Nitric oxide induces the alternative oxidase pathway in Arabidopsis seedlings deprived of inorganic phosphate

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Abstract

Phosphate starvation compromises electron flow through the cytochrome pathway of the mitochondrial electron transport chain, and plants commonly respond to phosphate deprivation by increasing flow through the alternative oxidase (AOX). To test whether this response is linked to the increase in nitric oxide (NO) production that also increases under phosphate starvation, Arabidopsis thaliana seedlings were grown for 15 d on media containing either 0 or 1 mM inorganic phosphate. The effects of the phosphate supply on growth, the production of NO, respiration, the AOX level and the production of superoxide were compared for wild-type (WT) seedlings and the nitrate reductase double mutant nia. Phosphate deprivation increased NO production in WT roots, and the AOX level and the capacity of the alternative pathway to consume electrons in WT seedlings; whereas the same treatment failed to stimulate NO production and AOX expression in the nia mutant, and the plants had an altered growth phenotype. The NO donor S-nitrosoglutathione rescued the growth phenotype of the nia mutants under phosphate deprivation to some extent, and it also increased the respiratory capacity of AOX. It is concluded that NO is required for the induction of the AOX pathway when seedlings are grown under phosphate-limiting conditions.

Key words: Alternative oxidase, Arabidopsis thaliana, inorganic phosphate, nitric oxide, phosphate stress, reactive oxygen species, respiration.

Introduction

Phosphorus is an important macronutrient and a shortage of inorganic phosphate (Pi) leads to biochemical, physiological and morphological changes in plants that reduce plant growth and yield (Wissuwa et al., 2005). Examples of such changes include an increase in root/shoot ratio, secretion of acid phosphatases and organic acids, increased capacity for Pi uptake, decreased uptake of nitrate, and changes in carbon metabolism (Lee et al., 1990; Lee and Ratcliffe, 1993; Gniazdowska et al., 1998; Rajgohthama, 1999; Plaxton, 2004; Hermans et al., 2006; Plaxton and Tran, 2011; Masakapalli et al., 2014). Respiratory metabolism undergoes several modifications in response to the reduced availability of Pi, including the increased use of inorganic pyrophosphate to conserve ATP, the reconfiguration of glycolysis and the induction of the alternative pathways of
mitochondrial electron transport to uncouple tricarboxylic acid (TCA) cycle activity from ATP synthesis under conditions where the latter is restricted by the reduced availability of ADP and Pi (Vance et al., 2003; Plaxton and Tran, 2011). The electron transport steps catalysed by the alternative dehydrogenases and the alternative oxidase (AOX) do not pump protons across the inner mitochondrial membrane and so provide a non-energy conserving alternative to the cytochrome pathway (Millar et al., 2011). Numerous studies have shown the importance of these pathways under Pi starvation (Rychter and Mikulška, 1990; Wanke et al., 1998), including observations on Phaseolus vulgaris mitochondria, which showed increased AOX activity and decreased cytochrome c oxidase (COX) activity when isolated from plants grown on a Pi-deficient medium (Rychter et al., 1992; Juszczyk et al., 2001). Similar observations have been made on tobacco (Nicotiana tabacum) cell suspension cultures, where Pi limitation caused a strong increase in AOX protein and the capacity for cyanide-resistant respiration (Parsons et al., 1999; Sieger et al., 2005); while in leaves, growth on low Pi increased the activity of the AOX pathway increased in P. vulgaris and Gliciridia sepium, but not in tobacco (González-Meler et al., 2001).

