heavy ($^{29}N_2$) and light ($^{28}N_2$) ions were detected in so-called Faraday cups and a ratio between both was calculated.

IRMS measurements always need the comparison with one or more standards with known isotope composition in the same range of the analyzed samples. For that reason, a lab standard (acetanilide), being part of every sequence in intervals, was used. A series of lab standards of different weights was measured to determine isotope linearity of the system. All standard measurements were also base for the calibration of N content calculation. The lab standard itself was calibrated against several suitable international isotope standards (International Atomic Energy Agency: IAEA; Vienna). International and lab isotope standards were also part of every sequence to create a final correction of ^{15}N: e.g. IAEA 600, USGS 40, IAEA N2, USGS 26, USGS 32, IAEA 310 B, IAEA 305 B covering all ^{15}N results of this sequence. ^{15}N results of higher enriched samples were finally corrected with enriched standards delivered from Fischer Analysen Instrumente (Leipzig, Germany).

Different to solid (plant and soil) samples, aliquots of DNA-, RNA- and protein samples, which exists in solution, were pipet to 4 × 6 mm tin capsules and dried over night at 60 °C. In this case Bovine serum albumin “BSA” was used as a lab standard: calibrated as a solid against international isotope standards but used in solution as lab standard in the measuring sequence, also in different amounts. Volume of sample aliquots were chosen depending on their expected N concentrations to get about 5 to 20 µg for each single measurement. For treating such small amounts of N, some modifications to the Elemental analyzer were done. E.g. using columns with smaller inner diameter and working with only about 30

ml/min. helium flow to increase the share of sample gas getting to the ion source. Final isotope correction was done with the same standards but all in solution with similar N concentration like the samples.

2.14 Stomatal conductance

Stomatal conductance (gs, mmol m^{-2} s^{-1}) of 20 days barley and 28 days *Arabidopsis* leaves were measured with a portable leaf porometer (SC-1 Leaf porometer, Decagon Devices, Pullman, USA) during midday (10:00-12:00). Measurements were done in the auto mode using the first 30 s of stomatal conductance data to predict the final stomatal conductance under true steady state conditions.

2.15 NO and NO_2 deposition measurement of trees

Rates of transpiration (FH_2O) (mmol m^{-2} s^{-1}) and exchange of NO (FNO) and NO_2 (FNO_2) (nmol m^{-2} s^{-1}) were calculated as $F= (Co-Ci)\cdot Q/A$ (Chaparro-Suarez *et al*., 2011), based on the concentration differences between the outlet ports of the branch cuvette and the empty Cuvette (Co and Ci, respectively, mmol m^{-3} or nmol m^{-3}), the enclosed leaf area (A, m^2), and the air flow rate through the cuvettes (Q) (m^3 s^{-1}). The linear relationship was made between FNO/FNO_2 and the fumigated NO/NO_2 concentration: $y = kx+b$ (x represents the fumigated NO/NO_2 concentration; y represents the transpiration rate of NO (FNO) or NO_2 (FNO_2)). The deposition potential is determined as the slope (k) value, and the compensation point is determined as the x value when y is zero.

2.16 Statistical analysis

All data were statistically analysed by student t-test or one way anova with Tukey's test ($P < 0.05$) using sigmaplot 12.0.

3 Results

3.1 NO-fixation by phytoglobins in *Arabidopsis*

In previous study, we found that treatment with up to 3000 ppb gaseous NO had no negative/toxic effects on plant growth and development but activated plant primary metabolism and improved plant growth in soil grown *Arabidopsis* (Kuruthukulangarakoola *et al*., 2017). Plants overexpressing Pgb 1 or Pgb 2 genes showed enhanced growth of rosette and vegetative shoot compared to WT controls under NO treatment. These results indicated that Pgb play significant role in the NO induced promoting effect in *Arabidopsis*.

To further demonstrate effect of NO on *Arabidopsis* and exclude the effect of soil microbes, hydroponic Pgb transgenic *Arabidopsis* were used for the NO fumigation and nitrate tracer experiment. Besides, ^{15}NO tracer experiment were also performed to verify the NO-fixation metabolism by Pgbs.

3.1.1 NO fumigation enhanced *Arabidopsis* growth

Arabidopsis WT plants and plants with altered Pgb1 and Pgb2 expression (Pgb 1 overexpression line (Pgb1+), phyroglobin 1 silence line (Pgb1-), Pgb 2 overexpression line (Pgb2+), and Pgb knockout line (Pgb2-) were hydroponically cultivated in N-containing medium for 12 days (Gilbert *et al*., 1997). Then, these plants were transferred to medium without any N-source and fumigated with ambient (as control) or 3000 ppb NO.

Phenotypes were analyzed after 20 days (Figure 9) and 30 days (Figure 10) treatments. Compared with ambient control plants, plants treated with 3000 ppb NO showed a better growth in all 5 lines (Figure 9 and Fig 10). However, the promoting effect differed in the 5 lines. For Pgb silence or knock out (Pgb1- or Pgb2-) *Arahidopsis*, NO fumigation had a slight promoting effect. While for Pgb

overexpression *Arabidopsis* (Pgb1+ and Pgb2+), NO fumigation had an obvious and significant promoting effect (Figure 9 and Fig 10). Especially in Pgb 2 overexpression plants, the red senescence phenotype was significantly delayed after 20 days 3000 ppb NO treatment, and the shoot looks much higher after 30 days 3000 ppb NO treatment.

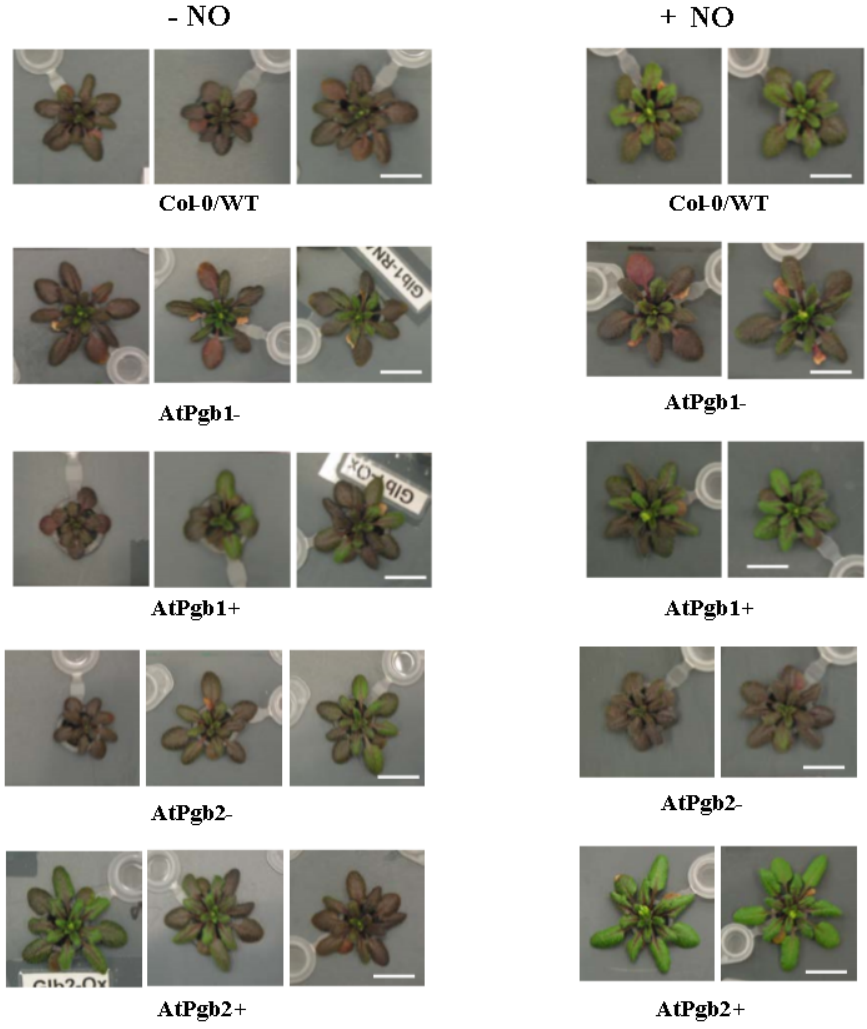


Figure 9 NO fumigation has different growth effect in phytoglobin transgenic lines.

Plants germinated and grew for twelve days in N-containing hydroponic medium. Afterwards, they were transferred in N-free medium and fumigated with air supplemented with 0 (-NO) or 3000 ppb NO (+NO). Photos were taken after ca. 20 days of treatment. Modified from Kuruthukulangarakoola *et al*., 2017.

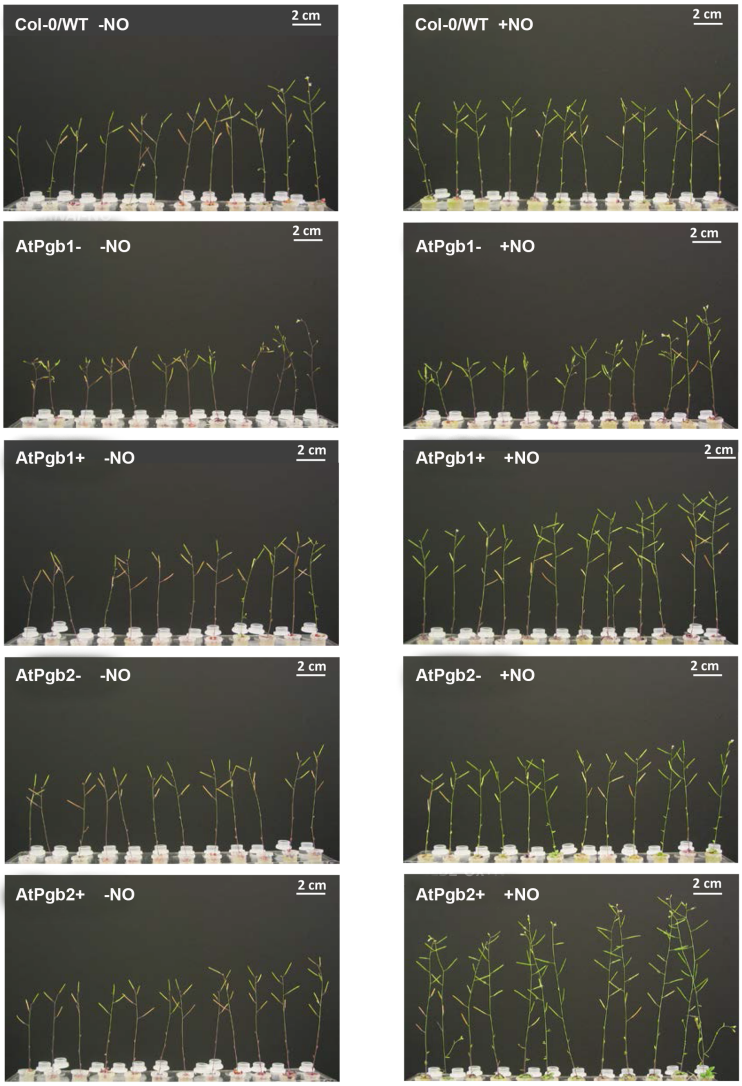


Figure 10 NO fumigation promote shoot growth after 30 days treatment.

Plants germinated and grew for twelve days in N-containing hydroponic medium. Afterwards, they were transferred in N-free medium and fumigated with air supplemented 0 (-NO) or with 3000 ppb NO (+NO). Photos were taken after ca. 30 days of treatment. Modified from Kuruthukulangarakoola *et al*., 2017.

After 30 days of treatment, the rosette size, shoot length, number of siliques and seed yield of *Arabidopsis* were measured (Figure 11). Generally, the rosette size, shoot length, number of siliques and seed yield was increased in NO-treated Pgb overexpressing lines, especially in Pgb2+ *Arabidopsis* (Figure 11). The rosette diameter was increased in WT, AtPgb1+ and AtPgb2- plants, but was not affected in AtPgb1- and AtPgb2+ plants. The siliques per plant was increased around 20% and 100% in AtPgb1+ and AtPgb2+ lines, respectively. The shoot length in

AtPgb2+ plants was increased from 60 mm to 100 mm after NO treatment. Moreover, the seed weight was significantly increased in AtPgb1+, AtPgb2+ and AtPgb2- lines after NO treatment, but no differences for WT and AtPgb1- plants.

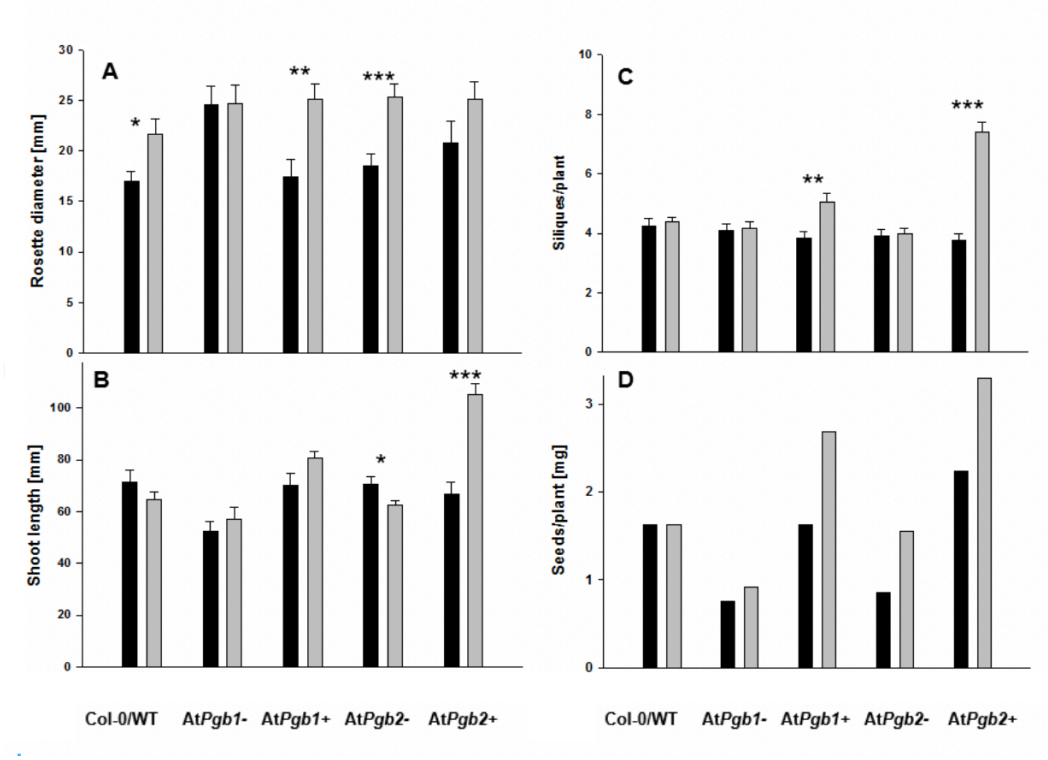


Figure 11 Phenotypical parameters of hydroponically cultivated *Arabidopsis* plants.

Rosette size of plants (a, 32-old-day plants), shoot length (b, 42-old-day plants), number of siliques (c, 42-old-day plants) and seed yield (d, 42-old-day plants) in hydroponically cultivated *Arabidopsis* plants with altered AtPgb1 or AtPgb2 expression. Plants were exposed to ambient (black) and 3000 ppb NO (grey). Data represent means ± SE of 15-20 plants for phenotypical parameters. Asterisks indicate statistically significant differences from WT (Student´s t-test; *P<0.05, **P<0.01, ***P<0.001). Modified from Kuruthukulangarakoola *et al*., 2017.

3.1.2 NO fumigation increased RSNO, nitrite and nitrate level

The uptake of NO by plants through stomata was proposed in the 1990s (Wellburn, 1990; Stulen *et al*., 1998), and this hypothesis was supported by a study that showed the expansion of the leaf disc in pea plants after NO fumigation (Leshem *et al*., 1998). To demonstrate the NO uptake by plant leaves, we measure the RSNO, nitrite and nitrate level in *Arabidopsis* leaves.

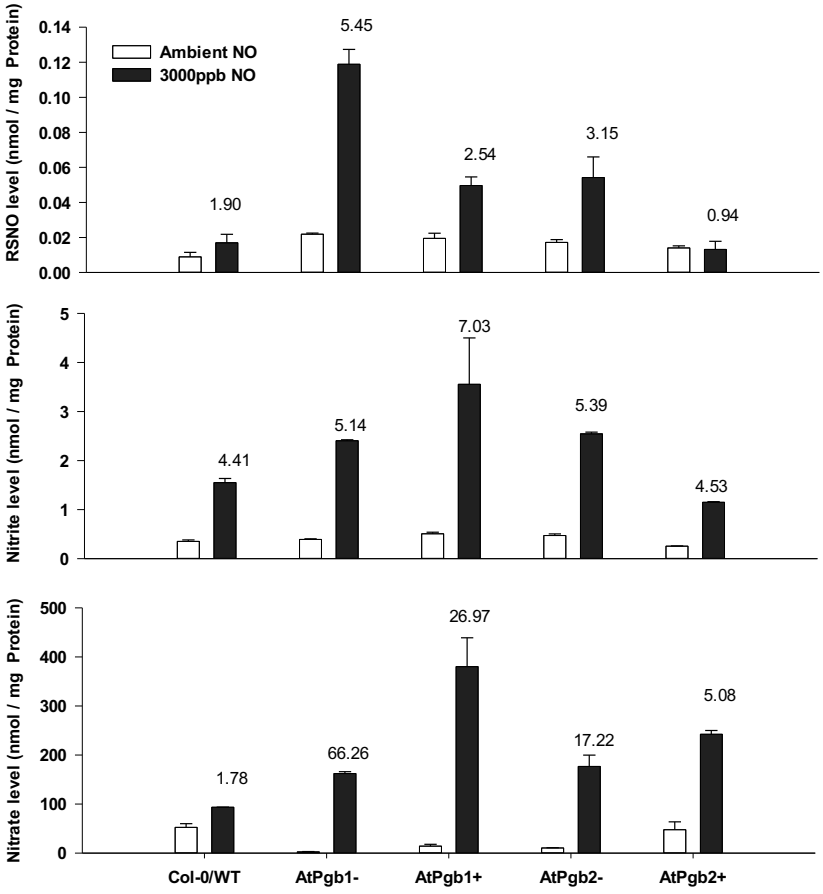


Figure 12 NO fumigation increases RSNO and N-containing ion levels in plant leaves.

Plants germinated and grew for twelve days in N-containing hydroponic medium. Afterwards, they were transferred in N-free medium and fumigated with air supplemented with 0 ppb or 3000 ppb NO. Leaf samples were harvested after 20 d of treatment and nitrosothiols, nitrite and nitrate contents were determined. White: Ambient NO; Black: 3000 ppb NO. Data represent means of 5 plants. The number above the bars for each plant line represents the ratio of the estimated quantity for the plants fumigated with 3000 ppb NO gas to that for plants fumigated with ambient NO.

Clearly, fumigation with 3000 ppb NO strongly increased RSNO, nitrite and nitrate level in plant leaves. For RSNO, Pgb 1 silence (Pgb1-) *Arabidopsis* showed the highest level (0.118 nmol /mg protein) after NO treatment, while for nitrite, Pgb 1 overexpression line (Pgb1-) showed the highest level (3.3 nmol/mg protein). Compared to RSNO and nitrite, the nitrate concentration in *Arabidopsis* were much higher (Figure 12). Among the 5 lines, nitrate level in NO treated Pgb1 and Pgb2 overexpressing *Arabidopsis* were around 360 nmol per mg protein and 250 nmol per mg protein, which is much higher than in WT (90 nmol per mg protein)

(Figure 12). These results support the existence of a NO-fixation mechanism, resulting in enhanced N-assimilation in *Arabidopsis* plants and better growth and development.

3.1.3 NO treatment and phytoglobins did not affect the nitrate uptake in *Arabidopsis*

NO was regard as an important regulator of N assimilation in previous study (Frungillo *et al*., 2014). To check whether NO fumigation and the changed internal NO levels in Pgb transgenic lines can affects root-dependent N uptake, we performed a ^{15}N- nitrate tracer experiment. Hydroponic *Arabidopsis* were grown in $^{15}NO_3^-$ containing medium under ambient NO and 3000 ppb NO fumigation, ^{15}N level in plant leaves were measured after 1, 4 and 11 days. From the results, we found that no significant differences between ambient NO and 3000 ppb NO treatment, and no significant differences among Pgb transgenic lines (Figure 13).

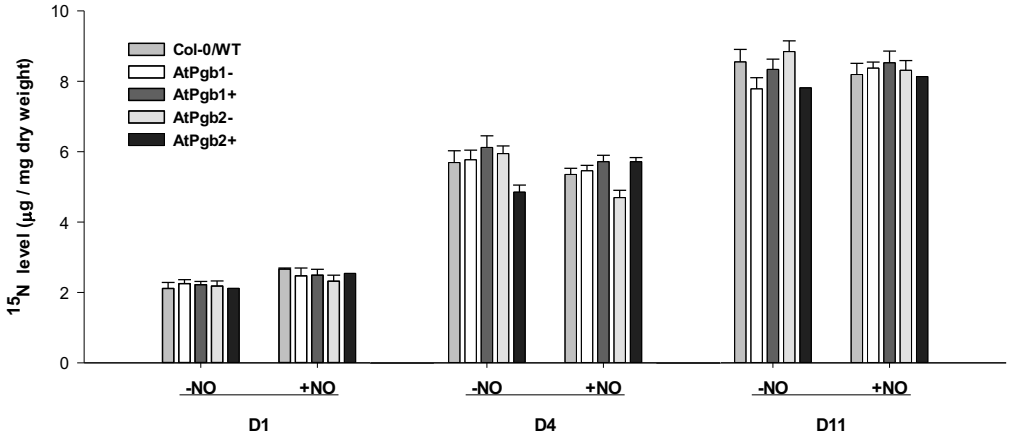


Figure 13 ^{15}N level in *Arabidopsis* leaves under ^{15}N nitrate medium or without 3000 ppb NO fumigation.

Plants germinated and grew for 12 days in N-containing hydroponic medium. Afterwards, they were transferred in medium with $^{15}NO_3^-$-containing medium under ambient NO (-NO) or 3000 ppb NO fumigation (+NO). ^{15}N content was determined in plant leaves after 1, 4- and 11-days treatment. Data represent means ±SE of 10 plants.

3.1.4 Phytoglobin dependent NO uptake by plant leaves

To further demonstrate NO uptake and the importance of Pgb proteins for N accumulation, we fumigated hydroponic cultures of *Arabidopsis* with 250 ppb ^{15}NO. Again, the plants were first cultivated in N-containing medium and transferred to N-free medium before ^{15}NO fumigation. Samples were harvested after 4, 6 and 11 days of fumigation and ^{15}N content in leaves was determined with an Isotope Ratio Mass Spectrometer (IRMS, delta V Advantage, Thermo Fisher, Dreieich, Germany) coupled to an Elemental Analyzer (Euro EA, Eurovector, Milano, Italy).

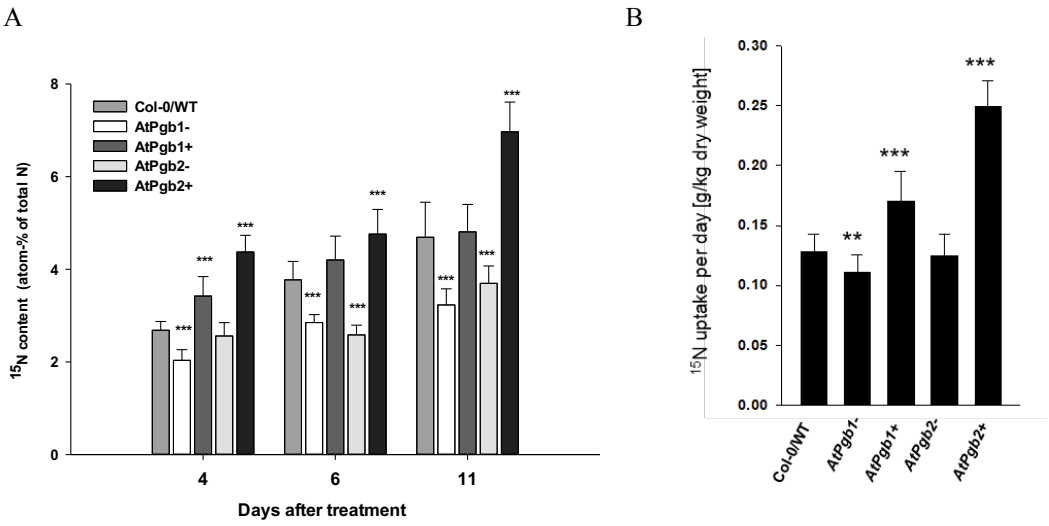


Figure 14 ^{15}N level in *Arabidopsis* leaves after ^{15}NO fumigation.