AOX is induced under many stress conditions (Van Aken et al., 2009) and it was recently shown that the response of AOX to hypoxia is mediated by nitric oxide (NO) (Gupta et al., 2012). NO is also involved in the induction of AOX under pathogen attack (Fu et al., 2010) and it activates the transcription of AOXIA in Arabidopsis thaliana cell cultures (Huang et al., 2002). More generally NO is a gaseous free radical that plays a role in biotic and abiotic stress responses, symbiotic interactions and plant development (Gupta et al., 2011; Yu et al., 2014). A prominent role for NO has been reported in the formation of cluster roots in P-deficient Lupinus albus (Wang et al., 2010) raising the possibility that NO may also be involved in the induction of AOX that occurs during Pi-deficiency. Since cytosolic nitrate reductase (NR), which produces nitrite from nitrate, is also usually the enzyme that converts nitrite to NO in plants under aerobic conditions (Planchet et al., 2005), it should be possible to test for the involvement of nitrite-dependent NO production in the induction of AOX during Pi deprivation by comparing the response of wild-type and NR double mutant (nia) lines of A. thaliana. NR is involved in the production of NO in response to a variety of physiological, developmental and stress conditions, including drought, temperature and pathogen attack (Gupta et al., 2011) and the nia mutant has been used to study the role of NO in iron deficiency (Chen et al., 2010) and salt stress (Xie et al., 2013). The approach used here is analogous to that previously used to analyse the role of NO in the induction of AOX under hypoxia (Gupta et al., 2012), and it leads to the conclusion that NR-derived NO is required for the induction of the AOX pathway when seedlings are grown under Pi-limiting conditions.

Materials and methods

Plant materials and growth conditions

Wild-type and nitrate reductase double mutant (nia1,2) seeds of Arabidopsis thaliana (L.) Heynh. (Col-0) were surface sterilized with 10% NaOCl and washed three times with autoclaved distilled water. The sterilized seeds were transferred to a medium that contained 1 mM NH₄NO₃, 250 µM CaCl₂, 100 µM FeEDTA, 1 mM MgSO₄, 100 µM H₂BO₃, 1.5 µM CuSO₄, 50 µM KCl, 10 mM MnSO₄, 0.1 µM Na₂MoO₄, 100 µM Na₂SiO₃, 2 µM ZnSO₄, 0.05 mM (+P) or 1 mM (+P) KH₂PO₄, 1% (w/v) sucrose, 100 mg/ml Murashige and Skoog vitamin powder (Sigma M-7150), and 1% (w/v) agar. The pH was adjusted to 5.8. Plates were kept overnight at 4°C to break dormancy, and then transferred to a growth chamber at 18°C, 60–70% relative humidity and long-day (16-h light: 8-h dark) illumination. The lengths of roots and shoots of vertically grown seedlings were measured from photographs taken at 8 and 15 d after germination. Seedlings for mitochondrial experiments were grown in liquid culture on half-strength medium without agar.

Respiration measurements

Plate-grown plants (2–3 seedlings; ~50 mg FW) were weighed and placed in a darkened oxygen electrode chamber that contained 2 ml of HEPES pH 7.2. KCN (1 mM) was added to measure COX-linked respiration, followed by salicylhydroxamic acid (SHAM) (2 mM) to monitor AOX-linked respiration.

Isolation of mitochondria

Mitochondria were isolated at 4°C from ~10 g fresh weight of 14-d-old Arabidopsis seedlings using a procedure similar to that described elsewhere (Day et al., 1985; Sweetlove et al., 2007). Seedlings were homogenized with a mortar and pestle in 200 ml of cold grinding medium [0.3 M sucrose, 25 mM tetrasodium pyrophosphate, 1% (w/v) PVP-40, 2 mM EDTA, 10 mM KH₂PO₄, 1% (w/v) BSA, 20 mM ascorbic acid, pH 7.5], followed by two 10 s bursts separated by 5–10 s in a kitchen blender. The homogenate was filtered through two layers of Miracloth (GE Healthcare) and centrifuged at 1500 × g for 5 min. The resulting supernatant was then centrifuged at 12 000 × g for 15 min and the organelle pellet was washed by repeating the 12 000 and 12 000 × g centrifugation steps twice in a sucrose wash medium containing 0.3 M sucrose, 0.1% (w/v) BSA, 2 mM MgCl₂, 1 mM EDTA, 0.1 mM KH₂PO₄, and 20 mM HEPES pH 7.6. The resulting pellet of crude organelles was carefully resuspended in 4 ml of sucrose wash medium and gently layered over a 35 ml continuous 28% Percoll density gradient consisting of 0–4.4% PVP-40. The gradient was then centrifuged at 40 000 × g for 45 min. The mitochondrial band was seen as a yellow-brown band near the bottom of the tube. The upper layers of the density gradient were removed, and the mitochondrial band was collected. The mitochondrial fraction was diluted ~5-fold with sucrose wash buffer and centrifuged at 24 000 × g for 10 min. The mitochondrial band was collected and washed three to four times with sucrose wash medium.