Plants germinated and grew for 12 days in N-containing hydroponic medium. Afterwards, they were transferred in N-free medium. ^{15}N content was determined in plant exposed to 250 ppb 15NO for 4, 6 and 11 days (A). The 15N uptake per day was calculated based on the 15N data after four days of 15NO fumigation (B). Data represent means ±SE of eight plants. Asterisks indicate statistically significant differences from WT (Student's t-test; *P<0.05, **P<0.01, ***P<0.001).

^{15}N accumulation could be observed in all lines during the treatment. The highest accumulation was detected in Pgb2+ plants (Figure 14A, up to almost 7% of total N content after 11 days of 15NO fumigation). After 6 days of treatment WT plants accumulated the same amount of ^{15}N as the Pgb1+ plants. The lowest ^{15}N accumulation was observed in Pgb1- and Pgb2- lines. These results demonstrate that overexpression of Pgb1 or Pgb2 positively affects ^{15}N accumulation in plants

and that both Pgb isoforms promote the use of NO as N source. Based on the ^{15}N data after 4 days of ^{15}NO treatment, we calculated a daily uptake of 250 mg N/kg dry matter for Pgb2 overexpressing plants and 170 mg N/kg dry matter for Pgb1 overexpressing plants, which is almost 100% and 35% more than in WT plants (Figure 14B).

Soil is recognized as an important source of tropospheric NO (Davidson and Kingerlee, 1997; Pilegaard, 2013). The estimated global NO emission inventories for soil ranged from 6.6 to 33 TgN yr-1 (above soil) or from 4.7 to 26.7 TgN yr^{-1} (above canopy) dependent on whether the studies considered canopy reduction factor, which is adopted to account for canopy uptake of NOx (Hudman *et al*., 2012; Huang and Li, 2014). The NO uptake by the plants was further demonstrated by 'scavenging' NO released from soil (Figure 15). In a similar experiment, reduction of soil-emitted NO has been already demonstrated in WT *Arabidopsis* plants (Mur *et al*., 2011).

The experiment was performed in a closed system/cuvette. When placing pots with soil and *Arabidopsis* rosettes in the cuvette, we detected lower NO levels than when the plants were cut and removed. When the excised plants were reapplied to the surface of the soil, NO levels were again reduced. In our experimental system ca. 100 ppb NO accumulated in the closed cuvette within 30 min (Figure 15A), when the plants were cut and removed (soil-released NO). In general, compared to WT *Arabidopsis*, Pgb overexpression plants removed higher levels of NO, while at the same time, Pgb knockdown or silence plants removed lower levels of NO. Pgb2+ plants reduced the levels of soil-released NO up to 4ppb per gram fresh weight within 30min, which is more than the double amount of NO removed by WT plants and four times more than by Pgb2- plants (Figure 15B).

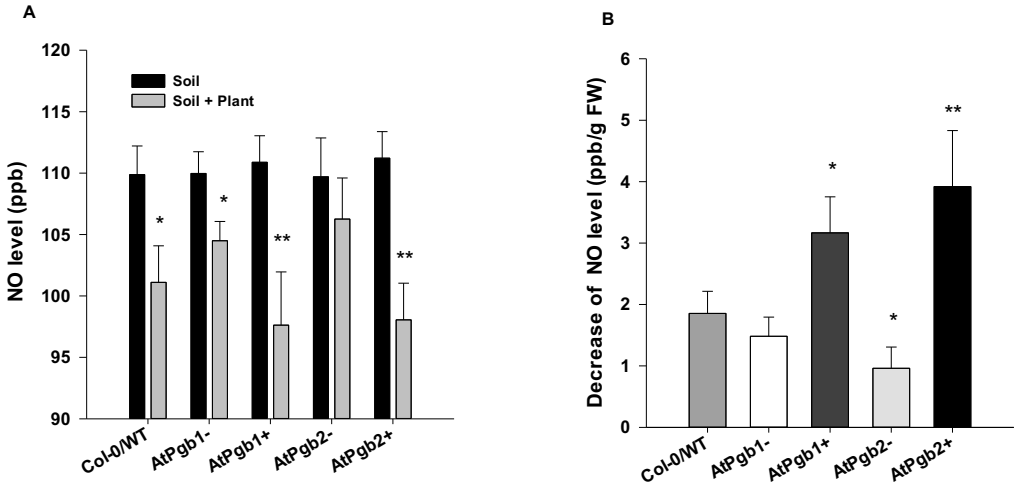


Figure 15 NO uptake of *Arabidopsis* plants.

A**.** NO level in reaction chamber with and without plant. In this experiment two pots containing 2 four-weeks-old *Arabidopsis* plants were placed in a closed reaction chamber and the NO level were measured after 30 min (grey, L1). Then the plants were cut at the soil surface and the NO levels were determined again after 30 min (black, L2). The experiment was done in 9 replicates. In all experiments, asterisks indicate statistically significant differences between the measured NO levels emitted from pots with plants (grey) and the same pots without plants (black) (Student´s t-test; *P<0.05, **P<0.01). B. NO uptake level of different *Arabidopsis*. The difference between both levels (L2–L1) reflects the amount of NO taken up in 30min by the different genotypes. Asterisks indicate statistically significant differences from WT (Student's t-test; *P<0.05, **P<0.01).

3.1.5 Nitrogen supply did not significantly affect the phytoglobin dependent NO uptake

The results above showed that NO uptake by *Arabidopsis* is Pgb dependent under N limited conditions. To analyze whether the N supply affect the NO uptake, we performed the ^{15}NO fumigation experiment with hydroponic *Arabidopsis* grown under N deficient (medium without N) or N sufficient (medium with N) conditions. ^{15}N levels were determined in plant leaves after 2 and 5 days fumigated with 200 ppb of ^{15}NO (Figure 16). We found that the ^{15}N level plant leaves is Pgb dependent in both N deficient and sufficient conditions. ^{15}N level are higher in Pgb overexpression *Arabidopsis* and lower in reduced Pgb lines compared to WT. Besides, we noted that the ^{15}N level in WT plants under N deficient conditions are higher compared to plants under N sufficient conditions after 5 days treatment

(Figure 16). However, we did not observe such differences in other lines (Figure 16).

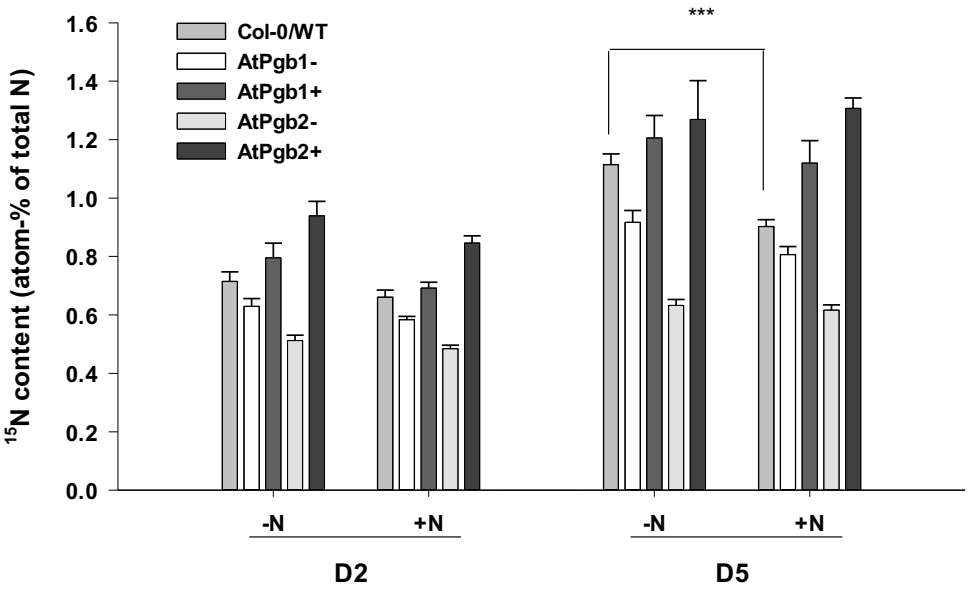


Figure 16 ^{15}N level in *Arabidopsis* leaves after ^{15}NO fumigation with or without N source.

Plants germinated and grew for 12 days in N-containing hydroponic medium. Afterwards, they were transferred in medium with (+N) or without N (-N). ^{15}N content was determined in plant exposed to 200 ppb ^{15}NO for 2 and 5 days. Data represent means ±SE of 5 plants. Asterisks indicate statistically significant differences (Student's t-test; *P<0.05, **P<0.01, ***P<0.001). Medium with or without N were prepared as Table 2.

In summary, these results support the existence of a Pgb dependent NO-fixation mechanism, enabling use of atmospheric NO as N source for plant growth. Moreover, in this way the level of atmospheric NO is reduced, which could be of importance in context of air quality.

3.2 NO-fixation by phytoglobins in barley

The results in *Arabidopsis* showed that overexpressing Pgb 1 or 2 genes resulted in an increase in rosettes size and weight, vegetative shoot thickness and seed yield than in wild-type plants under NO fumigation. Such growth promoting effects of

NO fumigation by Pgb dependent NO-fixation could be of importance for crop plants.

To investigate the effect of high atmospheric NO concentrations on crop plants and the role of Pgbs under these conditions, we performed a long-term study on barley "Golden Promise" wild type (WT), class 1 Pgb knockdown (HvPgb1.1-) and class 1 Pgb overexpression (HvPgb1.1+) lines.

3.2.1 Plant phytoglobins – relationships and expression pattern

In barley, the Pgb 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 Pgb gene in barley (Access number: HORVU1Hr1G076460.3 in IPK and AK376331.1 in NCBI).

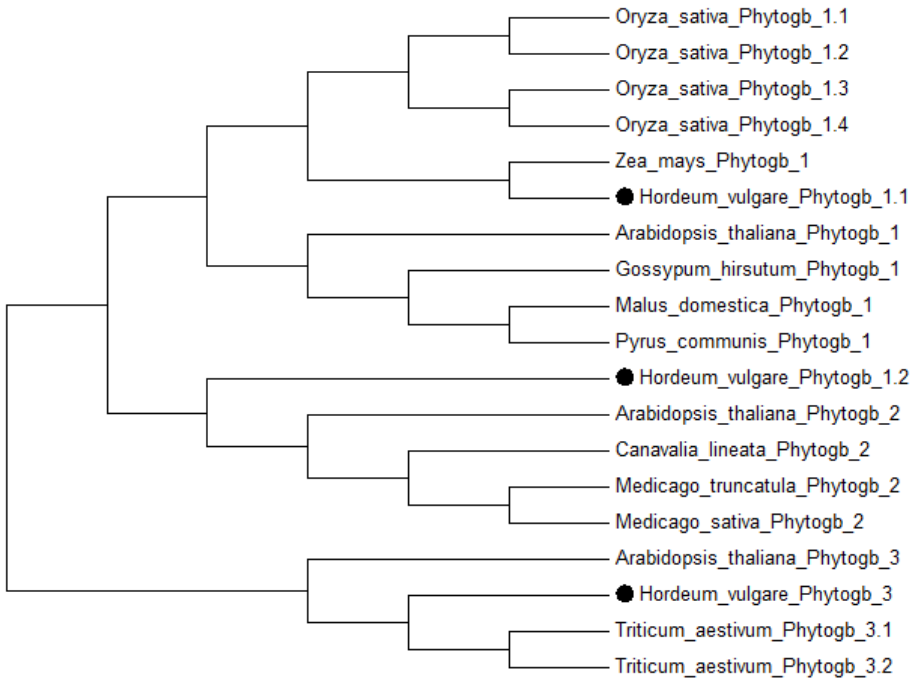


Figure 17 Phylogenetic tree of Pgbs.

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 Pgbs are listed in supplementary Table 1.

A phylogenetic tree was constructed with Pgb proteins in other plant species by the Neighbor–Joining method using MEGA 6.06). Based on the phylogenetic tree (Figure 17) and the rules for Pgb genes (Hill *et al*. 2016), the new gene was named *HvPgb1.2*. The amino acid sequences alignment analysis revealed a 74.7% homology of HvPgb1.2 to barley Pgb1.1 and a 70.1% homology to *Arabidopsis* Pgb1 (Figure 18).

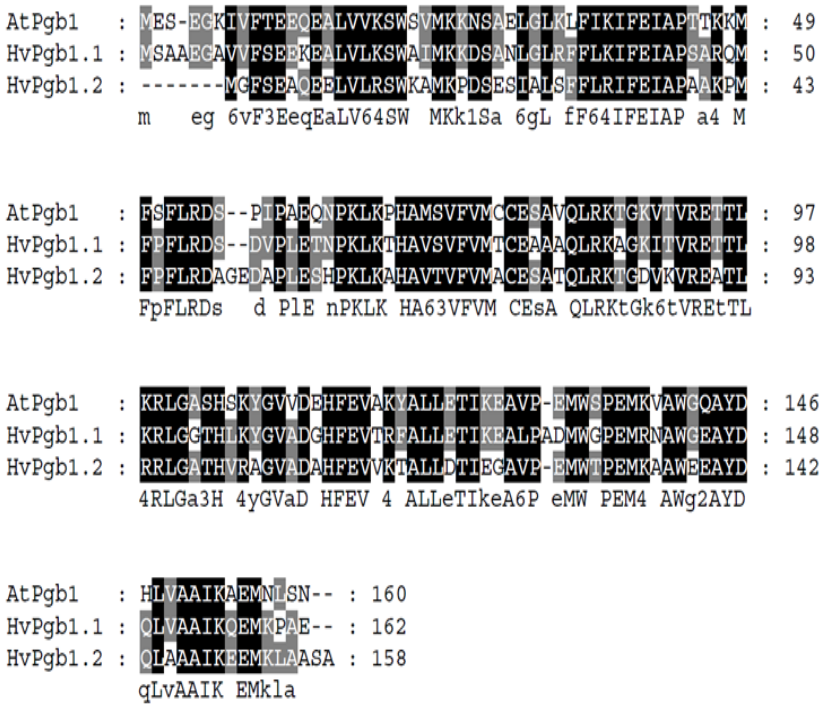


Figure 18 Amico acid sequences comparison of HvPgb1.1, HvPgb1.2 and AtPgb1.

The amino acid sequences alignment analysis was performed by online software - Pairwise Sequence Alignment. HvPgb1.2 shared 74.7% homology to HvPgb1.1 and 70.1% homology to AtPgb1.

We compared the expression patterns of the *HvPgb1.1*, *HvPgb1.2* and *HvPgb3* in different tissues according to the collected data from morexGenes-Barley RNA-seq Database. In general, the expression levels of *HvPgb1.2* and *HvPgb3* are much higher in all plant tissues compared to *HvPgb1.1* (Figure 19). The highest expression levels of HvPgb1.1 in roots (ROO1 and ROO2), etiolated seedlings (ETI) and shoots (LEA) compared to other tissues. *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* is relatively balanced in all tissues (Figure 19).

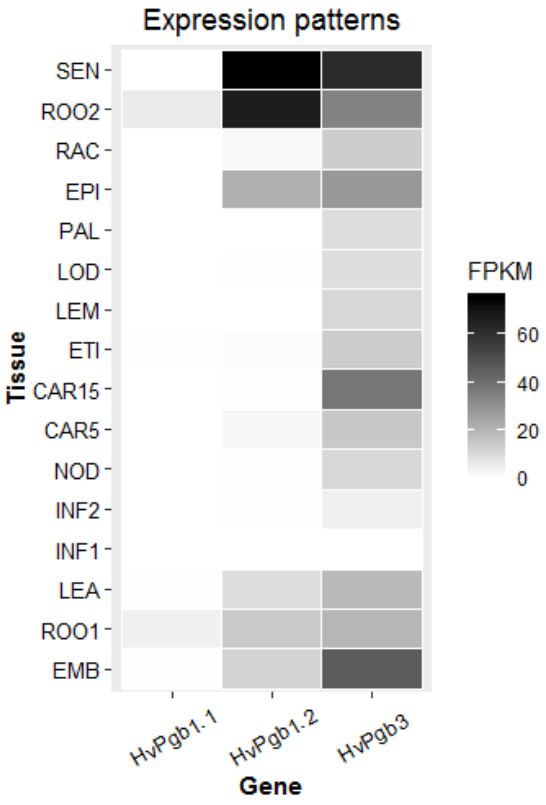


Figure 19 Expression patterns of *HvPgb1.1*, *HvPgb1.2* and *HvPgb3* in different tissues.

Data was collected from morexGenes-Barley RNA-seq Data, *HvPgb1.1* (HORVU4Hr1G066200.1), *HvPgb1.2* (HORVU1Hr1G076460.3), *HvPgb3* (HORVU0Hr1G021640.3). EMB: 4-day embryos; ROO1: Roots from seedlings (10 cm shoot stage); LEA: Shoots from seedlings (10 cm shoot stage); INF1: Young developing inflorescences (5mm); INF2: Developing inflorescences (1-1.5 cm); NOD: Developing tillers, 3rd internode (42 DAP); CAR5: Developing grain (5 DAP); CAR15: Developing grain (15 DAP); ETI: Etiolated seedling, dark cond. (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).

3.2.2 NO fumigation enhances expression level of *HvPgb1.1*

To analyze whether Pgb 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 days.

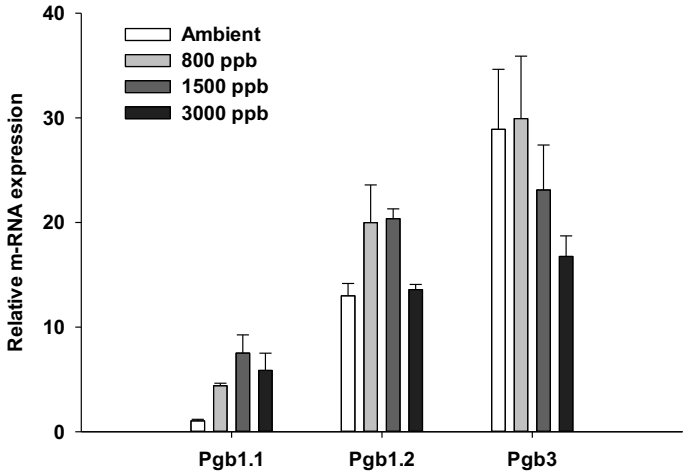


Figure 20 Transcription levels of of *HvPgb1.1*, *HvPgb1.2* and *HvPgb3* in barley leaves after NO fumigation.

Leaf samples were taken after 20 days of NO fumigation. *HvGADPH* and *Hvactin* were used as housekeeping gene. Each data represents means ± SE (n=3). The expression levels of *HvPgb1.2* and *HvPgb3* were normalized to *HvPgb1.1*.

Clearly, 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 were only slightly or not affected by NO fumigation, respectively (Figure 20). The expression level of *HvPgb1.2* increased only 1.5-fold in presence of 800 ppb and 1500 ppb of NO (Figure 20) and decreased to the control level if plants were fumigated with 3000 ppb. The transcript levels of HvPgb3 decreased in presence of NO concentrations higher than 800 ppb. Although accumulation of *HvPgb1.1* transcript is enhanced after NO fumigation, its transcript levels are still clearly lower than the levels of *HvPgb1.2* and *HvPgb3* (Figure 20). These results

indicated that *HvPgb1.1* might play an important role in conditions with enhanced levels of NO.

3.2.3 NO fumigation promotes growth of barley plants overexpressing HvPgb 1.1

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 analyzed in presence of different NO concentrations. Barley plants with class 1 Pgb overexpression (HvPgb1.1+) and silence (HvPgb1.1-) lines were obtained from Kim Hebelstrup (Aarhus University, Hebelstrup *et al.* 2014).

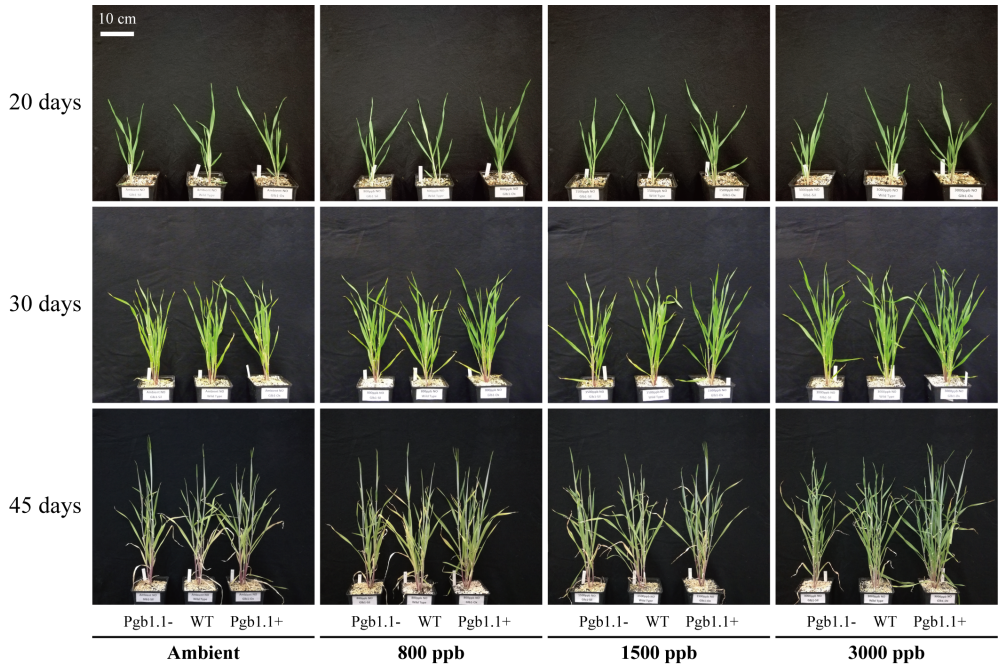


Figure 21 Phenotype of barley plants fumigated with different concentrations of NO for 20 days, 30 days and 45 days.

The plants were growth in climate chamber with different NO level (Ambient, 800 ppb, 1500 ppb and 3000 ppb). Photos were taken at 20, 30 and 45 days.

For 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 ppb, 1500 ppb and 3000 ppb of NO. Plants were grown in climate chambers under highly controlled conditions (Figure 7). During the

whole growth phase season, nutrient solutions without N were added every two weeks.