Mitochondrial protein preparation and immunoblotting

Mitochondrial protein concentration was determined by the Bradford method. For immunoblotting, protein samples (30 µg per lane) were mixed with 2 volumes SDS-PAGE sample buffer (10% SDS, 50% glycerol, 0.2% bromophenol blue and 1M Tris-HCl pH 6.8), and separated by SDS-PAGE. Separated proteins were stained with Coomassie Brilliant Blue R250 (Fisher Scientific, Loughborough, UK), or blotted on to Hybond ECL membrane (GE Healthcare). AOX1A primary antibody was obtained from Agrisera. The antibody (20 µl) was suspended in 20 ml of TBS-Tween-20 buffer (0.05% (v/v) Tween-20, 150 mM NaCl, and 10 mM Tris, pH 8) and 5% BSA and the membrane was incubated in the buffer for 24 hours, washed three times (5 min each) with TBS-Tween BSA buffer, and then incubated for 1 h with a secondary antibody [anti-mouse IgG horseradish peroxidase (HRP); Sigma Aldrich]. AOX protein was detected using a chemiluminescence HRP kit supplied by Bio-Rad using a Chemdoc scanner.
NO induces AOX under Pi deficiency

Pi was measured by a colorimetric assay using ammonium molybdate (Bozzo et al., 2006). Plant material (100 mg FW) was ground in 0.5 ml 10% perchloric acid and centrifuged at 13 000 rpm for 10 min. The supernatant was neutralized with 5 M KOH and the precipitate was removed by centrifugation. Free Pi was determined by adding an aliquot of the supernatant to 100 μl of a freshly prepared assay solution containing four parts 10% (w/v) ascorbate and one part 10 mM ammonium molybdate in 15 mM zinc acetate (pH 5.0). Samples were incubated for 60 min at 37°C and the absorbance was measured at 720 nm.

NO was measured by 4,5-diaminofluorescein diacetate (DAF-2DA) fluorescence. Roots were incubated in 1 ml of a detection buffer containing 2.5 mM HEPES and 10 μM DAF-2DA (Sigma) at pH 7.4. The formation of DAF-2T following the NO reaction with DAF-2DA was visualized at different time points using a Leica M165FC fluorescence microscope upon excitation at 488 nm with an Argon 2 laser. Fluorescence emission was recorded using a 505–530 nm band-pass filter coupled with a 515-nm long-pass filter. Images were analysed using Image J software.

Nitrite levels were measured by the Griess reagent assay. Roots (100 mg FW) were ground in 25 mM HEPES buffer, pH 7.2, and then centrifuged at 13 000 × g for 12 min. The supernatant was transferred to a solution containing 1% w/v sulphanalamide and 0.02% w/v N-(1)-(naphthyl) ethylene-diaminedihydrochloride and 10 μM zinc acetate and the absorbance was measured at 540 nm.

Superoxide levels were measured using the nitroblue tetrazolium chloride (NBT) staining method (Jambunathan, 2010). Seedlings were incubated in (0.1% NBT) for 24 h, destained using 96% ethanol at 40°C, and photographed using a Leica M165-FC microscope and Leica DFC310-FX camera. The staining was quantified using Image J.

Fig. 1. Effect of Pi supply on root growth of Arabidopsis seedlings. (A) Representative images of WT and nia seedlings grown on a medium containing 0 or 1 mM Pi at 8 d (upper row) and 15 d (lower row) after germination. For the GSNO treatment, 200 μM GSNO was added to the growth medium. (B) Length of WT and nia plants grown with or without Pi at 8 and 15 d after germination (n=32). Means with different letters are significantly different (one-way ANOVA, P<0.05).
Hydrogen peroxide levels were measured using the method described by Jambunathan (2010). Seedlings were immersed in a staining solution containing 1 mg/ml 3,3′-diaminobenzidine (DAB) solution, pH 3.8. The tissue was vacuum infiltrated and then incubated for 24 h in the staining solution. The tissue was destained using 96% (v/v) ethanol at 40°C, then fixed with a 3:1:1 solution of ethanol/lactic acid/glycerol and photographed. The staining was quantified using Image J.