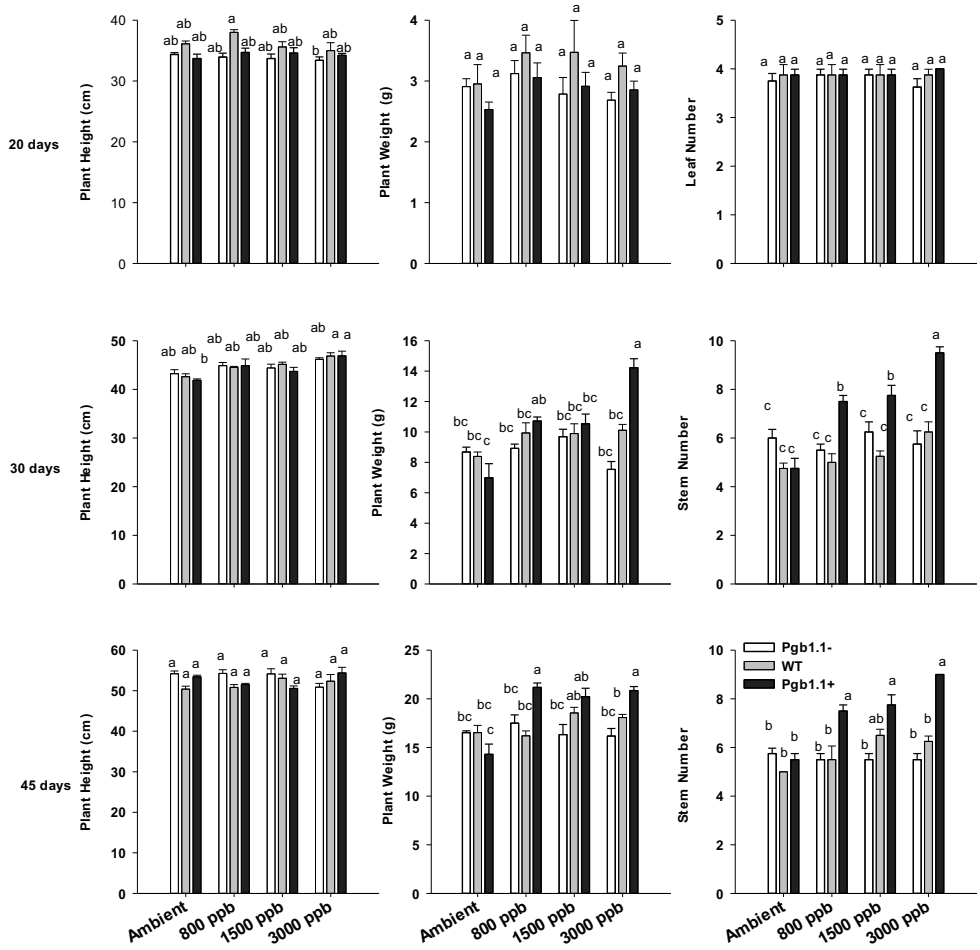


Figure 22 Growth parameters of of barley plants fumigated with different concentrations of NO for 20 days, 30 days and 45 days.

The growth prameters of plant height, plant weight and leaf numbers or stem numbers were measured. Each data represents means ± SE of at least 4 plants. Different letters indicate significant differences among treatments at $P < 0.05$, according to Tukey's test.

After 20 days of treatment, there were no obvious phenotypic differences – not only among the three different lines, but also among the different NO conditions (Figure 21). According to leaf number analysis during the first 16 days of growth, no obvious difference in development of the different barley lines could be seen (Figure S1). The expression level of *HvPgb1.2* and *HvPgb3* in the HvPgb1.1- and HvPgb1.1+ plants is only slightly different from the expression levels in WT plants

(Figure S2). Expression of both genes is 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 (Figure S2). 30 and 45 days 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 (Figure 22). In presence of 3000 ppb NO, the stem number and plant weight of HvPgb1.1+ plants increased about 2-fold in comparison to ambient conditions (Figure 22). The plant height of HvPgb1.1+ plants showed a slight increase with the increasing NO concentration after 30 days treatment, but no differences were observed after 45 days treatment (Figure 22). These results demonstrate that NO fumigation significantly promotes growth of HvPgb1.1+ plants, while the same NO concentration has no obvious effect on growth of WT and HvPgb1.1- plants.

3.2.4 NO fumigation increases barley yield in HvPgb1.1 overexpressing line

To analyze the effect of high concentrations of atmospheric NO on yield of barley plants expressing different levels of HvPgb1.1, we measured 8 yield parameters including dry matter weight per plant (DWP), plant height (PH), spike length excluding awns (SL), spikes per plant (SP), spike weight (SW), kernel numbers per plant (KNP), kernel weight (KW), and kernel weight per plant (KWP).

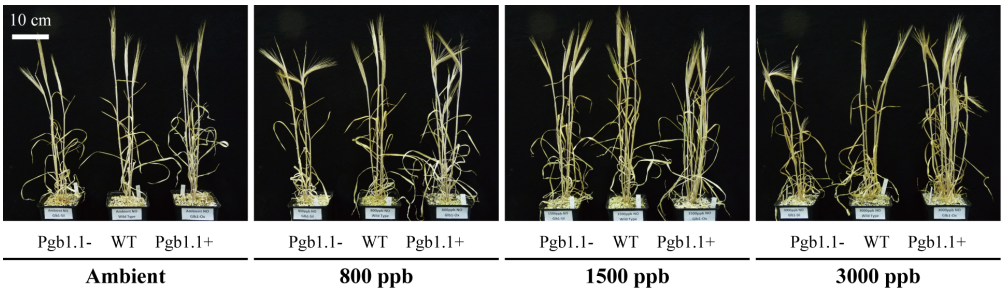


Figure 23 Phenotypical of barley plants fumigated with different concentrations of NO for 80 days.

The plants were growth in climate chamber with different NO level (Ambient, 800 ppb, 1500 ppb and 3000 ppb). Photos were taken at 80 days.

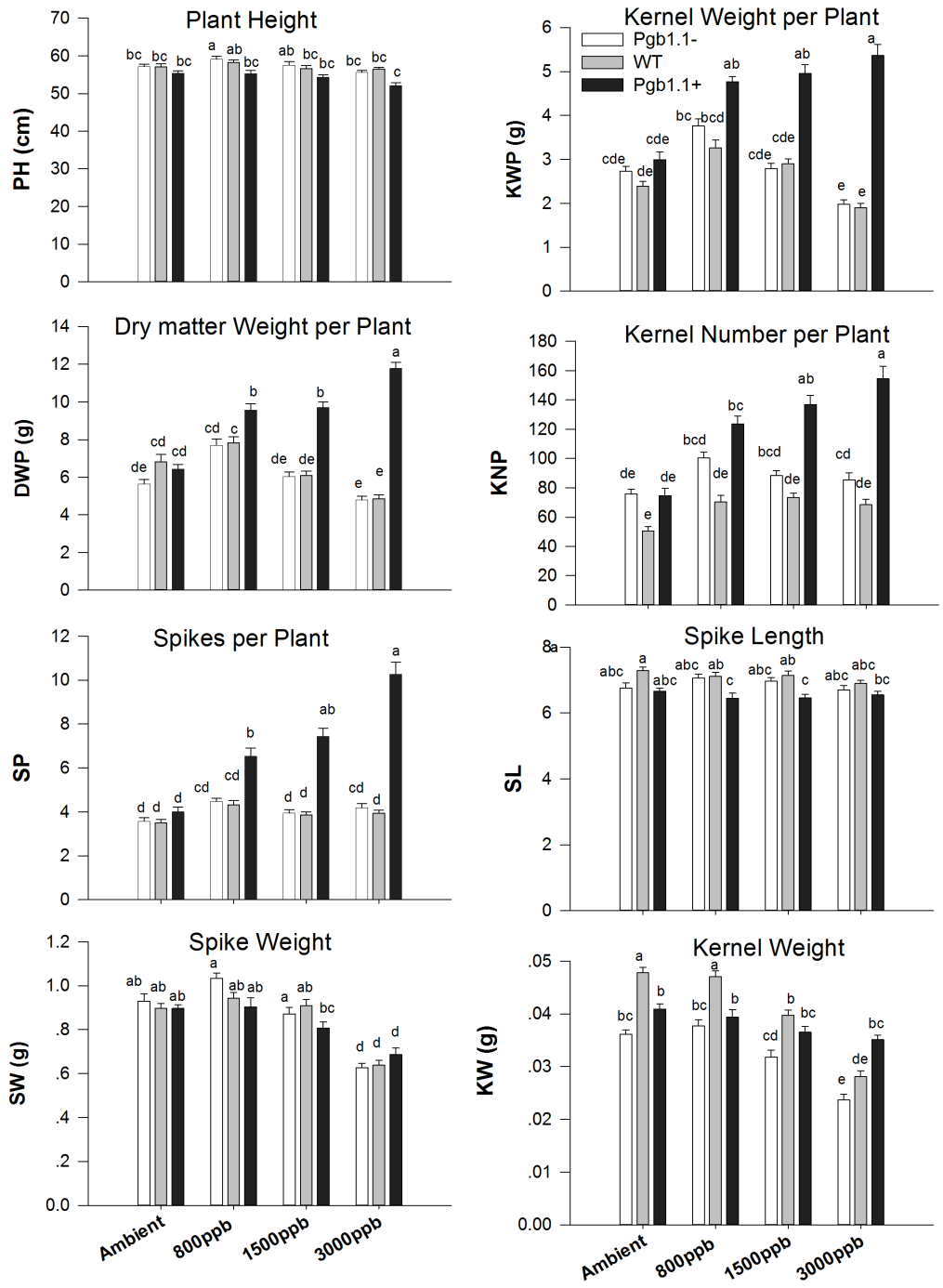


Figure 24 Yield parameters of barley after 80 days NO fumigation.

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) were measured after 80 days NO fumigation. Each data represents means ± SE (n=15). Different letters indicate significant differences among treatments at $P < 0.05$, according to Tukey's test.

After 80 days of treatment, we observed a clear promoting effect in Pgb overexpression barley (HvPgb1.1+) with increasing NO concentration application,

especially under 3000 ppb NO (Figure 23). No differences in PH and SL were observed, neither among the three barley lines (WT, HvPgb1.1+ and HvPgb1.1-) nor among the different NO conditions (Figure 24). However, NO fumigation significantly increased the DWP, KNP, and KWP level in HvPgb1.1+ plants. DWP, KNP and KWP in HvPgb1.1+ plants are up to two-fold higher when fumigated with 3000 ppb comparison to ambient conditions. In contrast, the SW and KW levels were decreased with increasing NO concentration (Figure 24). Such a negative correlation between the spike number and kernel weight is often observed (Dorostkar *et al*., 2015). From the results of DWP and KNP, we noted that in WT and HvPgb1.1- lines, 800 ppb fumigation had a promoting effect, while 3000 ppb fumigation led to a reduction of both parameters.

3.2.5 Effect of enhanced atmospheric NO on nitrogen metabolism in barley plants overexpressing HvPgb1.1

To analyse whether atmospheric NO affects the nitrogen metabolism in WT and transgenic Pgb barley, we measure the nitrite, and nitrate levels in leaves of barley plants fumigated for 30 days with 3000 ppb of NO. Under ambient conditions, no significant differences between the three different lines have been detected. However, 3000 ppb NO increased the nitrite and nitrate level in all 3 lines (Figure 25). The nitrate level in HvPgb1+ plants are increased 3.5-fold, while in HvPgb1- and WT plants the nitrate levels only 1.4 and 1.7- times increased, respectively (Figure 25B).

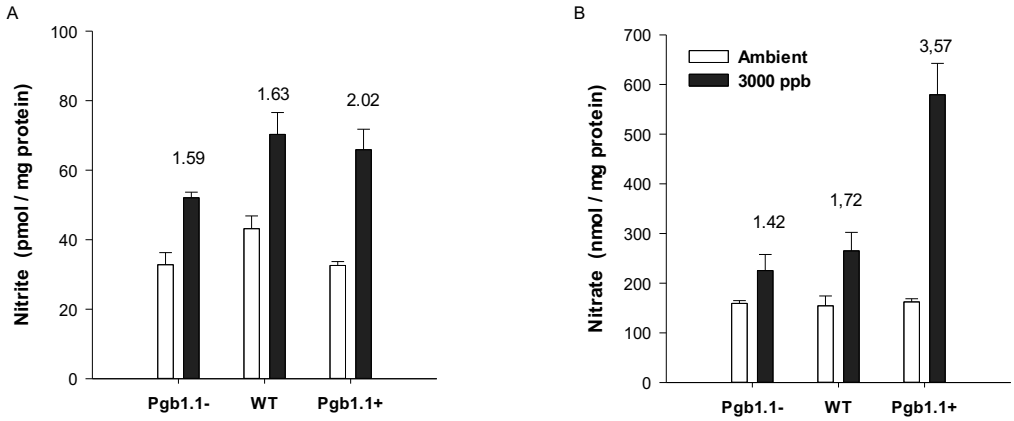


Figure 25 Nitrite and nitrate content of barley plants after 30 days of NO fumigation.

The number above the bars indicate the ratio of 3000 ppb NO and ambient NO fumigated plants. Each data represents means ± SE (n=4).

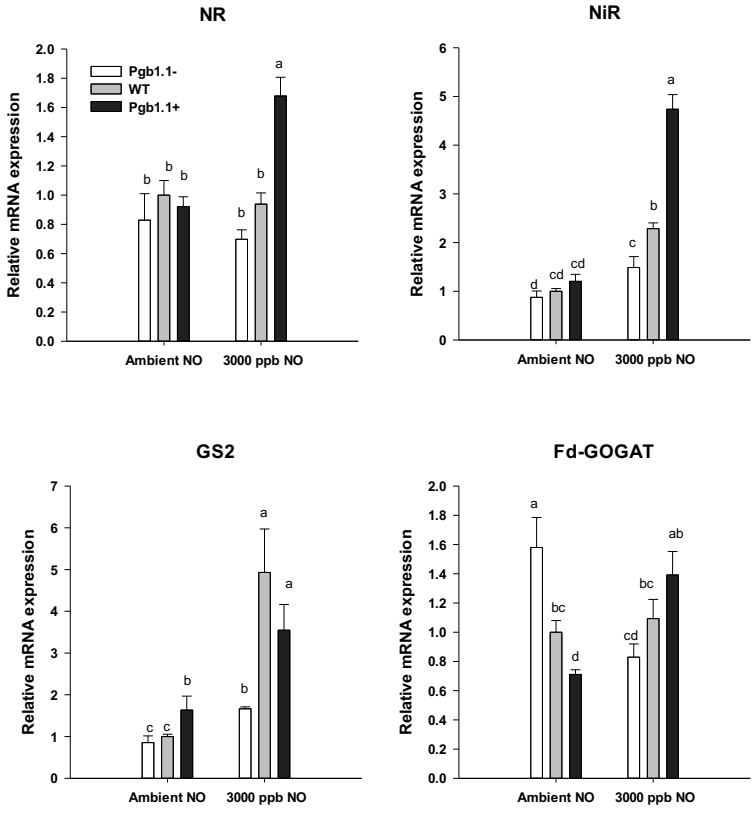


Figure 26 Transcription levels of of HvNR, HvNiR, HvGS2 and HvFd-GOGAT in barley leaves after NO fumigation.

Leaf samples were taken after 30 days of NO fumigation. HvGADPH and HvACTIN were used as housekeeping genes. Each data represents means ± 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.

Such an increase in N metabolites activated also genes of the N metabolism. Especially in HvPgb1.1+ plants, expression of NR, nitrite reductase (NiR), glutamine synthetase 2 (GS2) and ferredoxin-dependent glutamate-oxoglutarate-aminotransferase (Fd-GOGAT) is upregulated in NO fumigated plants in comparison to plant grown under ambient level of NO (Figure 26). In WT and HvPgb1.1- plants, transcript levels of nitrite reductase and glutamine synthetase were increased by NO treatment.

Since nitrogen supply correlates with leaf chlorophyll concentration (Ercoli *et al*. 1993), we measured the chlorophyll content in barley leaves after 20 and 35 days of NO fumigation. Twenty days of fumigation did not affect the chlorophyll content (Figure 27 and 28). However, as the plants are cultivated under N-limited conditions, the chlorophyll content was already decreased under ambient conditions from 35 µg cm^{-2} (20 days of treatment) to 22 µg cm^{-2} (35 days of treatment) (Figure 26A and Figure 27A). In plants fumigated for 35 days, the chlorophyll content correlated with increasing NO concentration. Especially in plants overexpressing HvPgb1.1 and treated with 3000 ppb of NO the chlorophyll content in the older plants (35 days of fumigation) is still as high as in the younger plants (20 days of fumigation) (Figure 27A and Figure 28A).

The effective quantum yield of PSII (ΔF/Fm') 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 (ΔF/Fm' = (Fm' – F)/Fm'). Similar to chlorophyll content, the effective quantum yield of PSII (ΔF/Fm') in HvPgb1.1+ lines increased with NO concentration increased only in older plants (35 days of fumigation, Figure 27B and Figure 28B).

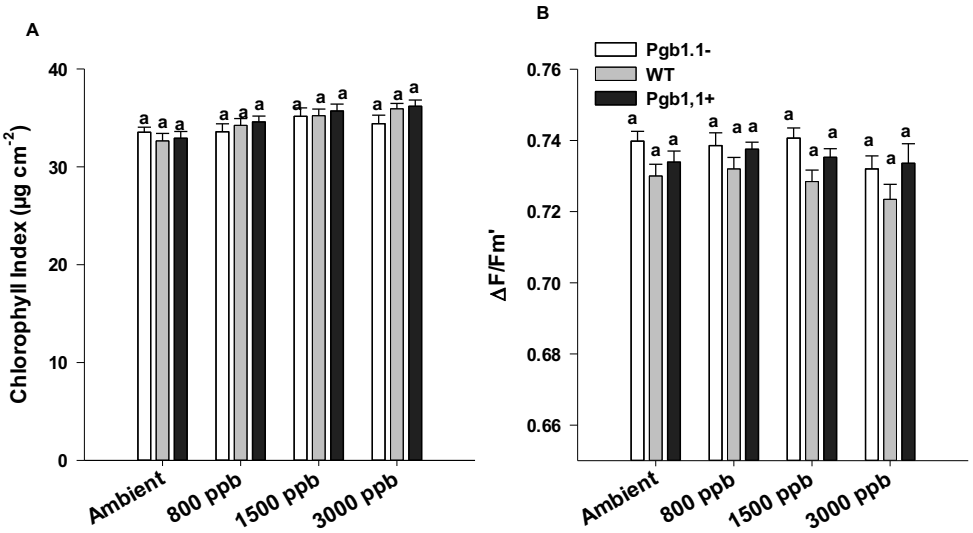


Figure 27 Chlorophyll index and effective quantum yield of PSII (ΔF/Fm') of barley leaves after 20 days NO fumigation.

Chlorophyll index was measured with Dualex Scientific+™, effective quantum yield of PSII was measured with MINI-PAM-II Photosynthesis Yield Analyzer. Both measurements were performed between 13:00 - 15:00. Each data represents means ± SE (n=15). Different letters indicate significant differences among treatments at $P < 0.05$, according to Tukey's test.

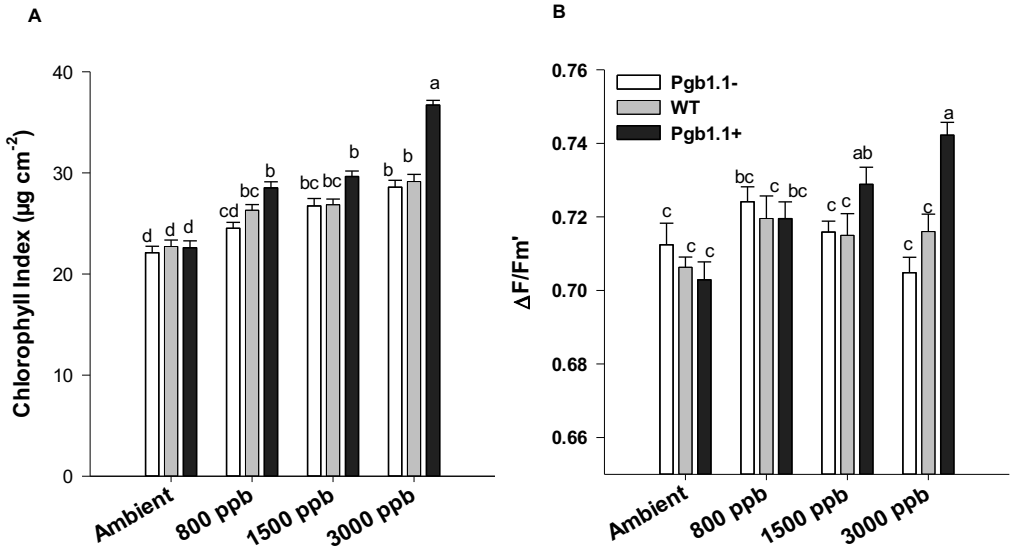


Figure 28 Chlorophyll index and effective quantum yield of PSII (ΔF/Fm') of barley leaves after 35 days NO fumigation.

Chlorophyll index was measured with Dualex Scientific+™, effective quantum yield of PSII was measured with MINI-PAM-II Photosynthesis Yield Analyzer. Both measurements were performed between 13:00 - 15:00. Each data represents means ± SE (n=15). Different letters indicate significant differences among treatments at $P < 0.05$, according to Tukey's test.

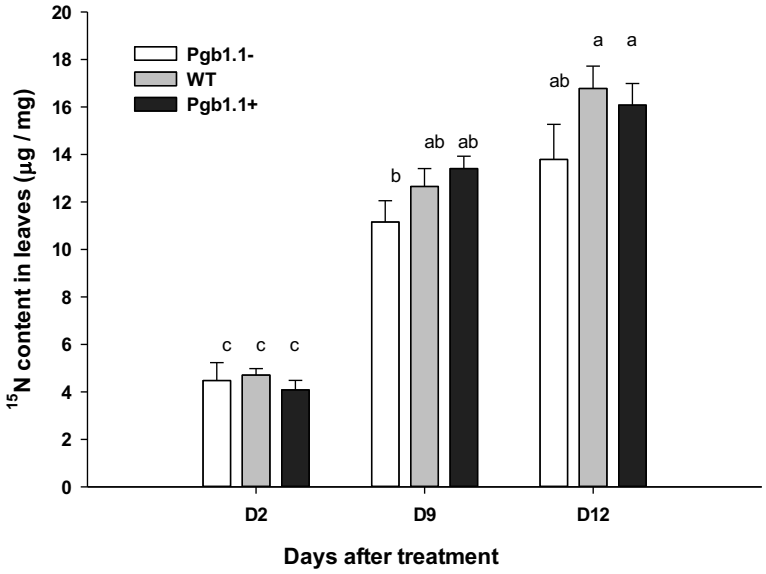


Figure 29 ^{15}N level in barley leaves after 2, 9 and 12 days under nutrient solutions containing $^{15}NO_3^-$.

Plants were grown in soilless matrix composed of vermiculite and sand. Nutrient solutions with 0.3 mM $^{15}NO3-$ were added every day. Leaves were harvested after 2, 9 and 12 days 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 Analyzer. Each data represents means ± SE (n=5). Different letters indicate significant differences among treatments at $P < 0.05$, according to Tukey's test.

To exclude that the enhanced nitrogen content in the HvPbg1.1+ plants is a result of absorption of nitrogen metabolites from the soil, we perform a $^{15}NO_3^-$ tracer application experiment. Barely plants were grown in soilless matrix with additional nutrient solution containing $^{15}NO_3^-$. ^{15}N level in barley leaves after 2, 9 and 12 days were compared among the 3 lines. No significant differences of the ^{15}N level among WT, HvPgb1.1- and HvPgb1.1+ plants (Figure 29).

Then we measured the nitrate and ammonia content in soil of plants treated for 30 days with 3000 ppb NO. Compared to the unused soil (control), both nitrate and ammonia content 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 (Figure 30A and B). Nitrogen ratio in the soil of the different barley

lines grown under different NO conditions were also compared. The nitrogen ratio is decreased after 30 days of treatment in all samples in comparison to unused soil. Interestingly, in the soil of the HvPgb1.1+ plants fumigated with 3000 ppb, the nitrogen ratio is higher than in the other samples (Figure 30C).

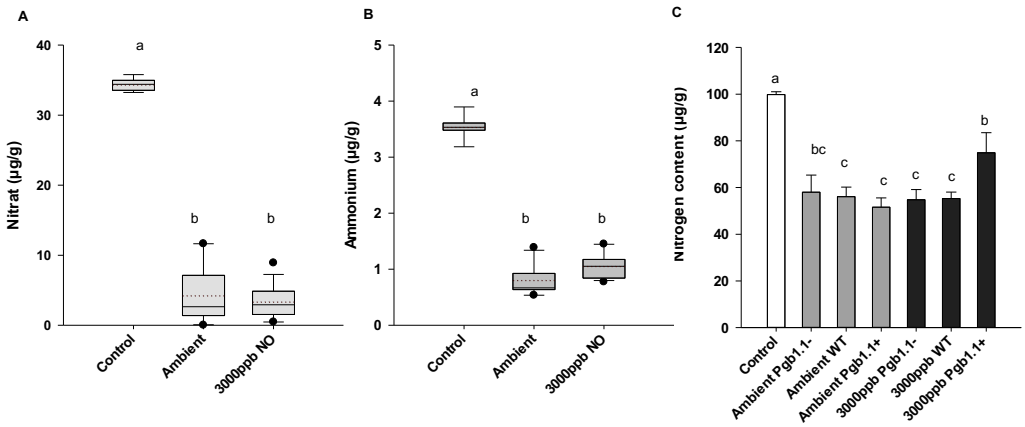


Figure 30 Nitrate, ammonium and nitrogen content in soil after 30 days of NO fumigation. Nitrate, ammonium and nitrogen content in soil were measured after harvesting plants and totally removing plant root. Control means the original soil. For nitrate and ammonium, 15 pots of soil were measured. For nitrogen content, each data represents means ± SE (n=5). Different letters indicate significant differences among treatments at $P < 0.05$, according to Tukey's test.

3.2.6 Uptaken NO is 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 additional N source. To further demonstrate the importance of Pgb 1.1 in NO fixation and N accumulation in barley, we fumigated 20-day old plants with 90 ppb of ^{15}NO for 7 days 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 during a day (Figure S3) dependent on weather conditions, season of the year, and/or activity of soil bacteria.

We found that in ^{15}NO fumigated barley leaves, more ^{15}N was detected in all 3 lines compared to the control fumigated with NO containing natural abundance ^{15}NO (Figure 31A). We calculated a daily uptake for HvPgb1.1+ barley of about

0.09g N/kg dry matter, which is around 2.5 times higher than in WT and HvPgb1.1- plants (Figure 31B).

DNA, RNA and protein are basic N-containing biological molecules. To demonstrate that the uptaken ^{15}N has been transferred to nitrogen metabolism and incorporated into N-containing compounds, we measured the ^{15}N level in DNA, RNA and protein of the barley leaves. We could detect increased ^{15}N amounts in RNA, DNA, and protein in plants fumigated with ^{15}NO. Consistent with the ^{15}N level in dry leaves, the increased ^{15}N level in RNA, DNA, and protein of HvPgb1.1+ lines are much higher compared to WT and HvPgb1.1- lines (Figure 31C, D and E). These results confirmed that the Pgb 1.1 dependent uptake NO can be used as nitrogen source in barley.

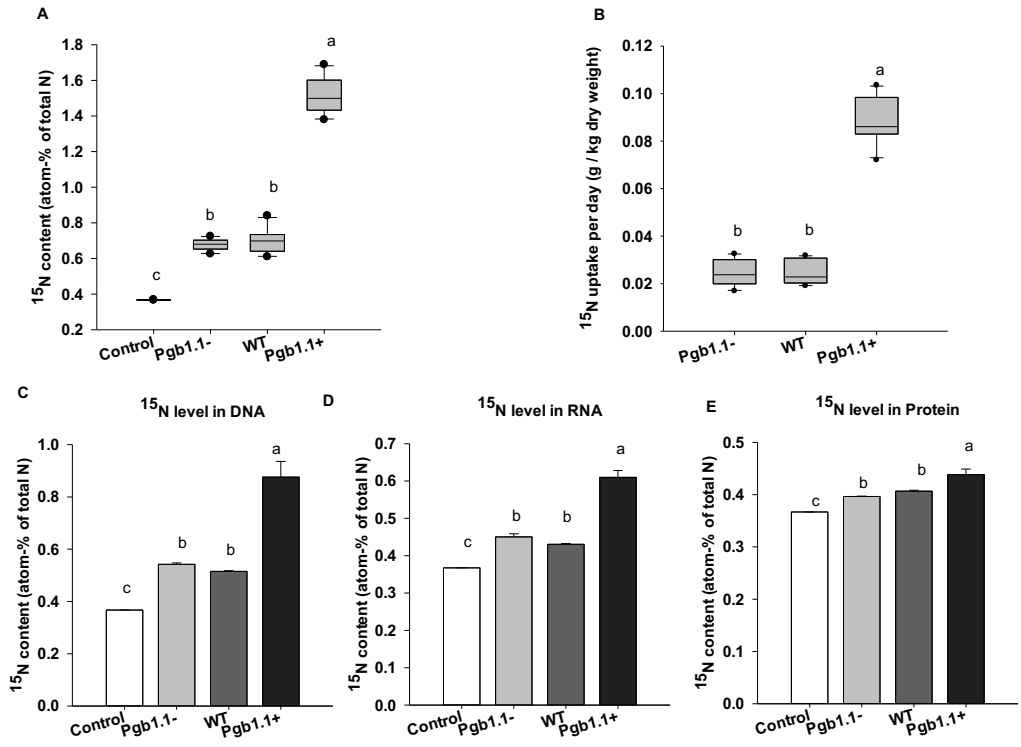


Figure 31 ^{15}N level in barley leaves, proteins and nucleic acids.

20 days barley plants were fumigated with 90 ppb ^{15}NO at daytime (8:00-20:00). ^{15}N content was determined in barley leaves from at least 10 plants after 7 days (A). The ^{15}N uptake per day (B) was calculated based on the ^{15}N data of A. ^{15}N level in DNA (C), RNA (D) and Protein (E) were measured from the extract solutions of barley leaves. Control means plants fumigated with 90 ppb NO. For graph C, D and E, each data represents means ± SE (n=3). Different letters indicate significant differences among treatments at $P < 0.05$, according to Tukey's test.

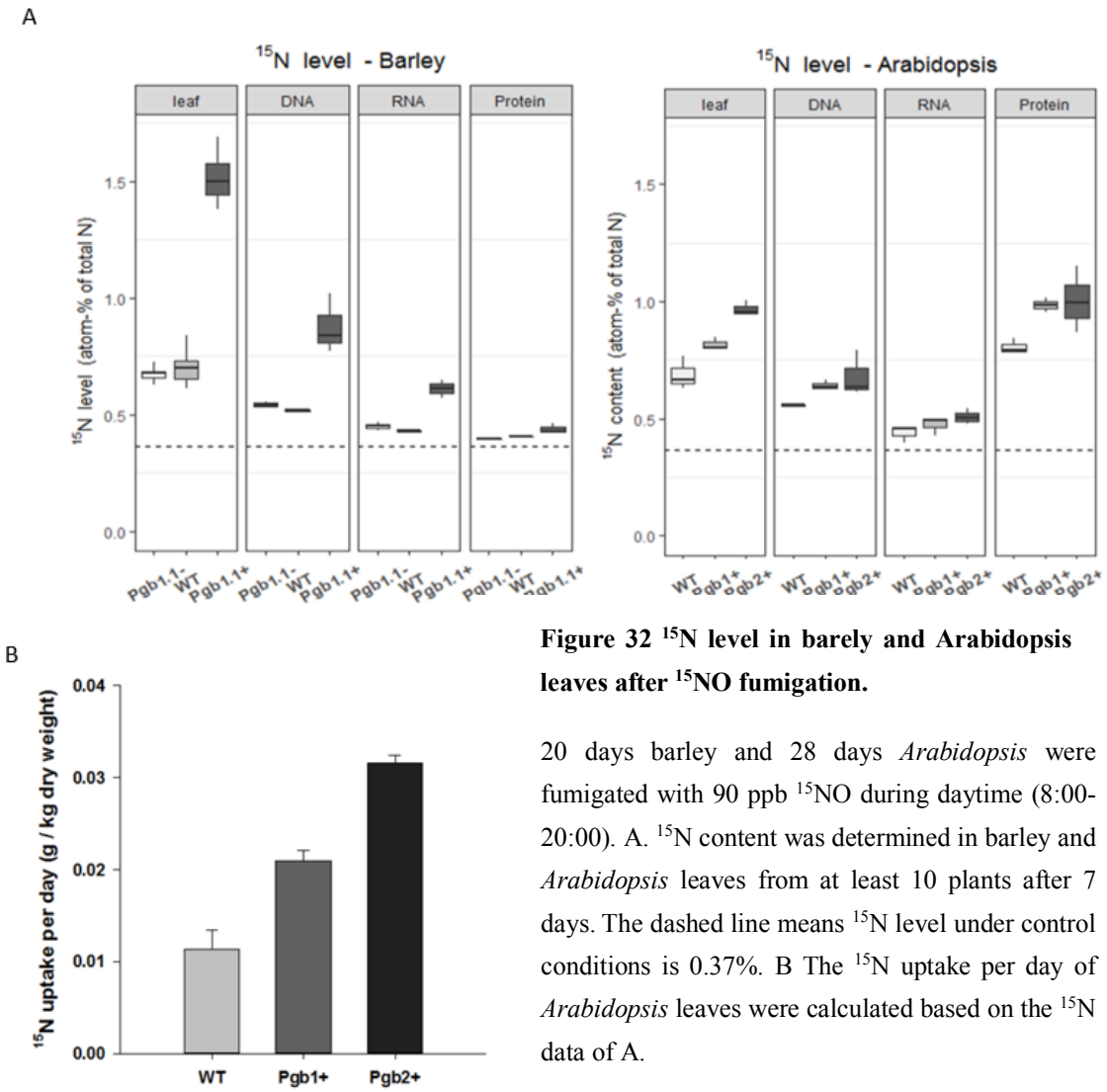


Figure 32 ^{15}N level in barely and Arabidopsis leaves after ^{15}NO fumigation.

20 days barley and 28 days *Arabidopsis* were fumigated with 90 ppb ^{15}NO during daytime (8:00-20:00). A. ^{15}N content was determined in barley and *Arabidopsis* leaves from at least 10 plants after 7 days. The dashed line means ^{15}N level under control conditions is 0.37%. B The ^{15}N uptake per day of *Arabidopsis* leaves were calculated based on the ^{15}N data of A.

The ^{15}NO uptake by barley was compared with the uptake by *Arabidopsis*. Consistent with barley, *Arabidopsis* plants overexpressing either class 1 Pgb (AtPgb1+) or class 2 Pgb *Arabidopsis* (AtPgb2+), contained more ^{15}N, in DNA, RNA and protein in comparison to WT plants (Figure 32A). Interestingly, in *Arabidopsis* most of the ^{15}N was found in the protein fraction, whereas in barley only low amounts of ^{15}N were detected in this fraction. Here most of the ^{15}N was found in the DNA fraction. A daily uptake for HvPgb1.1+ barley of about 0.09g

N/kg dry matter is even 2 times higher than Pgb2 overexpressing *Arabidopsis*, and almost 8 times higher than WT *Arabidopsis* (Figure 31 and 32).

Atmospheric NO is mainly taken up by plants through the stomata. To analyze whether Pgb dependent NO uptake is associated with changes in stomata opening, stomatal conductance was measured in WT and the different transgenic barley and *Arabidopsis* plants. No differences in stomatal conductance among the three barley lines could be observed (Figure 33). In the transgenic *Arabidopsis* plants, there were also no differences in stomata opening observed compared to WT plants, except in the class 1 knockdown line (AtPgb1-), which has a higher stomatal conductance than WT.

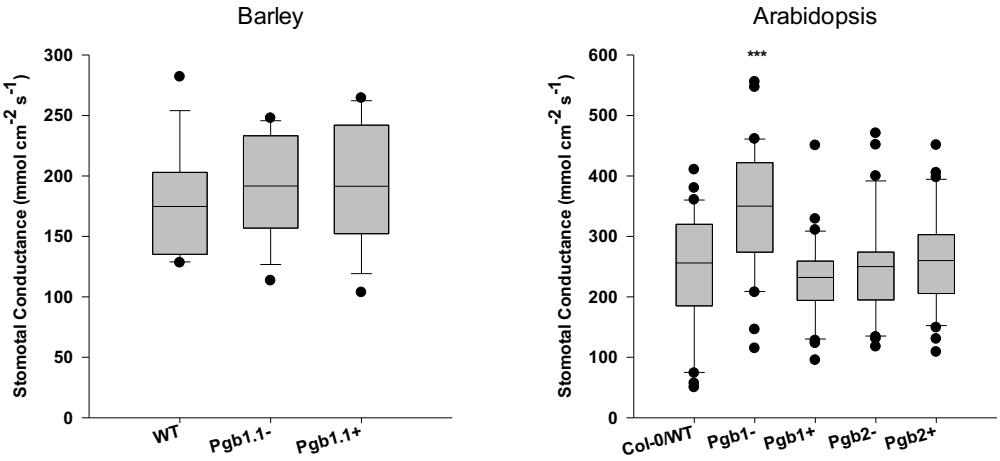


Figure 33 Stomatal conductance of barley and *Arabidopsis* plants.
Stomatal conductance was measured with a SC-1 Leaf porometer during 10:00-12:00 from at least 14 plants per line. Asterisks indicate statistically significant differences from WT (Student's t-test; ***P<0.001)

Plants can take up not only NO, but also NO_2 (Takahashi *et al.*, 2014). To analyse, whether Pgbs can also promote the use of NO_2 as N source, barley and *Arabidopsis* plants with different Pgb expression levels were fumigated with 90 ppb of $^{15}NO_2$ and ^{15}N content in leaves was determined after 3 and 7 days of fumigation (Figure 34). In general, the ^{15}N contents in leaves are up to six-fold higher when plants were fumigated for seven days with NO_2 in comparison to NO (see Figure 32 and

34). But Pgb overexpression did not enhance the NO_2 uptake demonstrating that the Pgb-dependent NO-fixation mechanism is NO-specific.

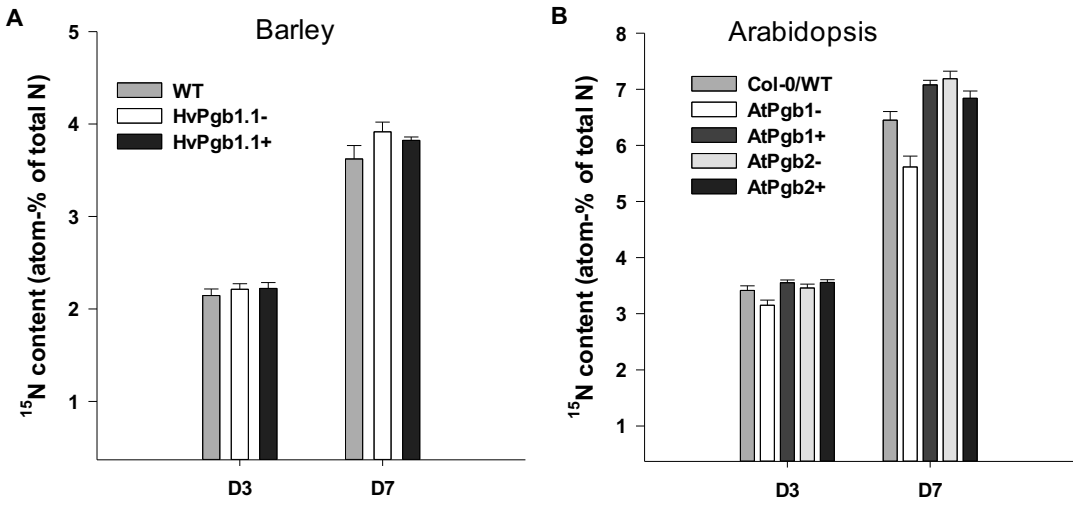


Figure 34 ^{15}N level in barely and *Arabidopsis* leaves after $^{15}NO_2$ fumigation.

20 days barley and 28 days *Arabidopsis* were fumigated with 90 ppb $^{15}NO_2$ during daytime (8:00-20:00). ^{15}N content was determined in barley (A) and *Arabidopsis* (B) leaves from at least 10 plants after 3 and 7 days.

In summary, 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. Short-term exposure to close to ambient levels of ^{15}NO (90 ppb) demonstrated that Pgbs allow barley plants efficiently using atmospheric NO as additional nitrogen source. Strengthening 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 or with less fertilization**.**

3.3 NO and NO_2 uptake capability of different trees

In *Arabidopsis* and barley, we demonstrated that the NO-fixing mechanism is a possibility for plants to use atmospheric NO as N source under N limited conditions. This process could be also of importance in context of air quality. In *Arabidopsis*, we found that the plant-based NO-fixation could lower the

concentration of atmospheric NOx. In this case, plants have a beneficial effect on air quality and human health. With regard to the air quality in cities with high concentrations of nitrogen oxides, the NO fixing capability of plants could contribute significantly to the reduction of NO and thus improve air quality. This finding may be especially significant for future urban planning in metropolitan areas and may contribute to improved living conditions there. Therefore, we analyzed the NO/NO_2-fixing capability of different city tree species and tried to explore the potential way to improve the NO-fixing ability using transgenic phytolobin plants (*Arabidopsis*, barley and poplar).

3.3.1 Deposition potential of NO and NO_2 in different trees

The NO and NO_2 deposition potential were measured in 4 different kinds of trees: *Carpinus betulus*, *Fraxinus omus*, *Fraxinus pennsyl* and *Ostrya carpinifolia*. Experiment was performed in the climate chamber with controlled growth conditions. Mature and healthy shoots were tightened together with a gas tube as air inlet in one side of the open plastic bag, the second gas tube were tightened at another side of plastic bag as air outlet (Figure 8). Different concentrations of NO and NO_2 were controlled and monitored for fumigation. Transpiration rate of NO (FNO) and NO_2 (FNO_2) (nmol m^{-2} s^{-1}) were calculated as described in method 2.3.4. The linear relationship was made between FNO/FNO_2 and the fumigated NO/NO_2 concentration: $y = kx+b$ (x represents the fumigated NO/NO_2 concentration; y represents the transpiration rate of NO (FNO) or NO_2 (FNO_2)). The deposition potential is determined as the slope (k) value.

We found that *Carpinus betulus* has a highest NO deposition potential, which is almost 3 times higher than the others. And no significant differences among the other 3 trees could be observed (Figure 35). Consistent with the NO deposition potential, the NO_2 deposition potential level in *Carpinus betulus* were also higher than that of the other trees. (Figure 35).

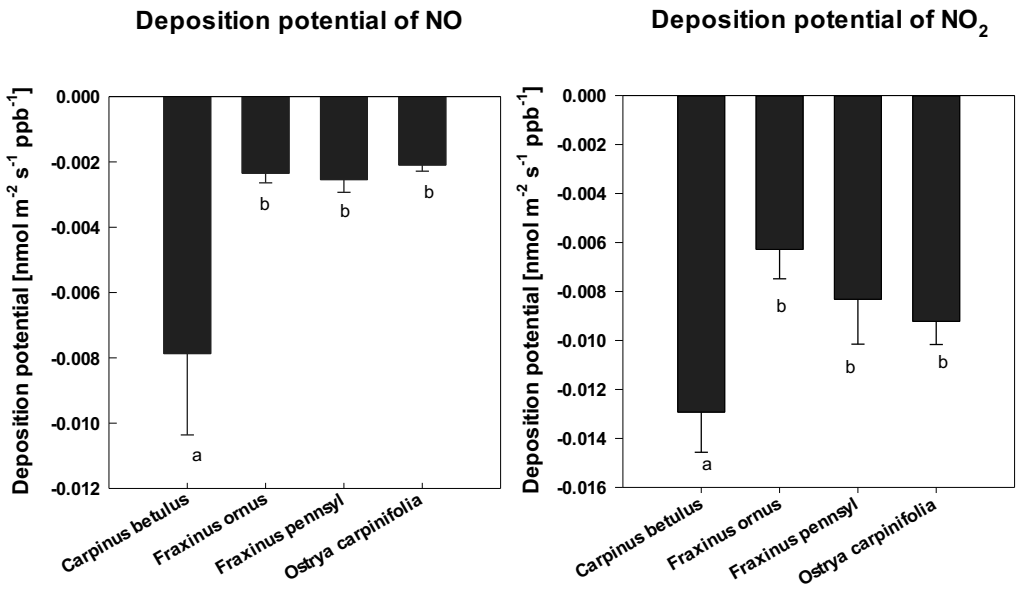


Figure 35 NO and NO_2 deposition potential of 4 different trees (*Carpinus betulus*, *Fraxinus omus*, *Fraxinus pennsyl* and *Ostrya carpinifolia*).

Trees were grown in the climate chamber. Mature and healthy shoots were chosen for the NO and NO_2 deposition potential measurement. Each data represents means ± SE (n=4). Different letters indicate significant differences among treatments at $P < 0.05$, according to Tukey's test.

3.3.2 NO uptake by different trees with ^{15}NO tracer experiment

The NO uptake capacity of trees was then examined by the ^{15}NO tracer experiment. Eight different trees (*Carpinus betulus*, *Fraxinus ornus*, *Fraxinus pennsylvanica*, *Ostrya carpinifolia*, *Celtis australis*, *Alnus spaethii*, *Alnus glutinosa*, and *Tilia henryana*) were chosen for the experiment based on the high resistance to climate change. Trees were fumigated with 50 ppb ^{15}NO, ^{15}N level in plant leaves was determined after 5 days treatment.

Clearly, *Alnus glutinosa* and *Carpinus betulus* showed a higher ^{15}N level than other plants, with the ^{15}N level 0.42% and 0.41% respectively. Followed are Tilia henryana, *Fraxinus pennsylvanica* and *Ostrya carpinifolia*, the ^{15}N level are between 0.39-0.40%. *Alnus spaethii* and *Fraxinus ornus* had the lowest level of ^{15}N, which is no more than 0.39% (Figure 36).

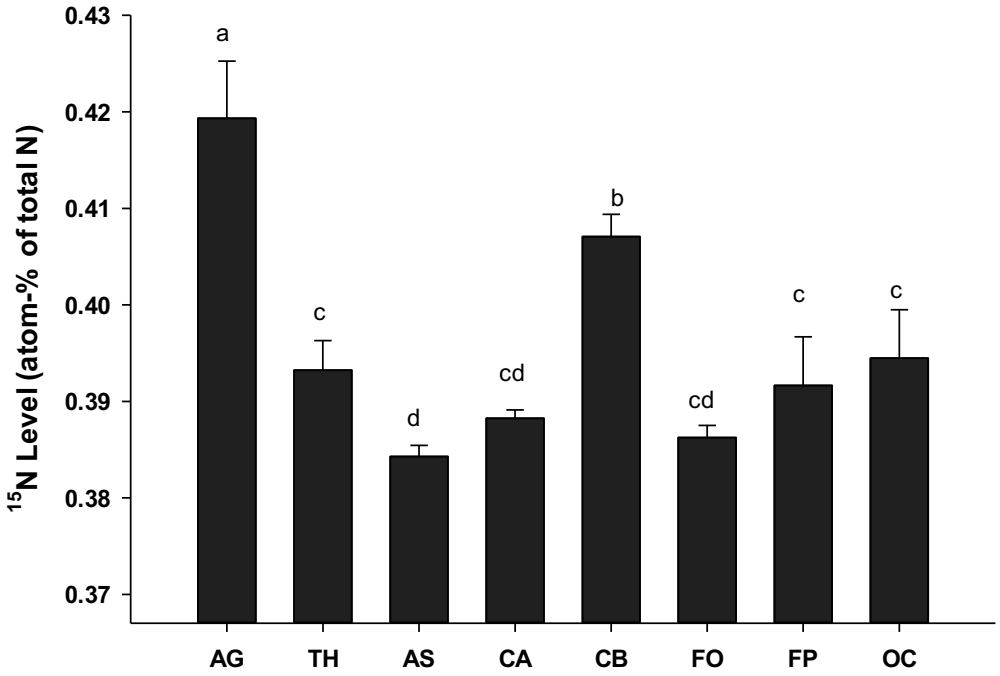


Figure 36 ^{15}N level in 8 different trees after 5 days 50 ppb ^{15}NO fumigation.