**Statistical analysis**

One-way analysis of variance (ANOVA) was performed using SPSS 21.0. All data were tested for normality and homogeneity of variance. Student-Newman-Keuls (SNK) or T3-Dunnett post hoc tests were used to discriminate between individual treatments. Comparisons for which $P<0.05$ were considered to be significantly different.

**Results**

**Increased sensitivity of the nia mutant to low Pi**

Omitting Pi from the growth medium reduced the total Pi content of both WT and nia seedlings, showing that the treatment was sufficient to cause the onset of P-deficiency (Supplementary Fig. S1). The overall growth of WT seedlings was unaffected by the absence of Pi from the growth medium over 15 d, with no significant difference in size between plants grown on media containing 0 or 1 mM Pi (Fig. 1). In contrast the growth of the nia mutants was significantly slower after 8 d in the absence of external Pi, and the effect was even more marked after 15 d (Fig. 1). Measurements of root/shoot ratios showed that omitting Pi from the growth medium increased the ratio for WT plants at days 8 and 15, but had no effect on the nia seedlings by day 15 (Supplementary Fig. S2). Thus the nia mutant is more sensitive to Pi deprivation than the WT plant, indicating the impairment of mechanisms that could contribute to adaptation to low Pi in the mutant.

**WT, but not nia, responded to low Pi with an increase in NO**

The effect of low Pi on NO production was measured using the fluorophore DAF-2DA. The advantage of this cell-permeant dye is that it diffuses to NO producing sites and reacts with NO to form a highly fluorescent product. WT roots had higher levels of NO than the nia mutant when the seedlings were grown on 1 mM Pi, but while the NO level increased substantially in the WT roots grown on 0 mM Pi, the level decreased slightly in the roots of the nia mutant (Fig. 2A; Supplementary Fig. S3). Similar results were obtained when NO production was analysed with the gas phase Griess reagent assay. These measurements showed that the rate of NO production increased substantially in WT roots grown on 0 mM Pi, whereas there was no change in the roots of the nia mutant (Fig. 2B). It is good practice to measure NO by more than one method (Gupta and Igamberdiev, 2013) and here the two assays show that Pi deprivation increased the capacity for NO production and the endogenous NO level in Arabidopsis roots.

**Effect of low Pi on respiration**

The respiration rate of WT and nia seedlings was the same for plants grown with 1 mM Pi (Fig. 3). In contrast there was a marked difference ($P<0.05$) between the lines grown on 0 mM Pi, with the respiration rate of the nia mutant dropping to about 50% of the WT value (Fig. 3). The capacity of the AOX pathway was investigated by the sequential addition of KCN and SHAM. The addition of SHAM had a greater effect on the respiration rate of WT seedlings grown on 0 mM Pi, reducing the KCN-independent respiration rate by 2.0 μmol O₂ g FW⁻¹ h⁻¹ at 1 mM Pi and by 2.9 μmol O₂ g FW⁻¹ h⁻¹ at 0 mM Pi (Fig. 3A, C); whereas the nia seedlings only showed an effect of SHAM on the seedlings were grown on 1 mM Pi, reducing the respiration rate by 2.5 μmol O₂ g FW⁻¹ h⁻¹ (Fig. 3B, D). The contrast between the WT and nia lines—specifically the absence of an effect of SHAM on the nia seedlings that were grown on 0 mM Pi—suggests that there could be a positive correlation between NO production and AOX induction during Pi deprivation. Note that the residual respiration rates in the presence of both KCN and SHAM were generally high in these experiments, but they did not decrease when the inhibitor concentrations were increased to 2 mM KCN and 5 mM SHAM, indicating that the high values could not be attributed to poor penetration by the inhibitors (data not shown).

**Fig. 2.** Effect of Pi supply on the NO level in Arabidopsis roots. NO was quantified in the roots of 14-d-old WT and nia seedlings grown on a medium containing 0 or 1 mM Pi by: (A) DAF-2DA fluorescence; and (B) a gas phase Griess reagent assay. Means (n=3) with different letters are significantly different (one-way ANOVA, $P<0.05$).
WT, but not nia, responded to low Pi with an increase in AOX.