Trees were grown in the climate chamber with 50 ppb ^{15}NO, leaf samples were taken for ^{15}N measurement after 5 days treatment. *Carpinus betulus* (CB), *Fraxinus ornus* (FO), *Fraxinus pennsylvanica* (FP), *Ostrya carpinifolia* (OC), *Celtis australis* (CA), *Alnus spaethii* (AS), *Alnus glutinosa* (AG), *Tilia henryana* (TH). Each data represents means ± SE (n=8). Different letters indicate significant differences among treatments at $P < 0.05$, according to Tukey's test.

3.3.3 NO uptake in phytoglobin transgenic *Arabidopsis*, barley and poplar

Poplar is one of the most important economical tree species in temperate regions of the world due to its desirable attributes in adaptability, growth rate, woody biomass, and versatility of its wood for industry (Confalonieri *et al.* 2003). In *Arabidopsis*, we already demonstrated the NO-fixation activity of class 1 and class 2 Pgb. To check whether the NO-fixation by Pgbs can be used to improve air quality in trees, we generate the Pgb transgenic poplar with overexpression *Arabidopsis* class 1 or class 2 *Pgb* gene. Transgenic poplars were identified by semi RT-PCR (Figure 37).

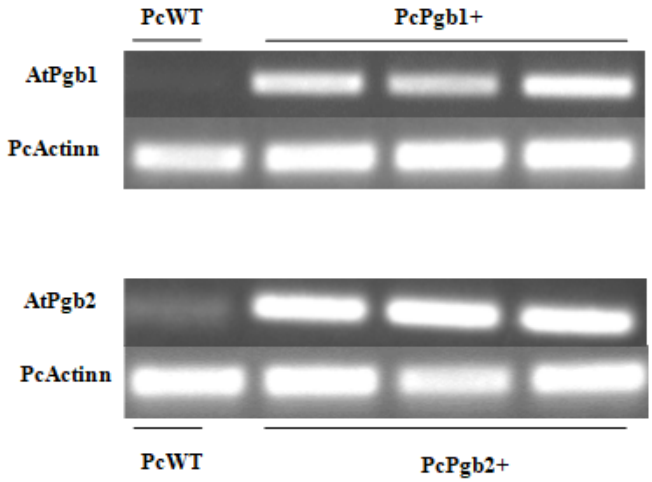


Figure 37 Semi RT-PCR detection of transgenic poplar with overexpression of AtPgb1 or AtPgb2.

RNA extracted from leaves were used for cDNA synthesis. A semi-quantitative reverse transcriptase PCR analysis was composed of 2 µl 20 mM dNTPs, 0.5 µl of 10 µM specific primers, 0.2 µl polymerase and 4 ul of 1:20 diluted cDNA template. PCR cycles of 32 were used to amplify transcripts of AtPgb1 or AtPgb2 from WT, PcPgb1+, and PcPgb2+ poplar. Transcripts of actin filaments serve as a positive loading control.

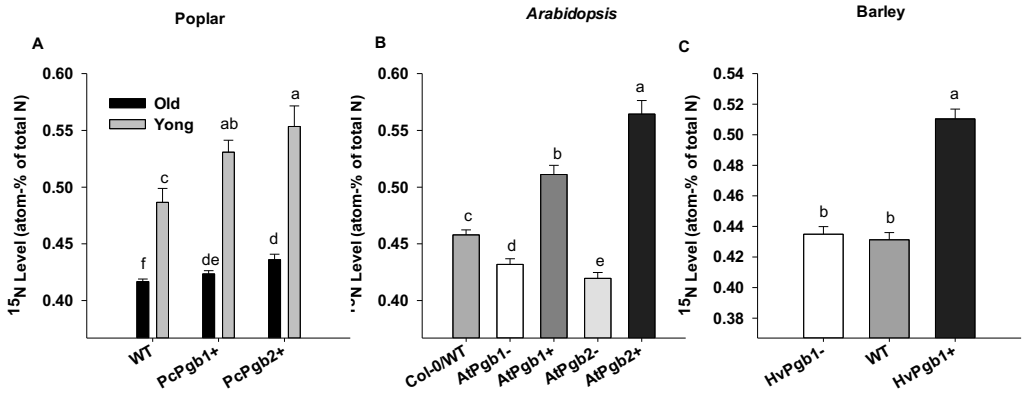


Figure 38 ^{15}N level transgenic poplar (A), *Arabidopsis* (B) and barley (C) after 5 days 50 ppb ^{15}NO fumigation.

All plants were grown in the climate chamber and fumigated with 50 ppb ^{15}NO. After 5 days, ^{15}N level in plant leaves were determined. In A, Old means 40 day-old poplar and young means 15 day-old poplar. Each data represents means ± SE (n=8). Different letters indicate significant differences among treatments at $P < 0.05$, according to Tukey's test.

NO uptake capacity was analyzed in Pgbs transgenic plants. Transgenic poplar, *Arabidopsis* and barley with altered Pgbs were used in the ^{15}NO fumigation

experiment. All plants were grown in the climate chamber and fumigated with 50 ppb ^{15}NO. After 5 days, ^{15}N level in plant leaves were determined.

Obviously, ^{15}N level in Pgb overexpression plants were higher than WT and Pgb knockdown/knockout plants (Figure 38). This confirms that overexpression Pgb can significantly increase NO uptake capacity. Interestingly, we observed a higher ^{15}N level in young poplar (15 day-old poplar) leaves in comparison to old poplar (40 day-old poplar) leaves (Figure 38A). Since the moisture content in young poplar leaves is higher than in old poplar leaves, we assumed that there is probably a positive relationship between leaf moisture content and the NO uptake capacity.

3.3.4 NO uptake capacity is related to leaf moisture content

To examine the relationship of NO uptake capacity and leaf water content, we did the linear regression analysis of ^{15}N uptake level with moisture content and ratio of fresh weight and dry weight.

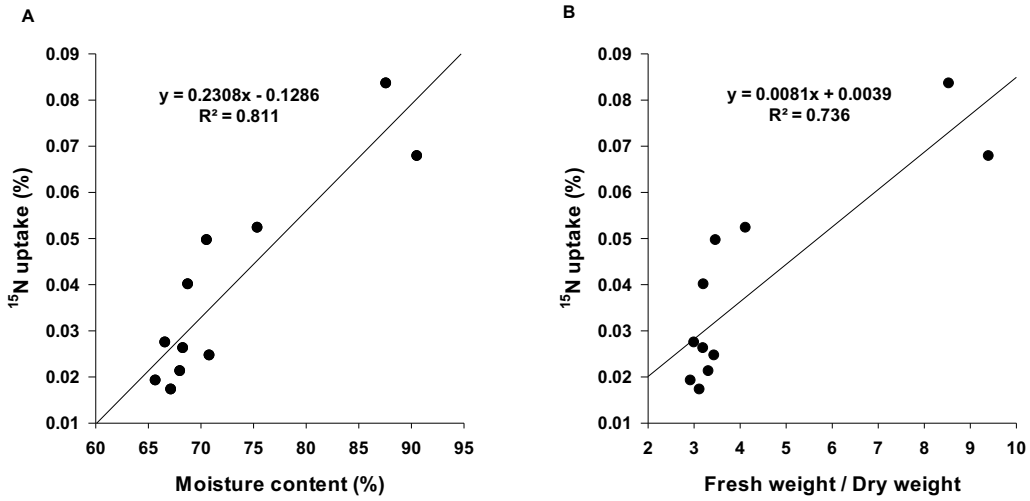


Figure 39 Linear analysis of ^{15}N uptake level with moisture content (A) and Fresh weight / Dry weight (B) in 8 city trees, poplar, *Arabidopsis* and barley.

^{15}N levels in plant leaves were determined after 5 days of 50 ppb ^{15}NO fumigation. The fresh weight was measured by weighing the freshly processed plant leaves after NO fumigation, dry weight was measured after keeping in oven at 60°C for 48 hours. The moisture content of leaves was calculated as: (fresh weight - dry weight) / fresh weight.

We found that ^{15}N uptake level showed a positive correlation with moisture content (R^2=0.811) and ratio of fresh weight and dry weight (R^2=0.736) (Figure 39).

In summary, we measured the NO uptake capacity of eight trees and NO_2 uptake capacity of 4 trees, *Alnus glutinosa* and *Carpinus betulus* showed higher potentiality of NO and NO_2 uptake capacity than other trees. Overexpression Pgb proteins signigicantly promote the NO uptake capacity in *Arabidopsis*, poplar and barley. Besides, we found that the NO uptake capacity in different plant species might have a positive correlation with moisture content.

4 Discussion

NO is naturally present in the atmosphere as part of earth's nitrogen cycle and is regard as a molecular signal in plant, which play significant role in the regulation of many biological processes. Pgbs are ubiquitously occurrence across all plant species and can scavenge NO.

Here, we describe the NO-fixation function of Pgbs in *Arabidopsis* and barley. NO fumigation experiments in *Arabidopsis* and barley demonstrated that a Pgb dependent NO-fixation allows the atmospheric NO into N metabolism and promote plant growth. Besides, the plant based NO uptake were also investigated in city trees, to analyze the function of Pgbs in reducing atmospheric NOx level and improving air quality.

4.1 NO-fixation by phytoglobins promote growth of *Arabidopsis*

In our previous studies, growth promoting effect of NO treatment was examined in soil grown *Arabidopsis*. The rosette sizes, fresh weight and dry weight of the rosettes of Pgb1+ and Pgb2+ plants fumigated with NO gas were significantly larger/higher than that of the fumigated WT control plants, the vegetative shoot length, shoot thickness and lateral shoot formation were more pronounced in Pgb1+ and Pgb2+ plants than in WT plants after NO fumigation (Kuruthukulangarakoola *et al*., 2017). Here, similar promoting effect were found in hydroponic *Arabidopsis*. Rosette size, shoot length, number of siliques and seed yield was increased in NO-treated Pgb1-overexpressing and/or Pgb2 overexpressing plants in comparison to the ambient control plant (Figure 9, 10 and 11). However, the differences are not as clear as in soil-grown plants. This is maybe because of plant growth is limited in our hydroponic system, where the medium completely free of an N-source. From this we can conclude that atmospheric NO alone cannot substitute N-uptake through the roots. But especially

in hydroponic cultures of Pgb2+ plants the red senescence phenotype was delayed (Figure 9) further demonstrating an N supply effect of NO fumigation.

A positive effect of NOx on plants growth and fruit yield has been already described previously (Leshem *et al*., 1998; Takahashi *et al*., 2005; Takahashi *et al*., 2011; Takahashi *et al*., 2014). In presence of up to 200 ppb nitrogen dioxide (NO_2), shoot biomass and total leaf area is increased in many different plant species (Takahashi and Morikawa, 2014). Moreover, cell proliferation and enlargement seem to be regulated by NO_2. A shoot biomass increase was also observed in *Arabidopsis* plants exposed to 50 ppb NO (Takahashi *et al*., 2014), and positive effects on vegetative growth was demonstrated in pea leaf discs and spinach (Leshem and Haramaty, 1996; Jin *et al*., 2009). Furthermore, vegetative growth could be enhanced in *Arabidopsis* seedlings treated with the NO donor sodium nitroprusside (He *et al*., 2004). However, the molecular mode of action underlying these effects has often remained elusive.

The SNO, nitrite, and nitrate content were increased in NO-fumigated plants compared to control plants in all lines (Figure 12) indicated that the NO can be taken up by plant leaves. In NO-fumigated plants, significantly higher nitrate levels were detected in Pgb1 and Pgb2 expression plants compared with WT plants confirming that Pgb protein converted NO to nitrate. In *Arabidopsis*, Pgb1 and Pgb2 can scavenge NO (Perazzolli *et al*., 2004; Hebelstrup *et al*., 2012). This NO scavenge ability of Pgbs has already been demonstrated to be important for limiting the loss of cellular N through NO gas emission from plants under hypoxic conditions (Hebelstrup *et al*., 2006; Hebelstrup *et al*., 2012). Moreover, the role of Pgbs in modulating NO metabolism and signalling by functioning as NO scavenger has been already discussed in different contexts such as seed germination, bolting and nitrogen-fixing symbiosis (Hebelstrup and Jensen, 2008; Shimoda *et al*., 2009; Hebelstrup *et al*., 2013). Because of its pentacoordinated heme iron, Pgb1 is known to convert NO to nitrate. Reducing equivalents were

supplied by NADPH (Gupta *et al*., 2011b). Metabolism of NO by Pgb2+ plants is surprising, because Pgb2 is not known for its NO metabolizing function because of its low oxygen affinity (Gupta *et al*., 2011b). But also for Pgb2, which contains a hexacoordinated heme iron, seems to be able to interact with NO, because an effective NO scavenging activity was already described for this protein (Hebelstrup and Jensen, 2008). Moreover, enhanced NO metabolism mediated by S-nitrosylation of Pgb2 cannot be ruled out. A similar function is suggested for haemoglobin proteins in animals (Foster *et al*., 2003).

We demonstrated that application of NO can promote plant growth through the Pgb dependent NO fixation mainly by *Arabidopsis* leaves, but we do not know whether external NO application and the inner changed NO level in transgenic Pgb plants can affect the N uptake in root. The $^{15}NO3^-$ experiment indicated that the application of 3000 ppb and Pgb had no significant effect in the N uptake by root. The importance of Pgb proteins for NO uptake was demonstrated by experiments using ^{15}NO (Figure 14). The highest ^{15}N uptake was observed in Pgb2+ plants. However, also WT plant accumulated already quite high amounts of ^{15}N, which was in the range of that in Pgb1+ plants. This is probably due to the NO-induced expression of Pgb1 in WT plants (Kuruthukulangarakoola *et al*., 2017). Of course, that would be also expected in Pgb1+ plants, but maybe the induction is higher in WT plants, because Pgb levels are already 'boosted' in Pgb1+ plants.

The uptaken ^{15}N can be present in both inorganic (non-as-similated) and organic (assimilated) forms. Especially, the composition of the N-containing organic form is very complex because it includes different types of compounds, such as amino acids/proteins, nucleic acids, secondary metabolites and pigments. Therefore, we presented here the total ^{15}N content. Based on the ^{15}N data after 4 days of ^{15}NO treatment, we calculated a daily uptake for Pgb2+ plants of 250 mg N/kg dry matter, which is almost 50% more than in WT plants (Figure 14B). We tried to extrapolate these results to field conditions. The average total N content in well-

grown healthy plants is ca. 2% of the plant dry matter (Epstein, 1965). This corresponds to 50 kg total N in 2500 kg plant dry matter – an amount that can be harvested per year on 1 ha grassland. Based on the NO-fixing capacity of Pgb2+ plants (250 mg N/kg dry matter) we calculated a NO-based N-uptake of 0.625 kg N/ha/year (250 mg N/kg dry matter ×2500). This is in the range of the N-fixation capacity of free-living bacteria (ca. 1–3 kg N/ha/year), whereas plant-associated N-fixing bacteria fix 100-300 kg N/ha/year. We further demonstrated the Pgb dependent NO uptake by plant leaves is not significantly affected under N sufficient conditions in short term. Of course, under N-limited conditions N-uptake via NO might be of greater importance, but the N-uptake in normal N conditions cannot be ignored.

NO emissions from microbial processes in soils represent around 15% of the modern global atmospheric NOx (NO + NO_2) source (∼50% in preindustrial times) and are a major contribution to the NOx budget outside of cities (Hudman *et al*., 2012). The NO uptake by *Arabidopsis* leaves prevented the loss of N from soil emissions. Besides, the NO scavenging by *Arabidopsis* showed us the potential function of the plant based NO uptake, which can reduce the atmospheric NO level and improve air quality.

4.2 Phytoglobin overexpression promotes barley growth in presence of NO

After the barley Pgb 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 Pgb *HvPgb1.2* gene (Figure 17 and 18). The expression pattern of *HvPgb1.2* in different tissues differed to *HvPgb1.1* and in general the expression levels of *HvPgb1.2* are much higher than *HvPgb1.1* (Figure 19). This indicates that HvPgb1.2 may have a different function in barley. Since little is known about HvPgb1.2, further research

is needed to analyze 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 (Figure 20), 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 Pgb synthesis might be a common mechanism in plant to protect differentiated plant cells from the cellular damage caused by excess NO. But Pgbs 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 to *Arabidopsis*, where five-week-old plants overexpressing class 1 Pgb are flowering earlier and have more progressed inflorescences than WT plants (Hebelstrup and Jensen, 2008; Hebelstrup *et al*., 2013). Surprisingly, we did not observe significant development differences among WT, HvPgb1.1+ and HvPgb1.1- lines, when growing the plants under a controlled climate (see growth parameter under ambient conditions in Figure 21 and S1), probably because of differences in the environment conditions of the two experiments. This is also similar to *Arabidopsis*, where the effect of Pgb overexpression was only very weak in a short-day regime (Hebelstrup and Jensen, 2008) in comparison to a longer day regime (Hebelstrup *et al*., 2013).

4.2.1 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 dry matter weight (DMW), kernel weight (KW) and kernel number (KN) of WT plants (Figure 23 and 24). In contrast, treatment with 1500 ppb and 3000 ppb NO did not increase or even decrease dry matter, kernel and spike development (Figure 24). 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 (Figure 21 and 23). The decrease in DWP and KWP at high NO (1500 and 3000 ppb) levels in WT and HvPgb1.1- plants could be explained by toxic effect of NO at these concentrations. Interestingly, when fumigated with 3000 ppb of NO for 9 weeks, the total seeds yield of *Arabidopsis* WT plants increased by 14% in comparison to ambient conditions (Kuruthukulangarakoola *et al.*, 2017), which means that barely 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 (Figure 19) to protect plants from such high concentration of NO.

Based on the results of plant weight and stem number, we observed a clear growth promoting effect after 30 and 45 days of NO fumigation in plants, especially in *HvPgb1.1* overexpressing barley treated with 3000 ppb of NO (Figure 21 and 22). This demonstrates that Pgb enabled a better growth especially in presence of high NO concentrations. However, the promoting effect was not observed in the early stage of development (20 days) (Figure 21 and 22). At this time, there was still enough nitrogen supply from the soil. However, after 30 days, nitrogen limitation in soil resulted in a nitrogen deficient state of the plants (Figure 30). Therefore, the nitrogen supply by Pgb-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 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 to WT and HvPgb1.1- lines (Figure 24), indicating that the NO promoting effect is Pgb-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 (Figure 19 and 20). 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 (Figure S2).

In NO-fumigated HvPgb1.1+ plants, higher nitrate levels were present compared to WT plants demonstrating that HvPgb1.1 converted NO to nitrate (Figure 25). Previous studies demonstrate that NO is an important regulator of N assimilation (Frungillo *et al*., 2014). In spinach, enhanced nitrate assimilation in 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 (Figure 30) indicating that NO fumigation had no obvious effect on nitrogen uptake from soil. Moreover, no differences in ^{15}N levels in barley leaves could be detected within the three barley lines grown in presence of $^{15}NO_3^-$ (Figure 31). This confirms that Pgbs did not affect the NO-induced N assimilation. In presence of 3000 ppb of NO, the higher nitrate level in HvPgb1+ plants (Figure 25) and the higher N content in HvPgb1+ grown soil (Figure 30) indicated that the Pgb dependent NO-fixation provided significant additional N for plant growth and the N absorption from soil was reduced. Thus, Pgb 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, for 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, and 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 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 (ΔF/Fm') could be observed after 20 days of exposure to different NO concentrations (Figure 27 and 28) concluding 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 days 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 presence of high concentrations of atmospheric NO. In WT and HvPgb1.1- plants the chlorophyll content is clearly lower in older plants in comparison to younger plants. However, with increasing NO concentrations the decreased is less pronounced (Figure 27 and 28). 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 to Pgb1.1+. The NO uptake by WT and Pgb1.1- barley plants was also demonstrated by ^{15}NO fumigation experiments (Figure 31 and 32). Besides the Pgb-dependent NO uptake, it cannot be excluded, that traces of NO dissolves in aqueous solutions

(e.g. in the apoplast) forming HNO_2, which can also "enter" the N metabolism. In conclusion, the observed higher biomass accumulation in HvPgb1.1+ plants (Figure 23 and 24) is mainly based on the additional N supply through HvPgb1.1-dependent NO-fixation.

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

The importance of Pgb 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 WT and HvPgb1.1- plants (Figure S2). However, also WT and HvPgb1.1- plants accumulated significant amounts of ^{15}N above the background level (Figure 31). This is probably due to the NO-induced expression of *HvPgb1.1* in these plants (Figure 20 and Figure S2) and due to the deposition of ^{15}NO in and on the plants. In 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 presence of 800 and 1500 ppb of NO (Figure 20). 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.*, 2016). 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 (Figure 31) and is even 2 times higher than the rate in the Pgb2+ *Arabidopsis* (Figure 32). This indicates that the Pgb-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 in

relation to climate gas emissions of agricultural soil. Loss of NO from soil means on one side loss of nitrogen and on the other side 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 a practical application improvement of the NO-fixing process is required. ^{15}N level in RNA, DNA, and protein demonstrated that the uptaken NO was used as additional N source in plants. 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 (Figure 31 and 32). 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 Pgb overexpressing plants (Pgb1+ and Pgb2+) in comparison to WT plants (Figure 32). 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 to $^{15}N/N_{total}$ in leaves, while the $^{15}N/N_{total}$ in *Arabidopsis* protein were more similar to the leaf values (Figure 32). 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 plants still undergo a strong vegetative growth with a need to allocate more N to chlorophyll and other biosynthetic processes, while 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-bisphosphat-carboxylase/-oxygenase (RuBisCo) 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 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 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-Pgb cycle in transgenic *Arabidopsis* and barley plants with alter Pgb 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 (Figure 33), 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 to WT *Arabidopsis* (Figure 32 and 33). This demonstrates that stomatal opening is not a key factor for the increased uptake of NO in leaves of Pgb overexpressing plants.

Based on the results above and integrate information from from literatures (Krapp, 2015; Lindermayr and Hebelstrup, 2016), we made a model to summarize how atmospheric NO enter in plant N metabolism in plants (Figure 40). Atmospheric NO can enter plant cell through stomatal, and first be converted to nitrate by phtoglobins in cytoplasm. Nitrate is reduced in the cytosolasm to nitrite by nitrate NR. Nitrite is then transported into the chloroplast and reduced to ammonium by NiR. Ammonium is incorporated into glutamine (Gln) and glutamate (Glu) by the GS/GOGAT cycle. Glutamate can be used for chlorophyll synthesis in chloroplast.

Glutamine and glutamate also contribute to protein, DNA, RNA and other N-contining compounds synthesis.

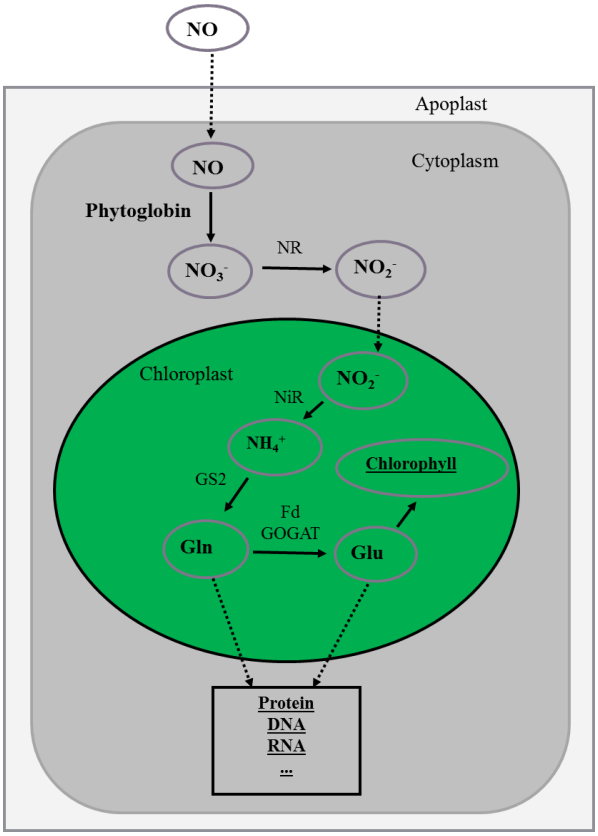


Figure 40 Proposed model of atmospheric NO enter in plant N metabolism.

Fd-GOGAT, ferredoxin-dependent glutamate-oxoglutarate-aminotransferase; GS, glutamine synthetase; NiR, nitrite reductase; NR, nitrate reductase.

In this part, we demonstrated that the Pgb-dependent uptake of NO allows a channeling of atmospheric NO into the plant N metabolism in the crop plant barley (Figure 40). In general, the effectiveness of this NO-fixation process depends on the availability of NO in the atmosphere, an increased content of Pgbs and the plant developmental stage triggering the N allocation. Improving the NO-fixing process to enable plants an efficient use of low-concentrated atmospheric NO, would be a promising approach allowing plants a 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 and 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 was already reported in the *HvPgb1.1* overexpressing line (Hebelstrup *et al*., 2014, Sørensen *et al*., 2018, Montilla-Bascón *et al*., 2017, Gupta *et al*., 2014).

4.3 NO and NO_2 uptake capacity in different plant species

Natural and artificial (agricultural) vegetation acts as a major 'sink' for atmospheric pollutants in terrestrial ecosystems (Hill, 1971). Through pollution removal and other tree functions (e.g., air temperature reductions), city trees can help improve air quality for many different air pollutants in cities, and consequently can help improve human health (Nowak *et al*., 2016). NO and NO_2 are regarded as environmental pollutants and are an important contributor to the formation of smog. Therefore, in this research we analyzed the NO and NO_2 uptake capacity of eight different city tree species, *Carpinus betulus*, *Fraxinus ornus*, *Fraxinus pennsylvanica*, *Ostrya carpinifolia*, *Celtis australis*, *Alnus spaethii*, *Alnus glutinosa*, and *Tilia henryana.* The tree species were chosen based on their high resistances to climate change.

The deposition potential of NO and NO_2 were measured in *Carpinus betulus*, *Fraxinus omus*, *Fraxinus pennsyl* and *Ostrya carpinifolia*. Obviously, the NO and NO_2 deposition potential differed in different tree species (Figure 35 and 36). The assimilation of NO_2 in different plant species is controlled by several factors (Morikawa *et al*., 1998), including the resistance to the entry these include resistance to the entry of NO_2 gas molecules through the stomata, cuticle layer and inter cellular cavity to reach the surface of mesophyll cells (Morikawa *et al*., 1998), permeability of nitrate and nitrite ions as well as neutral molecules through cell

walls and plasma membranes (Ammann *et al*. 1995; Lee and Schwartz, 1981; Ramge *et al*. 1993), and the activity in the primary nitrate assimilation pathway through which NO2-nitrogen is reported to be metabolized (Rogers *et al*., 1979; Wellburn, 1990; Yoneyama and Sasakawa 1979). Besides, in the same plant species, the NO_2 uptake by leaves is affected by by stomatal dynamics, rate of photosynthesis, and height within the canopy (Sparks *et al*., 2001; Chaparro-Suarez *et al*., 2011).

The deposition potential of the 4 different trees were used to predict the total deposition estimates in cities. Total deposition estimates of NO_2 and NO were caculated for the Mitte District /Berlin according to the climate and air pollution data of the year 2014 (data from the Berlin Senatsverwaltung). We found that if we replace the 4 dominant tree species grown in Mitte (maple, linden, beech, oak) with the 4 new species (*Carpinus betulus*, *Fraxinus ornus*, *Fraxinus Pennsylvania*, *Ostrya carpinifolia*), the total NO_2 and NO deposition estimates would be increased around 100% (Figure S4). Therefore, choosing better city tree species that has a higher NO and NO_2 uptake capacity could provide a viable means to reduce atmospheric NOx level and help meet clean air standards.

In *Arabidopsis* and barley, overexpression of Pgb enhanced the NO uptake capacity and did not affect the NO_2 uptake capacity. Overexpression of *Arabidopsis* Pgb 1 and 2 in poplar also significantly enhanced the NO uptake capacity compared to WT control (Figure 38). Recently, Zhang *et al* (2019) have developed a genetically modified a common houseplant, Epipremnum aureum, that can remove chloroform and benzene from the air around it. Therefore, the transgenic plants, especially city trees with overexpression of Pgb could be another potential means to reduce the atmospheric NOx level and improve air quality.

In this study, the NO uptake capacity showed a positive correlation linear relationship with leaf moisture content. However, the plant species are not enough for the analyses in this research. More plant species are needed to get a more

reliable result. Besides, more factors such as the stomatal conductance, photosynthesis rate, and the Pgb expression level should also be included for building a model to predict the NO uptake capacity of different plant species.

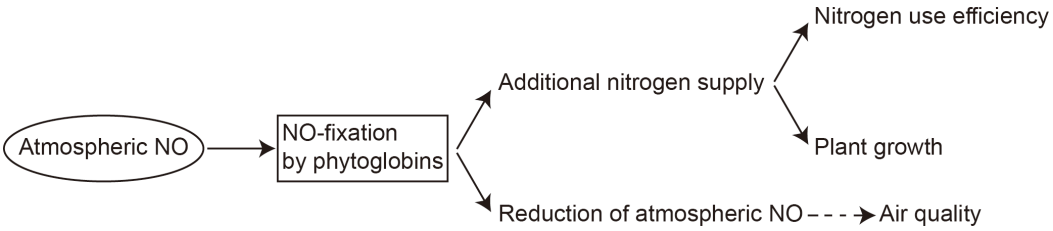


Figure 41 Proposed model of function of NO-fixation by phytoglobins in plant.

Atmospheric NO can be fixed by phytoglobins, which provide additional N supply and reduce the level of atmospheric NO, which is helpful to air quality. The additional N supply can enhance N use efficiency and promote plant growth.

To sum up, we studied the NO-fixation by Pgbs in plants, which can transfer the atmospheric NO into plant N metabolism as additional N supply. Under high NO concentrations, the additional N supply from NO-fixation increase nitrogen use efficiency and has an obvious promoting effect on plant growth, especially in Pgb overexpression lines (Figure 41). Besides, the NO-fixation by Pgbs can also result in reduction of atmospheric NO, which is helpful to the air quality and maybe more useful in city trees (Figure 41).

5 Outlook

It is well known that class 1 Pgbs are efficient NO scavengers converting NO to nitrate. Here we show that plants can fix NO from air and demonstrated Pgbs dependent NO fixation can promote plant growth under high concentrations of NO. Interestingly, we found that *Arabidopsis* class 2 Pgbs can also fix NO and are comparable compared to class 1 Pgbs. Therefore, it is necessary to study the structure of class 2 Pgbs and explore the mechanism of NO scavenger ability of class 2 Pgbs.

Besides, we found a new Pgb gene (HvPgb1.2) in barley, which have a higher expression level and may have different functions compared to HvPgb1.1. Transgenic barley with altered HvPgb1.2 expression should be obtained to check the possible NO-fixing ability and other unknown functions of barley Pgb1.2 protein.

Moreover, the plant based NO uptake showed a reduction of atmospheric NOx. The NOx uptake capacity should be checked in other city tree species. The possibility of using transgenic Pgb trees to enhance the NO uptake capacity and improve air quality in polluted city areas should also be examined.

6 Reference

Adam SEH, Abdel-Banat BMA, Sakamoto A, Takahashi M, Morikawa H. 2008. Effect of atmospheric nitrogen dioxide on mulukhiya (Corchorus olitorius) growth and flowering. American Journal of Plant Physiology **3**, 180–184.

Akhter MM, Hossain A, Timsina J, Teixeira da Silva JA, Islam MS. 2016. Chlorophyll meter – A decision-making tool for nitrogen application in wheat under light soils. International Journal of Plant Production **10**, 289–302.

Ammann M, von Ballmoos P, Stalder M, Suter M, Brunold C. 1995. Uptake and assimilation of atmospheric NO2 - N by spruce needles (Picea abies): A field study. Water, Air, and Soil Pollution **85**, 1497–1502.

Anderson CR, Jensen EO, LLewellyn DJ, Dennis ES, Peacock WJ. 1996. Synthesis and biological activity of s-bridge heterocyclic compounds. Proceedings of the National Academy of Sciences of the United States of America **93**, 5682–2687.

Anderson LS, Mansfield TA. 1979. The effects of nitric oxide pollution on the growth of tomato. Environmental Pollution (1970) **20**, 113–121.

Appleby CA. 1984. Leghemoglobin and Rhizobium respiration. Annu. Rev. Plant Physiol. **35**, 443–478.

Arasimowicz M, Floryszak-Wieczorek J. 2007. Nitric oxide as a bioactive signalling molecule in plant stress responses. Plant Science **172**, 876–887.

Arasimowicz M, Floryszak-Wieczorek J, Milczarek G, Jelonek T. 2009. Nitric oxide, induced by wounding, mediates redox regulation in pelargonium leaves. Plant biology (Stuttgart, Germany) **11**, 650–663.

Arc E, Sechet J, Corbineau F, Rajjou L, Marion-Poll A. 2013. ABA crosstalk with ethylene and nitric oxide in seed dormancy and germination. Frontiers in Plant Science **4**, 1–19.

Aréchaga-Ocampo E, Saenz-Rivera J, Sarath G, Klucas R V., Arredondo-Peter R. 2001. Cloning and expression analysis of hemoglobin genes from maize (Zea mays ssp. mays) and teosinte (Zea mays ssp. parviglumis). Biochimica et Biophysica Acta - Gene Structure and Expression **1522**, 1–8.

Arredondo-Peter R, Moran JF, Sarath G. 2014. Rice (Oryza) hemoglobins. F1000Research **3**, 1–16.

Asai S, Yoshioka H. 2009. Nitric oxide as a partner of reactive oxygen species participates in disease resistance to nectrotophic pathogen Botryis cinerea in Nicotiana benthamiana. Molecular plant-microbe interactions : MPMI **22**, 619–29.

Astier J, Rasul S, Koen E, Manzoor H, Besson-Bard A, Lamotte O, Jeandroz S, Durner J, Lindermayr C, Wendehenne D. 2011. S-nitrosylation: An emerging post-translational protein modification in plants. Plant Science **181**, 527–533.

Barroso JB, Corpas FJ, Carreras A, Sandalio LM, Valderrama R, Palma JM, Lupiánez JA, Del Río LA. 1999. Localization of nitric-oxide synthase in plant peroxisomes. Journal of Biological Chemistry **274**, 36729–36733.

Begara-Morales JC, Sánchez-Calvo B, Chaki M, Valderrama R, Mata-Pérez C, Padilla MN, Corpas FJ, Barroso JB. 2016. Antioxidant Systems are Regulated by Nitric Oxide-Mediated Post-translational Modifications (NO-PTMs). Frontiers in Plant Science **7**, 1–8.

Beligni MV, Lamattina L. 2000. Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. Planta **210**, 215–221.

Benamar A, Rolletschek H, Borisjuk L, Avelange-Macherel MH, Curien G, Mostefai HA, Andriantsitohaina R, Macherel D. 2008. Nitrite-nitric oxide control of mitochondrial respiration at the frontier of anoxia. Biochimica et Biophysica Acta - Bioenergetics **1777**, 1268–1275.

Berger A, Brouquisse R, Pathak PK, Hichri I, Inderjit I, Bhatia S, Boscari A,

Igamberdiev AU, Gupta KJ. 2018. Pathways of nitric oxide metabolism and operation of Pgbs in legume nodules: Missing links and future directions. Plant Cell and Environment **41**, 2057–2068.

Besson-Bard A, Gravot A, Richaud P, Auroy P, Duc C, Gaymard F, Taconnat L, Renou J-P, Pugin A, Wendehenne D. 2009. Nitric Oxide Contributes to Cadmium Toxicity in Arabidopsis by Promoting Cadmium Accumulation in Roots and by Up-Regulating Genes Related to Iron Uptake. Plant Physiology **149**, 1302–1315.

Besson-Bard A, Griveau S, Bedioui F, Wendehenne D. 2008. Real-time electrochemical detection of extracellular nitric oxide in tobacco cells exposed to cryptogein, an elicitor of defence responses. Journal of Experimental Botany **59**, 3407–3414.

Bethke PC, Gubler F, Jacobsen J V., Jones RL. 2004. Dormancy of Arabidopsis seeds and barley grains can be broken by nitric oxide. Planta **219**, 847–855.

Bethke PC, Libourel IGL, Aoyama N, Chung Y-Y, Still DW, Jones RL. 2007. The Arabidopsis Aleurone Layer Responds to Nitric Oxide, Gibberellin, and Abscisic Acid and Is Sufficient and Necessary for Seed Dormancy. Plant Physiology **143**, 1173–1188.

Bethke PC, Libourel IGL, Jones RL. 2006. Nitric oxide reduces seed dormancy in Arabidopsis. Journal of Experimental Botany **57**, 517–526.

Blokhina O, Fagerstedt K V. 2010. Oxidative metabolism, ROS and NO under oxygen deprivation. Plant Physiology and Biochemistry **48**, 359–373.

Bogusz D, Appleby CA, Landsmann J, Dennis ES, Trinick MJ, Peacock WJ. 1988. Functioning haemoglobin genes in non-nodulating plants. Nature **331**, 450.

Bojovic B. 2005. Effects of Fertilization on Chloroplasts Pigments Content , Leaf Surface and Dry Matter Weight in Some Wheat Cultivars. Acta Agriculturae Serbica **X**, 29–37.

Bojović B, Marković A. 2009. Correlation between nitrogen and chlorophyll

content in wheat (Triticum aestivum L.). Kragujevac Journal of Science **31**, 69–74.

Bright J, Desikan R, Hancock JT, Weir IS, Neill SJ. 2006. ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H2O2synthesis. Plant Journal **45**, 113–122.

Bruggink G, Wolting H, Dassen J, Bus V. 1988. The effect of nitric oxide fumigation at two CO2 concentrations on net photosynthesis and stomatal resistance of tomato (Lycopersicon lycopersicum L. cv. Abunda). New Phytologist **110**, 185–191.

Bustos-Sanmamed P, Tovar-Méndez A, Crespi M, Sato S, Tabata S, Becana M. 2011. Regulation of nonsymbiotic and truncated hemoglobin genes of Lotus japonicus in plant organs and in response to nitric oxide and hormones. New Phytologist **189**, 765–776.

Capron TM, Mansfield TA. 1976. Inhibition of net photosynthesis in tomato in air polluted with NO and NO2. Journal of Experimental Botany **27**, 1181–1186.

Carimi F, Zottini M, Costa A, Cattelan I, De Michele R, Terzi M, Lo Schiavo F. 2005. NO signalling in cytokinin-induced programmed cell death. Plant, Cell and Environment **28**, 1171–1178.

Chaki M, Álvarez De Morales P, Ruiz C, Begara-Morales JC, Barroso JB, Corpas FJ, Palma JM. 2015. Ripening of pepper (Capsicum annuum) fruit is characterized by an enhancement of protein tyrosine nitration. Annals of Botany **116**, 637–647.

Chaparro-Suarez IG, Meixner FX, Kesselmeier J. 2011. Nitrogen dioxide (NO2) uptake by vegetation controlled by atmospheric concentrations and plant stomatal aperture. Atmospheric Environment **45**, 5742–5750.

Chen J, Wu FH, Liu TW, Chen L, Xiao Q, Dong XJ, He JX, Pei ZM, Zheng HL. 2012. Emissions of nitric oxide from 79 plant species in response to simulated nitrogen deposition. Environmental Pollution **160**, 192–200.

Chen WW, Yang JL, Qin C, Jin CW, Mo JH, Ye T, Zheng SJ. 2010. Nitric Oxide Acts Downstream of Auxin to Trigger Root Ferric-Chelate Reductase Activity in Response to. Plant Physiology **154**, 810–819.

Cochrane DW, Shah JK, Hebelstrup KH, Igamberdiev AU. 2017. Expression of Pgb affects nitric oxide metabolism and energy state of barley plants exposed to anoxia. Plant Science **265**, 124–130.

Confalonieri M, Balestrazzi A, Bisoffi S, Carbonera D. 2003. In vitro culture and genetic engineering of Populus spp.: Synergy for forest tree improvement. Plant Cell, Tissue and Organ Culture **72**, 109–138.

Corpas FJ, Leterrier M, Valderrama R, Airaki M, Chaki M, Palma JM, Barroso JB. 2011. Nitric oxide imbalance provokes a nitrosative response in plants under abiotic stress. Plant Science **181**, 604–611.

Corpas FJ, Palma JM, del Río LA, Barroso JB. 2016. Nitric Oxide Emission and Uptake from Higher Plants. In: Lamattina L., García-Mata C. (eds) Gasotransmitters in Plants. Signaling and Communication in Plants. Springer, Cham. DOI: 10.1007/978-3-319-40713-5_4

Corpas FJ, Palma JM, Rı´o LA del, Beltrán-Brown F, Juan B. Barroso. 2009. Evidence supporting the existence of L-arginine- dependent nitric oxide synthase activity in plants. New Phytologist **184**, 9–14.

Correa-Aragunde N, Graziano M, Lamattina L. 2004. Nitric oxide plays a central role in determining lateral root development in tomato. Planta **218**, 900–905.

Correa-Aragunde N, Lombardo C, Lamattina L. 2008. Nitric oxide: An active nitrogen molecule that modulates cellulose synthesis in tomato roots. New Phytologist **179**, 386–396.

Crawford NM, Galli M, Tischner R, Heimer YM, Okamoto M, Mack A. 2006. Response to Zemojtel et al: Plant nitric oxide synthase: back to square one. Trends in Plant Science **11**, 526–527.

Reference

D'Angelo P, Lucarelli D, Della Longa S, Benfatto M, Hazemann JL, Feis A, Smulevich G, Ilari A, Bonamore A, Boffi A. 2004. Unusual heme iron-lipid acyl chain coordination in Escherichia coli flavohemoglobin. Biophysical Journal **86**, 3882–3892.

Dean J V, Harper JE. 1986. Nitric Oxide and Nitrous Oxide Production by Soybean and Winged Bean during the in Vivo Nitrate Reductase Assay. Plant Physiology **82**, 718–723.

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

Dordas C. 2009. Nonsymbiotic hemoglobins and stress tolerance in plants. Plant Science **176**, 433–440.

Dorostkar S, Pakniyat H, Kordshooli MA. 2015. Study of relationship between grain yield and yield components using multivariate analysis in barley cultivars (Hordeum vulgare L .). International Journal of Agronomy and Agriculture Research **6**, 240–250.

Du J, Li M, Kong D, Wang L, Lv Q, Wang J, Bao F, Gong Q. 2014. Nitric oxide induces cotyledon senescence involving signalling pathways in Arabidopsis. Journal of Experimental Botany **65**, 4051–4063.

Durner J, Wendehenne D, Klessig DF. 1998. Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. Proceedings of the National Academy of Sciences of the United States of America **95**, 10328–10333.

Elhiti M, Hebelstrup KH, Wang A, Li C, Cui Y, Hill RD, Stasolla C. 2013. Function of type-2 Arabidopsis hemoglobin in the auxin-mediated formation of embryogenic cells during morphogenesis. Plant Journal **74**, 946–958.

Epstein E. 1965. Typical concentrations sufficient for plant growth. Plant Biochemistry. 438–466.

Fancy NN, Bahlmann AK, Loake GJ. 2017. Nitric oxide function in plant abiotic stress. Plant Cell and Environment **40**, 462–472.

Farquhar GD, Ehleringer JR, Hubick KT. 1989. Carbon Isotope Discrimination and Photosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology **40**, 503–537.

Fernandez-Marcos M, Sanz L, Lewis DR, Muday GK, Lorenzo O. 2011. Nitric oxide causes root apical meristem defects and growth inhibition while reducing PIN-FORMED 1 (PIN1)-dependent acropetal auxin transport. Proceedings of the National Academy of Sciences **108**, 18506–18511.

Foster MW, McMahon TJ, Stamler JS. 2003. S-nitrosylation in health and disease. Trends in Molecular Medicine **9**, 160–168.

Freschi L. 2013. Nitric oxide and phytohormone interactions: current status and perspectives. Frontiers in Plant Science **4**, 1–22.

Freschi L, Rodrigues MA, Domingues DS, Purgatto E, Van Sluys MA, Magalhaes JR, Kaiser WM, Mercier H. 2010. Nitric Oxide Mediates the Hormonal Control of Crassulacean Acid Metabolism Expression in Young Pineapple Plants. Plant Physiology **152**, 1971–1985.

Frey AD, Kallio PT. 2005. Nitric oxide detoxification - A new era for bacterial globins in biotechnology? Trends in Biotechnology **23**, 69–73.

Frungillo L, Skelly MJ, Loake GJ, Spoel SH, Salgado I. 2014. S-nitrosothiols regulate nitric oxide production and storage in plants through the nitrogen assimilation pathway. Nature Communications **5**, 5401.

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

Garrocho-Villegas V, Arredondo-Peter R. 2008. Molecular cloning and characterization of a moss (Ceratodon purpureus) nonsymbiotic hemoglobin provides insight into the early evolution of plant nonsymbiotic hemoglobins. Molecular Biology and Evolution **25**, 1482–1487.

Garrocho-Villegas V, Gopalasubramaniam SK, Arredondo-Peter R. 2007. Plant hemoglobins: What we know six decades after their discovery. Gene **398**, 78–85.

Gayatri G, Agurla S, Raghavendra AS. 2013. Nitric oxide in guard cells as an important secondary messenger during stomatal closure. Frontiers in Plant Science **4**, 1–11.

Genty B, Briantais JM, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta - General Subjects **990**, 87–92.

Gilbert GA, Wilson C, Madore MA. 1997. Root-Zone Salinity Alters Raffinose Oligosaccharide Metabolism and Transport in Coleus. Plant Physiology **115**, 1267–1276.

Grün S, Lindermayr C, Sell S, Durner J. 2006. Nitric oxide and gene regulation in plants. Journal of Experimental Botany **57**, 507–516.

Guo FQ, Okamoto M, Crawford NM. 2003. Identification of a Plant Nitric Oxide Synthase Gene Involved in Hormonal Signaling. Science **302**, 100–103.

Gupta KJ, Fernie AR, Kaiser WM, van Dongen JT. 2011*a*. On the origins of nitric oxide. Trends in Plant Science **16**, 160–168.

Gupta KJ, Hebelstrup KH, Mur LAJ, Igamberdiev AU. 2011*b*. Plant hemoglobins: Important players at the crossroads between oxygen and nitric oxide. FEBS Letters **585**, 3843–3849.

Hancock JT, Neill SJ, Wilson ID. 2011. Nitric oxide and ABA in the control of plant function. Plant Science **181**, 555–559.

Hargrove MS, Brucker EA, Stec B, Sarath G, Arredondo-Peter R, Klucas RV, Olson JS, Phillips GN. 2000. Crystal structure of a nonsymbiotic plant hemoglobin. Structure **8**, 1005–1014.

Harper JE. 1981. Evolution of Nitrogen Oxide(s) during In Vivo Nitrate Reductase Assay of Soybean Leaves. Plant physiology **68**, 1488–1493.

He Y, Tang R-H, Hao Y, Stevens RD, Cook CW. 2004. Nitric Oxide Represses the Arabidopsis Floral Transition. Science **305**, 1968–1971.

Hebelstrup KH, Hunt P, Dennis E, Jensen SB, Jensen EØ. 2006. Hemoglobin is essential for normal growth of Arabidopsis organs. Physiologia Plantarum **127**, 157–166.

Hebelstrup KH, Jensen EØ. 2008. Expression of NO scavenging hemoglobin is involved in the timing of bolting in Arabidopsis thaliana. Planta **227**, 917–927.