There was a substantial increase in the AOX level in WT plants grown on 0 mM Pi (Fig. 4), which correlated with the increased capacity of the AOX pathway and the effect of SHAM on the respiration rate of the KCN-treated seedlings (Fig. 3A, C). However Pi deprivation had no effect on the AOX protein level in the nia mutant (Fig. 4), suggesting that the induction of AOX under low Pi required an increase in NO.

S-nitrosoglutathione (GSNO) improved the growth and AOX capacity of nia mutants under low Pi conditions.

To confirm the involvement of NO in the response to low Pi in the growth medium nia mutant plants were grown on a medium containing 200 µM GSNO. This compound is an effective and reliable NO donor (Mur et al., 2013) and its inclusion in the medium improved the growth of the plants on 0 mM Pi (Fig. 1) and increased the root/shoot ratio (Supplementary Fig. S2). In contrast GSNO had no effect on the growth of WT plants in a medium lacking Pi (Supplementary Fig. S4). GSNO also increased the effect of SHAM on the respiration of the nia seedlings (Fig. 3D, E) suggesting that NO is indeed required for AOX induction and growth under low Pi conditions.

Superoxide levels increased in nia plants under low Pi conditions but H₂O₂ levels did not alter.

AOX helps to minimize ROS production under conditions that lead to over-reduction of ubiquinone (Maxwell et al., 1999). While only low levels of superoxide were detected in WT and nia roots grown on 1 mM Pi, the level increased in nia plants grown on 0 mM Pi (Fig. 5A; Supplementary Fig. S5). Thus the inability of the nia mutant to induce AOX under low Pi conditions has a deleterious effect on one of the mechanisms controlling ROS levels in the roots. Increased levels of superoxide can increase H₂O₂, but DAB staining showed

Fig. 3. Effect of Pi supply on the respiration rate of Arabidopsis seedlings. Oxygen consumption rates of 14-d-old seedlings were measured for: (A) WT seedlings grown on 1 mM Pi; (B) nia seedlings grown on 1 mM Pi; (C) WT seedlings grown on 0 mM Pi; (D) nia seedlings grown on 0 mM Pi; (E) nia seedlings grown on 0 mM Pi plus 200 µM GSNO. The measurements were repeated after the addition of 1 mM KCN, and again after adding 2 mM SHAM. Means (n=6–9) with different letters are significantly different (one-way ANOVA, P<0.05) within a treatment.
no change in root H$_2$O$_2$ levels in all treatments (Fig. 5B; Supplementary Fig. S6).

**Discussion**

Understanding the mechanisms that allow plants to acclimate to Pi deprivation is a prerequisite for optimizing Pi use efficiency in crop plants (Wu et al., 2013). This task is important because current agronomic practice relies heavily on the use of the finite and dwindling reserves of rock phosphate to compensate for low Pi availability in the soil (Cordell et al., 2011). Induction of AOX, which is commonly observed in response to Pi starvation, provides additional flexibility in the coordination of the TCA cycle and respiration under Pi-limiting conditions, and the experiments reported here show that this acclimatory response is mediated by NO. The same molecule has been observed to act upstream of AOX induction during the response of tomato leaves to tobacco mosaic virus (Fu et al., 2010), and it is likely, although yet to be established, that the increase in NO triggers a signal transduction pathway that culminates in increased AOX gene expression. In principle, NO can be synthesized by several oxidative and reductive pathways in plants (Gupta et al., 2011; Yu et al., 2014), but the lower NO levels in the nia mutants, and the failure to increase the level in a growth medium lacking Pi, demonstrate the importance of the NR pathway for NO production under these conditions. Given that the formation of cluster roots by white lupin during Pi deficiency is also regulated by NO (Wang et al., 2010), it may be concluded that Pi deficiency is another example of an abiotic stress that elicits responses that depend on NO.

The nitrite produced by NR can either be further metabolized by NR to NO (Rockel et al., 2002), or it can be reduced to NO by the mitochondrial electron transport chain (Gupta et al., 2005). NO production can be limited by the availability of nitrite (Planchet et al., 2005) and interestingly it has been found that Pi starvation caused a 4-fold down-regulation of nitrite reductase (NiR) gene expression in roots of *Arabidopsis* (Wu et al., 2003). This is likely to result in reduced NiR activity, elevated nitrite levels and hence increased NO production in WT plants during Pi starvation. In agreement with this prediction, nitrite levels were found to increase in WT roots, but not the nia mutants, when seedlings were grown in a medium lacking Pi (Supplementary Fig. S7).

AOX reduces the production of ROS in plant mitochondria, including superoxide (Cvetkovska and Vanlerberghe, 2012), and under stress conditions the induction of the alternative pathway leads to reduced ROS levels (Van Aken et al., 2009). In keeping with this, the failure to elevate NO in the nia mutant grown on the Pi-free medium resulted in higher levels of superoxide than the WT, reflecting both the very
low respiratory capacity of the AOX pathway in the *nma* seedlings under these conditions (Fig. 3D) and the inability of the mutant to increase the AOX level in response to the stress. The increased capacity of the alternative pathway in the *nma* mutant grown with the NO donor (Fig. 3F) emphasizes the pivotal role for NO in the recruitment of the alternative pathway under Pi deficiency.

Observations on barley seedlings overexpressing a non-symbiotic haemoglobin-1 to scavenge NO led to the conclusion that the NO level regulates respiration, internal oxygen, carbohydrate consumption and ROS levels in aerobic barley roots (Gupta et al., 2014). COX is inhibited by competitive binding of NO to the Fe²⁺-heme group at the O₂-binding site (Cleeter et al., 1994) and the inverse correlation between NO and oxygen consumption in barley roots was attributed to decreased inhibition of COX by NO (Gupta et al., 2014). The interpretation of the changes in oxygen consumption observed during P-deficiency is less straightforward because of the induction of the AOX pathway (Rychter and Mikulska, 1990; Wanke et al., 1998). Thus the increased NO levels observed in WT grown in a Pi-free medium did not cause the expected inhibition of respiration (Fig. 3A), presumably reflecting the NO-induced expression of AOX and an increased contribution of the alternative pathway to respiration. Moreover total respiration in the *nma* mutant was indistinguishable from the WT when the plants were grown on 1 mM Pi, despite the lower NO level, and the rate decreased when the plants were grown on 0 mM Pi even though the NO level remained low. Thus while the *nma* mutant data provide evidence that NO is required for the increase in the AOX level when seedlings are grown under Pi-limiting conditions, it seems that the NO level is not a major factor in determining the respiratory behaviour of the mutant. The increase in superoxide level observed in the *nma* mutant roots under Pi deficiency (Fig. 5A), with the potential for oxidative damage and lipid peroxidation to the mitochondria (Taylor et al., 2002), might also be relevant, and the decrease in superoxide in response to incubation with the NO donor strengthens the conclusion that NO stimulates the AOX pathway when Pi availability is reduced.

It has been shown previously that AOX plays an important role in the response of plants to cold, drought stress, hypoxia, ozone injury and Pi deficiency (Van Aken et al., 2009; Plaxton and Tran, 2011; Gupta et al., 2012). The pathway that leads to increased AOX activity under Pi deficiency has yet to be fully elucidated, but it is now clear that NO provides the signal that triggers the process.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1** Effect of Pi supply on the Pi content of *Arabidopsis* seedlings.

**Supplementary Fig. S2** Effect of Pi supply on the root/shoot ratio of *Arabidopsis* seedlings.

**Supplementary Fig. S3** Effect of Pi supply on DAF-2DA fluorescence of *Arabidopsis* roots.

**Supplementary Fig. S4** Effect of GSNO on WT *Arabidopsis* seedlings grown on a medium containing 0 mM Pi.

**Supplementary Fig. S5** Effect of Pi supply on NBT staining of *Arabidopsis* roots.

**Supplementary Fig. S6** Effect of Pi supply on DAB staining of *Arabidopsis* roots.

**Supplementary Fig. S7** Effect of Pi supply on nitrite levels in *Arabidopsis* roots.

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