Hebelstrup KH, Shah JK, Igamberdiev AU. 2013. The role of nitric oxide and hemoglobin in plant development and morphogenesis. Physiologia Plantarum **148**, 457–469.

Hebelstrup KH, Shah JK, Simpson C, Schjoerring JK, Mandon J, Cristescu SM, Harren FJM, Christiansen MW, Mur LAJ, Igamberdiev AU. 2014. An assessment of the biotechnological use of hemoglobin modulation in cereals. Physiologia Plantarum **150**, 593–603.

Hendriks T, Scheer I, Quillet MC, Randoux BE, Delbreil B, Vasseur J, Hilbert JL. 1998. A nonsymbiotic hemoglobin gene is expressed during somatic embryogenesis in Cichorium. Biochimica et Biophysica Acta - Gene Structure and Expression **1443**, 193–197.

Hill AC. 1971. Vegetation: A Sink for Atmospheric Pollutants. Journal of the Air Pollution Control Association **21**, 341–346.

Hill RD. 1998. What are hemoglobins doing in plants ? Can. J. Microbiol, 707–712.

Hill RD. 2012. Non-symbiotic haemoglobins--What's happening beyond nitric oxide scavenging? AoB Plants **2012**, pls004-pls004.

Hill R, Hargrove M, Arredondo-Peter R. 2016. Pgb: a novel nomenclature for plant globins accepted by the globin community at the 2014 XVIII conference on Oxygen-Binding and Sensing Proteins. F1000Research **5**, 212.

Hoy JA, Hargrove MS. 2008. The structure and function of plant hemoglobins.

Plant Physiology and Biochemistry **46**, 371–379.

Hu HW, Chen D, He JZ. 2015. Microbial regulation of terrestrial nitrous oxide formation: Understanding the biological pathways for prediction of emission rates. FEMS Microbiology Reviews **39**, 729–749.

Hu X, Neill SJ, Tang Z, Cai W. 2005. Nitric Oxide Mediates Gravitropic Bending in Soybean Roots. Plant Physiology **137**, 663–670.

Hu Y, Sun G. 2010. Leaf nitrogen dioxide uptake coupling apoplastic chemistry, carbon/sulfur assimilation, and plant nitrogen status. Plant Cell Reports **29**, 1069–1077.

Huang Y, Li D. 2014. Soil nitric oxide emissions from terrestrial ecosystems in China: A synthesis of modeling and measurements. Scientific Reports **4**, 4–11.

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

Hudman RC, Moore NE, Mebust AK, Martin R V., Russell AR, Valin LC, Cohen RC. 2012*a*. Steps towards a mechanistic model of global soil nitric oxide emissions: Implementation and space based-constraints. Atmospheric Chemistry and Physics **12**, 7779–7795.

Hudman RC, Moore NE, Mebust AK, Martin R V, Russell AR, Valin LC, Cohen RC. 2012*b*. Steps towards a mechanistic model of global soil nitric oxide emissions : implementation and space based-constraints. Atmos. Chem. Phys, 7779–7795.

Hung KT, Kao CH. 2003. Nitric oxide counteracts the senescence of rice leaves induced by abscisic acid. Journal of Plant Physiology **160**, 871–879.

Hung KT, Kao CH. 2004. Nitric oxide acts as an antioxidant and delays methyl jasmonate-induced senescence of rice leaves. Journal of Plant Physiology **161**, 43–52.

Hunt PW, Watts RA, Trevaskis B, Llewelyn DJ, Burnell J, Dennis ES,

Peacock WJ. 2001. Expression and evolution of functionally distinct haemoglobin genes in plants. Plant Molecular Biology **47**, 677–692.

Igamberdiev AU, Bykova N V., Hill RD. 2006. Nitric oxide scavenging by barley hemoglobin is facilitated by a monodehydroascorbate reductase-mediated ascorbate reduction of methemoglobin. Planta **223**, 1033–1040.

Igamberdiev AU, Hill RD. 2004. Nitrate, NO and haemoglobin in plant adaptation to hypoxia: An alternative to classic fermentation pathways. Journal of Experimental Botany **55**, 2473–2482.

Jasid S, Simontacchi M, Bartoli CG, Puntarulo S. 2006. Chloroplasts as a Nitric Oxide Cellular Source. Effect of Reactive Nitrogen Species on Chloroplastic Lipids and Proteins. Plant Physiology **142**, 1246–1255.

Jeandroz S, Wipf D, Stuehr DJ, Lamattina L, Melkonian M, Tian Z, Zhu Y, Carpenter EJ, Wong GKS, Wendehenne D. 2016. Occurrence, structure, and evolution of nitric oxide synthase-like proteins in the plant kingdom. Science Signaling **9**.

Jin CW, Du ST, Zhang YS, Tang C, Lin XY. 2009. Atmospheric nitric oxide stimulates plant growth and improves the quality of spinach (Spinacia oleracea). Annals of Applied Biology **155**, 113–120.

Kanter D, Mauzerall DL, Ravishankara AR, Daniel JS, Portmann RW, Grabiel PM. 2013. A post-Kyoto partner: Considering the stratospheric ozone regime as a tool to manage nitrous oxide. Proceedings of the National Academy of Sciences of the United States of America **110**, 4451–4457.

Kapoor K, Mira MM, Ayele BT, Nguyen T, Robert N, Claudio DH. 2018. Pgbs regulate nitric oxide ‐ dependent abscisic acid synthesis and ethylene ‐ induced program cell death in developing maize somatic embryos. Planta **247**, 1277–1291.

Klepper L. 1979. Nitric oxide and nitrogen dioxide (NO2) emissions from herbicide- treated soybean plants. Atmospheric Environment **13**, 237–542.

Klepper L. 1990. Comparison between NO(x) Evolution Mechanisms of Wild-

Type and nr(1) Mutant Soybean Leaves. Plant Physiology **93**, 26–32.

Kolbert Z, Ortega L, Erdei L. 2010. Involvement of nitrate reductase (NR) in osmotic stress-induced NO generation of Arabidopsis thaliana L. roots. Journal of Plant Physiology **167**, 77–80.

Kolbert Z, Petô A, Szôllôsi R, Erdei L, Tari I. 2011. Nitric oxide (NO) generation during vegetative/generative transition of the apical meristem in wheat. Acta Biologica Szegediensis **55**, 95–97.

Kopyra M, Gwóźdź EA. 2003. Nitric oxide stimulates seed germination and counteracts the inhibitory effect of heavy metals and salinity on root growth of Lupinus luteus. Plant Physiology and Biochemistry **41**, 1011–1017.

Krapp A. 2015. Plant nitrogen assimilation and its regulation: A complex puzzle with missing pieces. Current Opinion in Plant Biology **25**, 115–122.

Krasuska U, Ciacka K, Andryka-Dudek P, Bogatek R. 2015. "Signaling and communication in plants," in Reactive Oxygen and Nitrogen Species Signaling and Communication in Plants, eds K. J. Gupta and A. U. Igamberdiev (New York City, NY: Springer International Publishing), 316.

Krizman M, Jakse J, Baricevic D, Javornik B, Prosekm M. 2006. Robust CTAB-activated charcoal protocol for plant DNA extraction. Acta agriculturae Slovenica **87**, 427–433.

Kubo H. 1939. Uber hamoprotein aus den wurzelknollchen von leguminosen, Acta Phytochim. (Tokyo), 11 ,195-200

Kundu S, Trent JT, Hargrove MS. 2003. Plants, humans and hemoglobins. Trends in Plant Science **8**, 387–393.

Kuruthukulangarakoola GT, Zhang J, Albert A, *et al.* 2017. Nitric oxide-fixation by non-symbiotic haemoglobin proteins in Arabidopsis thaliana under N-limited conditions. Plant Cell and Environment **40**, 36–50.

Lamattina L, García-Mata C, Graziano M, Pagnussat G. 2003. NITRIC OXIDE: The Versatility of an Extensive Signal Molecule. Annual Review of Plant

Biology **54**, 109–136.

LANE PI, BELL JNB. 1984. the Effects of Simulated Urban Air-Pollution on Grass Yield .2. Performance of Lolium-Perenne, Phleum-Pratense and Dactylis-Glomerata Fumigated With So2, No2 and/or No. Environmental Pollution Series a-Ecological and Biological **35**, 97–124.

Lanteri L, Lombardo C, Pagnussat GC, Lamattina L. 2004. Nitric Oxide Mediates the Indole Acetic Acid Induction Activation of a Mitogen-Activated Protein Kinase Cascade Involved in Adventitious Root Development 1. Plant Physiology **135**, 279–286.

Lanteri ML, Pagnussat GC, Lamattina L. 2006. Calcium and calcium-dependent protein kinases are involved in nitric oxide- and auxin-induced adventitious root formation in cucumber. Journal of Experimental Botany **57**, 1341–1351.

Larsen K. 2003. Molecular cloning and characterization of cDNAs encoding hemoglobin from wheat (Triticum aestivum) and potato (Solanum tuberosum). Biochimica et Biophysica Acta - General Subjects **1621**, 299–305.

Lee YN, Schwartz SE. 1981. Evaluation of the rate of uptake of nitrogen dioxide by atmospheric and surface liquid water. Journal of Geophysical Research **86**, 11971–11983.

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

Leshem YY, Pinchasov Y. 2000. Non-invasive photoacoustic spectroscopic determination of relative endogenous nitric oxide and ethylene content stoichiometry during the ripening of strawberries Fragaria anannasa (Duch.) and avocados Persea americana (Mill.)1. Journal of Experimental Botany **51**, 1471–1473.

Leshem YY, Wills RBH, Ku VVV. 1998. Evidence for the function of the free

radical gas - nitric oxide (NO·) as an endogenous maturation and senescence regulating factor in higher plants. Plant Physiology and Biochemistry **36**, 825–833.

Lindermayr C, Hebelstrup KH. 2016. Growth-Promoting Effect of NO Fumigation and Hemoglobins. In: Lamattina L., García-Mata C. (eds) Gasotransmitters in Plants. Signaling and Communication in Plants. Springer, Cham. Doi: 10.1007/978-3-319-40713-5_7

Lindermayr C, Sell S, Muller B, Leister D, Durner J. 2010. Redox Regulation of the NPR1-TGA1 System of Arabidopsis thaliana by Nitric Oxide. The Plant Cell **22**, 2894–2907.

Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS. 2001. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. Nature **410**, 490–494.

Liu Y, Shi L, Ye N, Liu R, Jia W, Zhang J. 2009. Nitric oxide-induced rapid decrease of abscisic acid concentration is required in breaking seed dormancy in Arabidopsis. New Phytologist **183**, 1030–1042.

LIU M chen, SONG W hong, ZHU S hua, ZHOU J. 2007. Effects of Nitric Oxide and Exogenous Ethylene Treatments on Ethylene Biosynthesis in Feicheng Peach. Agricultural Sciences in China **6**, 290–295.

Liu Y, Ye N, Liu R, Chen M, Zhang J. 2010. H2O2mediates the regulation of ABA catabolism and GA biosynthesis in Arabidopsis seed dormancy and germination. Journal of Experimental Botany **61**, 2979–2990.

Lombardo MC, Graziano M, Polacco JC, Lamattina L. 2006. Nitric Oxide Functions as a Positive Regulator of Root Hair Development. Plant Signaling and Behavior **1:1**, 28–33.

Lozano-Juste J, Leon J. 2011. Nitric Oxide Regulates DELLA Content and PIF Expression to Promote Photomorphogenesis in Arabidopsis. Plant Physiology **156**, 1410–1423.

MA C yan, XU X, HAO L, CAO J. 2007. Nitrogen Dioxide-Induced Responses in Brassica campestris Seedlings: The Role of Hydrogen Peroxide in the Modulation of Antioxidative Level and Induced Resistance. Agricultural Sciences in China **6**, 1193–1200.

Manjunatha G, Gupta KJ, Lokesh V, Mur LAJ, Neelwarne B. 2012. Nitric oxide counters ethylene effects on ripening fruits. Plant Signaling and Behavior **7**, 476–483.

Manjunatha G, Lokesh V, Neelwarne B. 2010. Nitric oxide in fruit ripening: Trends and opportunities. Biotechnology Advances **28**, 489–499.

Meyer C, Lea US, Provan F, Kaiser WM, Lillo C. 2005. Is nitrate reductase a major player in the plant NO (nitric oxide) game? Photosynthesis Research **83**, 181–189.

Meyer C, Stöhr C. 2002. Soluble and Plasma Membrane-bound Enzymes Involved in Nitrate and Nitrite Metabolism. Photosynthetic Nitrogen Assimilation and Associated Carbon and Respiratory Metabolism, 49–62.

Mira MM, Hill RD, Stasolla C. 2016. Pgbs Improve Hypoxic Root Growth by Alleviating Apical Meristem Cell Death. Plant Physiology **172**, 2044–2056.

Mira MM, Huang S, Kapoor K, Hammond C, Hill RD, Stasolla C. 2017. Expression of Arabidopsis class 1 Pgb (AtPgb1) delays death and degradation of the root apical meristem during severe PEG-induced water deficit. Journal of Experimental Botany **68**, 5653–5668.

Moche M, Stremlau S, Hecht L, Göbel C, Feussner I, Stöhr C. 2010. Effect of nitrate supply and mycorrhizal inoculation on characteristics of tobacco root plasma membrane vesicles. Planta **231**, 425–436.

Montilla-Bascón G, Rubiales D, Hebelstrup KH, Mandon J, Harren FJM, Cristescu SM, Mur LAJ, Prats E. 2017. Reduced nitric oxide levels during drought stress promote drought tolerance in barley and is associated with elevated polyamine biosynthesis. Scientific Reports **7**, 1–15.

Morikawa H, Higaki A, Nohno M, *et al.* 1998. More than a 600-fold variation in nitrogen dioxide assimilation among 217 plant taxa. Plant, Cell and Environment **21**, 180–190.

Mukhi N, Dhindwal S, Uppal S, Kapoor A, Arya R, Kumar P, Kaur J, Kundu S. 2016. Structural and Functional Significance of the N- and C-Terminal Appendages in Arabidopsis Truncated Hemoglobin. Biochemistry **55**, 1724–1740.

Mukhi N, Kundu S, Kaur J. 2017. NO dioxygenase- and peroxidase-like activity of Arabidopsis Pgb 3 and its role in Sclerotinia sclerotiorum defense. Nitric Oxide - Biology and Chemistry **68**, 150–162.

Mur LAJ, Mandon J, Cristescu SM, Harren FJM, Prats E. 2011. Methods of nitric oxide detection in plants: A commentary. Plant Science **181**, 509–519.

Mur LAJ, Mandon J, Persijn S, Cristescu SM, Moshkov IE, Novikova G V., Hall MA, Harren FJM, Hebelstrup KH, Gupta KJ. 2013. Nitric oxide in plants: An assessment of the current state of knowledge. AoB PLANTS **5**, 1–17.

Nawaz F, Shabbir RN, Shahbaz M, Majeed S, Raheel M, Hassan W, Sohail MA. 2017. Cross Talk between Nitric Oxide and Phytohormones Regulate Plant Development during Abiotic Stresses. Phytohormones - Signaling Mechanisms and Crosstalk in Plant Development and Stress Responses.

Needoba JA, Sigman DM, Harrison PJ. 2004. The mechanism of isotope fractionation during algal nitrate assimilation as illuminated by the 15N/14N of intracellular nitrate. J Phycol **40**, 517–522.

Neill SJ. 2002. Hydrogen peroxide and nitric oxide as signalling molecules in plants. Journal of Experimental Botany **53**, 1237–1247.

Neill S, Barros R, Bright J, Desikan R, Hancock J, Harrison J, Morris P, Ribeiro D, Wilson I. 2008. Nitric oxide, stomatal closure, and abiotic stress. Journal of Experimental Botany **59**, 165–176.

Nienhaus K, Dominici P, Astegno A, Abbruzzetti S, Viappiani C, Nienhaus GU. 2010. Ligand migration and binding in nonsymbiotic hemoglobins of

arabidopsis thaliana. Biochemistry **49**, 7448–7458.

Nowak DJ, Crane DE, Stevens JC. 2006. Air pollution removal by urban trees and shrubs in the United States. Urban Forestry and Urban Greening **4**, 115–123.

Ohwaki Y, Kawagishi-Kobayashi M, Wakasa K, Fujihara S, Yoneyama T. 2005. Induction of class-1 non-symbiotic hemoglobin genes by nitrate, nitrite and nitric oxide in cultured rice cells. Plant and Cell Physiology **46**, 324–331.

Omasa K, Tobe K, Kondo T. 2002. Absorption of Organic and Inorganic Air Pollutants by Plants. In: Omasa K., Saji H., Youssefian S., Kondo N. (eds) Air Pollution and Plant Biotechnology. Springer, Tokyo. DOI: 10.1007/978-4-431-68388-9_8

Ötvös K, Pasternak TP, Miskolczi P, Domoki M, Dorjgotov D, Szucs A, Bottka S, Dudits D, Fehér A. 2005. Nitric oxide is required for, and promotes auxin-mediated activation of, cell division and embryogenic cell formation but does not influence cell cycle progression in alfalfa cell cultures. Plant Journal **43**, 849–860.

Pagnussat GC, Lanteri ML, Lombardo MC, Lorenzo Lamattina. 2004. Nitric Oxide Mediates the Indole Acetic Acid Induction Activation of a Mitogen-Activated Protein Kinase Cascade Involved in Adventitious Root Development 1. Plant Physiology **135**, 279–286.

Pagnussat GC, Lanteri ML, Lorenzo Lamattina. 2003. Nitric Oxide and Cyclic GMP Are Messengers in the Indole Acetic Acid-Induced Adventitious Rooting Process. Plant Physiology **132**, 1241–1248.

Pagnussat GC, Simontacchi M, Puntarulo S, Lamattina L. 2002. Nitric Oxide Is Required for Root Organogenesis. Plant Physiology **129**, 954–956.

Perazzolli M, Dominici P, Romero-Puertas MC, Zago E, Zeier J, Sonoda M, Lamb C, Delledonne M. 2004. Arabidopsis Nonsymbiotic Hemoglobin AHb1 Modulates Nitric Oxide Bioactivity. The Plant Cell **16**, 2785–2794.

Pii Y, Crimi M, Cremonese G, Spena A, Pandolfini T. 2007. Auxin and nitric

oxide control indeterminate nodule formation. BMC Plant Biology **7**, 1–11.

Pilegaard K. 2013. Processes regulating nitric oxide emissions from soils. Philosophical Transactions of the Royal Society B: Biological Sciences **368**, 20130126–20130126.

Prado AM, Colacxo R, Moreno N, Silva AC, Feijó JA. 2008. Targeting of pollen tubes to ovules is dependent on nitric oxide (NO) signaling. Molecular Plant **1**, 703–714.

Procházková D, Haisel D, Wilhelmová N, Pavlíková D, Száková J. 2013. Effects of exogenous nitric oxide on photosynthesis. Photosynthetica **51**, 483–489.

Qu ZL, Zhong NQ, Wang HY, Chen AP, Jian GL, Xia GX. 2006. Ectopic expression of the cotton non-symbiotic hemoglobin gene GhHbd1 triggers defense responses and increases disease tolerance in Arabidopsis. Plant and Cell Physiology **47**, 1058–1068.

RAMGE P, BADECK F -W, PLÖCHL M, KOHLMAIER GH. 1993. Apoplastic antioxidants as decisive elimination factors within the uptake process of nitrogen dioxide into leaf tissues. New Phytologist **125**, 771–785.

Ribeiro EA, Cunha FQ, Tamashiro WMSC, Martins IS. 1999. Growth phase-dependent subcellular localization of nitric oxide synthase in maize cells. FEBS Letters **445**, 283–286.

Rinaldi AC, Bonamore A, Macone A, Boffi A, Bozzi A, Di Giulio A. 2006. Interaction of Vitreoscilla hemoglobin with membrane lipids. Biochemistry **45**, 4069–4076.

Robinson D. 2001. delta15N as an integrator of the nitrogen cycle. Trends Ecol Evol **16**,153–162.

Rockel P, Strube F, Rockel A, Wildt J, Kaiser WM. 2002. Regulation of NO produced by plant NR.pdf. Journal of Experimental Botany **53**, 103–110.

Rodríguez-Ruiz M, Mioto P, Palma JM, Corpas FJ. 2017. S-nitrosoglutathione reductase (GSNOR) activity is down-regulated during pepper (Capsicum annuum

L.) fruit ripening. Nitric Oxide - Biology and Chemistry **68**, 51–55.

Rogers HH, Campbell JC, Volk RJ. 1979. Nitrogen-15 dioxide uptake and incorporation by phaseolus vulgaris (L.). Science **206**, 333–335.

Rogers HH, Jeffries HE, Heck WW, Witherspoon AM, Ripperton LA. 1977. Measuring air pollutant uptake by plants: A Direct kinetic technique. Journal of the Air Pollution Control Association **27**, 1192–1197.

Sami F, Faizan M, Faraz A, Siddiqui H, Yusuf M, Hayat S. 2018. Nitric oxide-mediated integrative alterations in plant metabolism to confer abiotic stress tolerance, NO crosstalk with phytohormones and NO-mediated post translational modifications in modulating diverse plant stress. Nitric Oxide - Biology and Chemistry **73**, 22–38.

Sandhu R, Gian Gupta. 1989. Effects of Nitrogen Fertilizer on Growth and Yield of Spring Wheat. Environmental Pollution **59**, 337–344.

Sang J, Jiang M, Lin F, Xu S, Zhang A, Tan M. 2008. Nitric oxide reduces hydrogen peroxide accumulation involved in water stress-induced subcellular anti-oxidant defense in maize plants. Journal of Integrative Plant Biology **50**, 231–243.

Sanz L, Albertos P, Mateos I, Sánchez-Vicente I, Lechón T, Fernández-Marcos M, Lorenzo O. 2015. Nitric oxide (NO) and phytohormones crosstalk during early plant development. Journal of Experimental Botany **66**, 2857–2868.

Saxe H. 1994. Relative sensitivity of greenhouse pot plants To long-term exposures of NO- and NO2-containing air. Environmental Pollution **85**, 283–290.

Seregélyes C, Igamberdiev AU, Maassen A, Hennig J, Dudits D, Hill RD. 2004. NO-degradation by alfalfa class 1 hemoglobin (Mhb1): A possible link to PR-1a gene expression in Mhb1-overproducing tobacco plants. FEBS Letters **571**, 61–66.

Shankar A, Fernandes JL, Kaur K, Sharma M, Kundu S, Pandey GK. 2018. Rice Pgbs regulate responses under low mineral nutrients and abiotic stresses in Arabidopsis thaliana. Plant Cell and Environment **41**, 215–230.

Shen Q, Wang YT, Tian H, Guo FQ. 2013. Nitric oxide mediates cytokinin

functions in cell proliferation and meristem maintenance in arabidopsis. Molecular Plant **6**, 1214–1225.

Shi FM, Li YZ. 2008. Verticillium dahliae toxins-induced nitric oxide production in Arabidopsis is major dependent on nitrate reductase. Bmb Reports **41**, 79–85.

Shi S, Wang G, Wang Y, Zhang L, Zhang L. 2005. Protective effect of nitric oxide against oxidative stress under ultraviolet-B radiation. Nitric Oxide - Biology and Chemistry **13**, 1–9.

Shimoda Y, Shimoda-Sasakura F, Kucho KI, Kanamori N, Nagata M, Suzuki A, Abe M, Higashi S, Uchiumi T. 2009. Overexpression of class 1 plant hemoglobin genes enhances symbiotic nitrogen fixation activity between Mesorhizobium loti and Lotus japonicus. Plant Journal **57**, 254–263.

Simontacchi M, Galatro A, Ramos-Artuso F, Santa-María GE. 2015. Plant Survival in a Changing Environment: The Role of Nitric Oxide in Plant Responses to Abiotic Stress. Frontiers in Plant Science **6**, 1–19.

Simontacchi M, García-Mata C, Bartoli CG, Santa-María GE, Lamattina L. 2013. Nitric oxide as a key component in hormone-regulated processes. Plant Cell Reports **32**, 853–866.

Singh A, Agrawal M. 2008. Acid rain and its ecological consequences. Journal of Environmental Biology **29**, 15–24.

Singh HP, Kaur S, Batish DR, Sharma VP, Sharma N, Kohli RK. 2009. Nitric oxide alleviates arsenic toxicity by reducing oxidative damage in the roots of Oryza sativa (rice). Nitric Oxide - Biology and Chemistry **20**, 289–297.

Smagghe BJ, Hoy JA, Percifield R, *et al.* 2009. Correlations between oxygen affinity and sequence classifications of plant hemoglobins. Biopolymers - Peptide Science Section **91**, 1083–1096.

Smagghe BJ, Trent JT, Hargrove MS. 2008. NO dioxygenase activity in hemoglobins is ubiquitous in vitro, but limited by reduction in vivo. PLoS ONE **3**.

Soegiarto L, Wills RBH, Seberry JA, Leshem YY. 2003. Nitric oxide

degradation in oxygen atmospheres and rate of uptake by horticultural produce. Postharvest Biology and Technology **28**, 327–331.

Sørensen CK, Carciofi M, Hasler-Sheetal H, Zafari S, Andrzejczak O, Hovmøller MS, Møller IM, Hebelstrup KH. 2018. Overexpression of Pgb in barley alters both compatible and incompatible interactions with the mildew pathogen *Blumeria graminis*. Plant Pathology.

Sparks JP, Monson RK, Sparks KL, Lerdau M. 2001. Leaf uptake of nitrogen dioxide (NO2) in a tropical wet forest: Implications for tropospheric chemistry. Oecologia **127**, 214–221.

Srivastava N, Gonugunta VK, Puli MR, Raghavendra AS. 2009. Nitric oxide production occurs downstream of reactive oxygen species in guard cells during stomatal closure induced by chitosan in abaxial epidermis of Pisum sativum. Planta **229**, 757–765.

Stasolla C, Hill RD. 2017. Determining Cellular Responses: Pgbs May Direct the Traffic. Trends in Plant Science **22**, 820–822.

Stöhr C, Strube F, Marx G, R WU, Rockel P. 2001. Achievable rate and fairness in coordinated base station transmission. Planta **212**, 835–841.

Stoimenova M, Igamberdiev AU, Gupta KJ, Robert D, Planta S, July N, Stoimenova M, Igamberdiev AU. 2007. Plant defense response against Fusarium oxysporum and strategies to develop tolerant genotypes in PubMed Commons. Planta **226**, 465–474.

Stulen I, Perez-Soba M, Kok LJ DE, Eerden L Vander. 2017. Impact of gaseous nitrogen deposition on plant functioning. New Phytol **139**, 61–70.

Sun C, Lu L, Liu L, Liu W, Yu Y, Liu X, Hu Y, Jin C, Lin X. 2014. Nitrate reductase-mediated early nitric oxide burst alleviates oxidative damage induced by aluminum through enhancement of antioxidant defenses in roots of wheat (Triticum aestivum). New Phytologist **201**, 1240–1250.

Takahashi M, Adam SEH, Konaka D, Morikawa H. 2008. Nitrogen dioxide at

an ambient level improves the capability of kenaf (Hibiscus cannabinus) to decontaminate cadmium. International Journal of Phytoremediation **10**, 73–76.

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

Takahashi M, Morikawa H. 2014. Nitrogen dioxide is a positive regulator of plant growth. Plant Signaling and Behavior **9**, 8–11.

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

Taylor ER, Nie XZ, MacGregor AW, Hill RD. 1994. A cereal haemoglobin gene is expressed in seed and root tissues under anaerobic conditions. Plant Molecular Biology **24**, 853–862.

Terrile MC, París R, Calderón-Villalobos LIA, Iglesias MJ, Lamattina L, Estelle M, Casalongué CA. 2012. Nitric oxide influences auxin signaling through S-nitrosylation of the Arabidopsis TRANSPORT INHIBITOR RESPONSE 1 auxin receptor. Plant Journal **70**, 492–500.

Thomson AJ, Giannopoulos G, Pretty J, Baggs EM, Richardson DJ. 2012. Biological sources and sinks of nitrous oxide and strategies to mitigate emissions. Philosophical Transactions of the Royal Society B: Biological Sciences **367**, 1157–1168.

Tonón C, Cecilia Terrile M, José Iglesias M, Lamattina L, Casalongué C. 2010. Extracellular ATP, nitric oxide and superoxide act coordinately to regulate hypocotyl growth in etiolated Arabidopsis seedlings. Journal of Plant Physiology **167**, 540–546.

Tossi V, Lamattina L, Cassia R. 2009. An increase in the concentration of

abscisic acid is critical for nitric oxide-mediated plant adaptive responses to UV-B irradiation. New Phytologist **181**, 871–879.

Trevaskis B, Watts RA, Andersson CR, Llewellyn DJ, Hargrove MS, Olson JS, Dennis ES, Peacock WJ. 1997. Two hemoglobin genes in Arabidopsis thaliana: the evolutionary origins of leghemoglobins. Proceedings of the National Academy of Sciences of the United States of America **94**, 12230–4.

Tun NN, Holk A, Scherer GFE. 2001. Rapid increase of NO release in plant cell cultures induced by cytokinin. FEBS Letters **509**, 174–176.

Tun NN, Livaja M, Kieber JJ, Scherer GFE. 2008. Zeatin-induced nitric oxide (NO) biosynthesis in Arabidopsis thaliana mutants of NO biosynthesis and of two-component signaling genes. New Phytologist **178**, 515–531.

Tun NN, Santa-Catarina C, Begum T, Silveira V, Handro W, Segal Floh EI, Scherer GFE. 2006. Polyamines induce rapid biosynthesis of nitric oxide (NO) in Arabidopsis thaliana seedlings. Plant and Cell Physiology **47**, 346–354.

Uchida A, Jagendorf AT, Hibino T, Takabe T, Takabe T. 2002. Effects of hydrogen peroxide and nitric oxide on both salt and heat stress tolerance in rice. Plant Science **163**, 515–523.

Vázquez-Limón C, Hoogewijs D, Vinogradov SN, Arredondo-Peter R. 2012. The evolution of land plant hemoglobins. Plant Science **191–192**, 71–81.

Vigeolas H, Huhn D, Geigenberger P. 2011. Nonsymbiotic Hemoglobin-2 Leads to an Elevated Energy State and to a Combined Increase in Polyunsaturated Fatty Acids and Total Oil Content When Overexpressed in Developing Seeds of Transgenic Arabidopsis Plants. Plant Physiology **155**, 1435–1444.

Vinken GCM, Boersma KF, Maasakkers JD, Adon M, Martin R V. 2014. Worldwide biogenic soil NOxemissions inferred from OMI NO2observations. Atmospheric Chemistry and Physics **14**, 10363–10381.

Vinogradov SN, Fernández I, Hoogewijs D, Arredondo-Peter R. 2011. Phylogenetic relationships of 3/3 and 2/2 hemoglobins in Archaeplastida genomes

to bacterial and other eukaryote hemoglobins. Molecular Plant **4**, 42–58.

Vinogradov SN, Hoogewijs D, Bailly X, Arredondo-Peter R, Gough J, Dewilde S, Moens L, Vanfleteren JR. 2006. A phylogenomic profile of globins. BMC Evolutionary Biology **6**, 1–17.

Wang YH, Kochian L V., Doyle JJ, Garvin DF. 2003. Two tomato non-symbiotic haemoglobin genes are differentially expressed in response to diverse changes in mineral nutrient status. Plant, Cell and Environment **26**, 673–680.

Wang BL, Tang XY, Cheng LY, *et al.* 2010. Nitric oxide is involved in phosphorus deficiency-induced cluster-root development and citrate exudation in white lupin. New Phytologist **187**, 1112–1123.

Watts RA, Hunt PW, Hvitved AN, Hargrove MS, Peacock WJ, Dennis ES. 2001. A hemoglobin from plants homologous to truncated hemoglobins of microorganisms. Proceedings of the National Academy of Sciences **98**, 10119–10124.

Wellburn AR. 1990. Why are atmnospheric oxides of nitrogen usually phytotoxic and not alternative fertilizers ? New Phytologist **115**, 395–429.

Wellburn AR, Wilson J, Aldridge PH. 1980. Biochemical responses of plants to nitric oxide polluted atmospheres. Environmental Pollution. Series A, Ecological and Biological **22**, 219–228.

Wilkinson JQ, Crawford NM. 1993. Identification and characterization of a chlorate-resistant mutant of Arabidopsis thaliana with mutations in both nitrate reductase structural genes NIA1 and NIA2. MGG Molecular and General Genetics **239**, 289–297.

Wills RBH, Ku VV V, Leshem YY. 2000. Fumigation with nitric oxide to extend the postharvest life of strawberries. Postharvest Biology and Technology **18**, 75–79.

Wimalasekera R, Tebartz F, Scherer GFE. 2011. Polyamines, polyamine oxidases and nitric oxide in development, abiotic and biotic stresses. Plant Science

181, 593–603.

Wittenberg J, Bergersen F, Appleby C, Turner G. 1974. Facilitated oxygen diffusion: the role of leghemoglobin in nitrogen fixation by bacteroids isolated from soybean root nodules. Journal of Biological Chemistry **249**, 4057–4066.

Xiao-Ping S, Xi-Gui S. 2006. Cytokinin- and auxin-induced stomatal opening is related to the change of nitric oxide levels in guard cells in broad bean. Physiologia Plantarum **128**, 569–579.

Xu J, Wang W, Yin H, Liu X, Sun H, Mi Q. 2010. Exogenous nitric oxide improves antioxidative capacity and reduces auxin degradation in roots of Medicago truncatula seedlings under cadmium stress. Plant and Soil **326**, 321–330.

Yamamoto-Katou A, Katou S, Yoshioka H, Doke N, Kawakita K. 2006. Nitrate reductase is responsible for elicitin-induced nitric oxide production in Nicotiana benthamiana. Plant and Cell Physiology **47**, 726–735.

Yang J, Zhang J, Liu K, Wang Z, Liu L. 2006. Abscisic acid and ethylene interact in wheat grains in response to soil drying during grain filling. New Phytologist **171**, 293–303.

Yoneyama T, Sasakawa H. 1979. Transformation of atmospheric NO2 absorbed in spinach leaves. Plant and Cell Physiol. **20**, 263–266.

Zhang L, Routsong R, Strand SE. 2019. Greatly Enhanced Removal of Volatile Organic Carcinogens by a Genetically Modified Houseplant, Pothos Ivy (Epipremnum aureum) Expressing the Mammalian Cytochrome P450 2e1 Gene. Environ. Sci. Technol. **53 (1)**, 325-331

Zhu S, Liu M, Zhou J. 2006. Inhibition by nitric oxide of ethylene biosynthesis and lipoxygenase activity in peach fruit during storage. Postharvest Biology and Technology **42**, 41–48.

7 Supplements

Table S1: Accession numbers of Pgb sequences employed in the multiple alignments and used to generate the phylogenetic tree.

Protein	Species	Accession number
Class 1	*Arabidopsis thaliana* Pgb 1	AAD26949.1
	Malus domestica Pgb 1	AAP57676.1
	Pyrus communis Pgb 1	AAP57677.1
	Gossypum hirsutum Pgb 1	AAL09463.1
	Zea mays Pgb 1	AAG01375.1
	Oryza sativa Pgb 1.1	AAC49882.1
	Oryza sativa Pgb 1.4	AAK72231.1
	Oryza sativa Pgb 1.2	NM_001055972.1
	Oryza sativa Pgb 1.3	NM_001056012.1
	Hordeum vulgare Pgb 1.1	AAB70097.1
	Hordeum vulgare Pgb 1.2	BAK07526.1
Class 2	*Arabidopsis thaliana* Pgb 2	AAM65188.1
	Brassica napus Pgb 2	AAK07741.1
	Grossypium hirsurtum Pgb 2	AAK21604.1
	Beta vulgaris Pgb 2	NP_001290022
Class3	*Arabidopsis thaliana* Pgb 3	AEE86104.1
	Triticum aestivum Pgb 3.1	ACH86231.1
	Triticum aestivum Pgb 3.2	ACH86230.1
	Hordeum vulgare Pgb 3	AAK55410.1

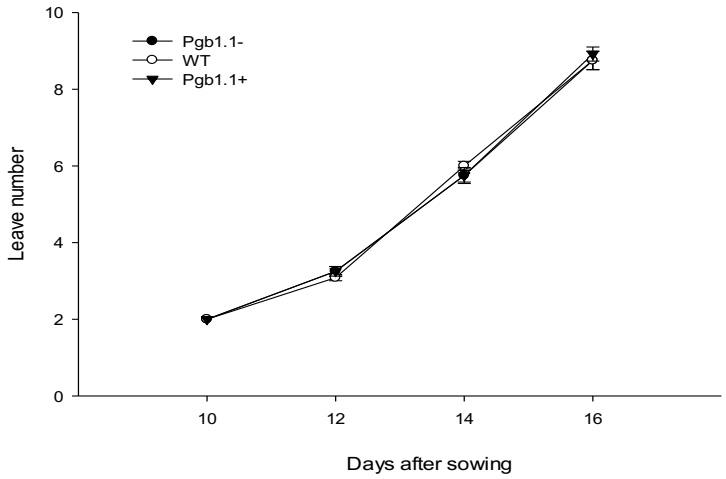


Figure S1 Number of leaves during plant development.

Leaf numbers of WT, HvPgb1.1- and HvPgb1.1+ were determined at 10, 12, 14 and 16 days after sowing. 12 plants per line were analyzed.

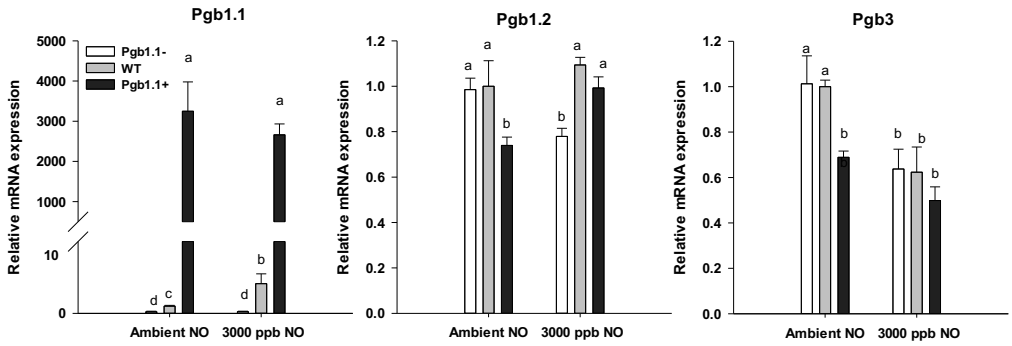


Figure S2 Transcript levels of HvPgb1.1, HvPgb1.2 and HvPgb3 in barley leaves of Pgb1.1-, WT and Pgb1.1+ plants after NO fumigation.

Leaf samples were taken after 20 days of NO fumigation. HvGADPH and HvACTIN were used as housekeeping genes. Each data represents means ± SE (n=4). Different letters indicate significant differences among treatments at $P < 0.05$, according to Tukey's test.

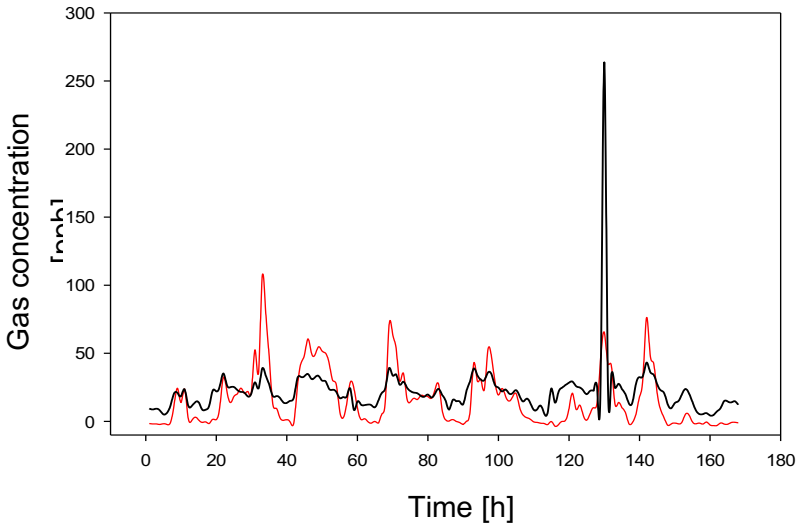


Figure S3 NO and NO2 measurements from 13.02.2019 – 19.02.2019 at the Helmholtz Zentrum München.

NO (red) and NO_2 (black) concentrations were monitored hourly using an Ecophysics chemiluminescence NOx Analyzer. Measurements started on 13.02.2019 at 0:00.

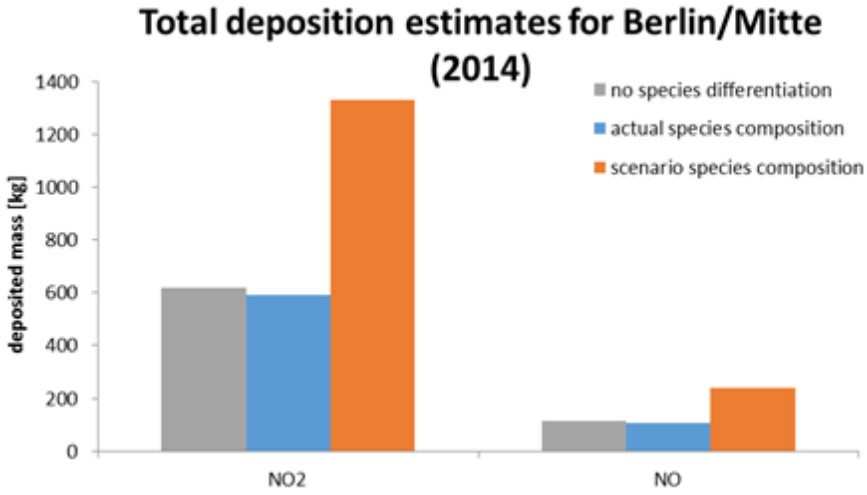


Figure S4 Total deposition estimates for Berlin/Mitte (2014).

The calculations are done with NO_2 and NO for the Mitte District only (appox.65 km^2, 78000 trees, (Tigges *et al*, 2017)) and with climate and air pollution data of the year 2014 (data from the Berlin Senatsverwaltung). The no species differentiation means actual plant composition based on one single standard deposition velocity parameter; actual plant composition means literature-based species-specific deposition velocities; scenario species composition means trees are replaced by "urban greening" plants, measured deposition velocities for the 4 new species (Carpinus betulus, Fraxinus ornus, Fraxinus Pennsylvania, Ostrya carpinifolia) that replace the 4 dominant tree species (maple, linden, beech, oak).

8 Acknowledgements

Firstly, I would like to thank my supervisor PD. Dr. Christian Lindermayr for giving me the opportunity to finish my PhD project work in their laboratory at BIOP, Helmholtz Zentrum München, Germany. PD. Dr. Christian Lindermayr gave me most help and advices during the whole project, his conscientious academic spirit and optimistic attitude inspired me in both academic research and daily life.

Secondly, I would like to thank Prof. Dr. Jörg Durner, my supervisor in TUM. His valuable suggestions and critical comments made me successfully complete my thesis.

I give my gratitude to China Scholarship Council (CSC) for the financial support.

I would like to thank Prof. Dr. Ralph Hückelhoven for being my thesis committee member and the second examiner. His comments provided a new perspective on my work.

Further, I am grateful to Prof. Dr. Jörg-Peter Schnitzler, Dr. Ghirardo Andrea, Felix Antritter Dr. Albert Andreas, and Dr. Winkler, J. Barbro, for their constant help in setting up the NO and NO_2 fumigation systems.

Thanks to Dr. Buegger Franz for performing all the ^{15}N tracer measurement and providing great advices for the data analysis.

I would like to thank Asst. Prof. Dr. Kim Hebelstrup from Aarhus University, Denmark for generously providing me with the barley seeds for my thesis project.

I would also like to thank Ms. Elke Mattes, Ms. Rosina Ludwig, Ms. Lucia Gößl, in our group for their technical suggestions and help during my work. Thank all lab members for their continuous help and providing a pleasant working atmosphere at the institute.

Acknowledgements

Specially, I feel grateful to you, my girlfriend Gao Jin. Thanks for your warm companionship and support during the last years.

Finally, I would like to thank my whole family for their trust and optimism during my studies and this PhD-thesis.

9 Curriculum Vitae

Name	Jiangli Zhang
Email	Zhangjiangli1988@gmail.com
Date of birth	16. 07. 1988
Place of birth	Henan, China

Dissertation

Since 01.09. 2014	Helmholtz Zentrum Munich (Technical University Munich)
	Institute for Biochemical Plant Pathology
	Title: Nitric oxide-fixation by phytoglobin proteins in plants

University studies

2011-2014	M. Sc. of Agriculture from Northwest A and F University, China
2007-2011	B. Sc. of Agriculture from Northwest A and F University, China

Conferences

2016/09: 6th Plant International NO Meeting, Granada, Spain, **Poster**

2018/10: 7th Plant International NO Meeting, Nice, France, **Oral presentation**

Publications

Jiangli Zhang, Franz Buegger, Andreas Albert, Andrea Ghirardo, Barbro Winkler, Jörg-Peter Schnitzler, Kim Henrik Hebelstrup, Jörg Durner, Christian Lindermayr: *Phytoglobin overexpression promotes barley growth in the presence of enhanced level of atmospheric nitric oxide*. *Journal of Experimental Botany*, erz249, Doi:10.1093/jxb/erz249

Gitto Thomas Kuruthukulangarakoola, **Jiangli Zhang**, Andreas Albert, Barbro Winkler, Hans Lang, Franz Buegger, Frank Gaupels, Werner Heller, Bernhard Michalke, Hakan Sarioglu, Jörg-Peter Schnitzler, Kim Henrik Hebelstrup, Jörg

Durner, Christian Lindermayr: *Nitric oxide-fixation by non-symbiotic hemoglobin proteins in Arabidopsis thaliana under N-limited conditions*. Plant Cell and Environment 06/2016; DOI:10.1111/pce.12773

Jiangli Zhang, Changsheng Chen, Di Zhang, Houhua Li, Pengmin Li, Fengwang Ma: *Reactive oxygen species produced via plasma membrane NADPH oxidase regulate anthocyanin synthesis in apple peel*. Planta 07/2014; 240(5). DOI:10.1007/s00425-014-2120-4

Xiuli Bi, **Jiangli Zhang** (co-author), Changsheng Chen, Di Zhang, Pengmin Li, Fengwang Ma: *Anthocyanin contributes more to hydrogen peroxide scavenging than other phenolics in apple peel*. Food Chemistry 06/2014; 152. DOI: 10.1016/j.foodchem.2013.11.088

Jiangli Zhang, Junping Niu, Ying Duan, Mengxia Zhang, Jingying Liu, Pengmin Li, Fengwang Ma: *Photoprotection mechanism in the 'Fuji' apple peel at different levels of 'photooxidative sunburn'*. Physiologia Plantarum 09/2014; 154(1). DOI:10.1111/ppl.12272

Rui Zhai, Xiao-Ting Liu, Wen-Ting Feng, Sha-Sha Chen, Ling-Fei Xu, Zhi-Gang Wang, **Jiang-Li Zhang**, Peng-Min Li, Feng-Wang Ma: *Different Biosynthesis Patterns among Flavonoid 3-glycosides with Distinct Effects on Accumulation of Other Flavonoid Metabolites in Pears (Pyrus bretschneideri Rehd.)*. PLoS ONE 03/2014; 9(3). DOI: 10.1371/journal.pone.0091945