Title: The alternative respiratory pathway mediates carboxylate synthesis in white lupin cluster roots under phosphorus deprivation

Running title: Alternative pathway in white lupin cluster roots

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ABSTRACT
Plant adaptations associated with a high efficiency of phosphorus (P) acquisition can be used to increase productivity and sustainability in a world with a growing population and decreasing rock phosphate reserves. White lupin (*Lupinus albus*) produces cluster roots that release carboxylates to efficiently mobilize P from P-sorbing soils. It has been hypothesized that an increase in the activity of the alternative oxidase (AOX) would allow for the mitochondrial oxidation of NAD(P)H produced during citrate synthesis in cluster roots at a developmental stage when there is a low demand for ATP. We used the oxygen-isotope fractionation technique to study the *in vivo* respiratory activities of the cytochrome oxidase pathway (COP) and the alternative oxidase pathway (AOP) in different root sections of white lupins grown hydroponically with and without P. In parallel, AOX protein levels and internal carboxylate concentrations were determined in cluster and non-cluster roots. Higher *in vivo* AOP activity was measured in cluster roots when malate and citrate concentrations were also high, thus confirming our hypothesis. AOX protein levels were not always correlated with *in vivo* AOP activity, suggesting post-translational regulation of AOX.
Keyword index: alternative oxidase, oxygen-isotope fractionation, root exudation, phosphorous deficiency
INTRODUCTION

We are gradually running out of non-renewable rock phosphate resources in an era that requires increased application of phosphorus (P) fertilizer to produce sufficient food and fiber to sustain a growing global population (Vance, Uhde-Stone & Allan 2003; Cordell et al. 2009; Gilbert 2009). Developing crop and pasture systems that are more efficient at acquiring or utilizing P is an essential strategy to deal with this problem (Lambers et al. 2011). Plant traits that enhance the capacity to acquire P include the formation of mycorrhizal associations and the production of cluster roots (Lambers et al. 2006).

The root systems of species that develop cluster roots have a unique capacity for altered branch-root development; large numbers of determinate branch roots (‘rootlets’) are initiated in compact segments along the axes of growing roots (Shane & Lambers 2005). In white lupin (Lupinus albus L.), the rootlets of a cluster emerge along the root axis in a proximal to distal direction, so that each cluster represents a progression of rootlet development (Gardner, Parbery & Barber 1981). During a short period in their development, cluster roots produce and then release vast amounts of carboxylates, e.g., citrate (Gardner, Barber & Parbery 1983; Johnson, Allan & Vance 1994). These carboxylates solubilize P that is sorbed onto soil particles (Lambers et al. 2006). The combination of cluster-root structure and their functioning maximizes the amount of P that can be ‘mined’ from soils with a low P availability (Lambers et al. 2008).

The production of vast amounts of citrate in cluster rootlets is inexorably associated with the production of NADH; this happens at a time when little ATP is required, because the growth of cluster roots ceases soon after their initiation (Shane et al. 2004). It has, therefore, been hypothesized that the alternative oxidase (AOX) of the mitochondrial respiratory chain may be important for the oxidation of NADH produced during citrate synthesis in cluster roots (Kania et al. 2002). Indeed, in Hakea prostrata,
increased expression of AOX has been found at the same developmental stage that citrate accumulation takes place (Shane et al. 2004). However, AOX protein and transcript levels are often poorly correlated with AOX pathway (AOP) activity in vivo due to post-translational control of AOP flux (Guy & Vanlerberghe 2005; Vidal et al. 2007; Grant et al. 2008; Rasmusson, Fernie & van Dongen 2009; Florez-Sarasa et al. 2011, Miller et al. 2011). Whilst the capacity of the AOP can be measured in vivo using appropriate concentrations of inhibitors, these inhibitors are not appropriate to measure \textit{in vivo} activity of the AOP (Day et al. 1996). Instead, activity can be measured based on the difference in $^{18}$O-isotope fractionation between AOX and cytochrome oxidase (Ribas-Carbo, Robinson & Giles, 2005).

The aim of our study was to determine the \textit{in vivo} activity of AOP in cluster roots of white lupin which were induced by growing plants in the absence of a P supply. We compared AOX protein levels, the \textit{in vivo} activities of AOP and the cytochrome oxidase pathway (COP), and the internal carboxylate concentrations in juvenile and mature segments of cluster roots with those in the tips and mature root zones of P-deprived and P-supplied plants. Our hypothesis was that the activity of COP would decrease as the growth rate of the cluster rootlets declined during development. The activity of the AOP, on the other hand, was hypothesized to remain high in mature segments of cluster roots, when production of carboxylates was high.

\textbf{MATERIAL AND METHODS}

\textbf{Plant material and growth conditions}

Seeds of \textit{Lupinus albus} L. cv Kiev mutant were germinated and grown in vermiculite for two weeks in a growth chamber at 25°C, a photosynthetically active photon flux density of 400 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) and with a 12h/12h light/dark regime. Plants were then
transferred to 20-L plastic tanks (nine plants per tank) containing an aerated nutrient solution of the following composition: 5 mM KNO$_3$, 1 mM MgSO$_4$, 1.5 mM Ca(NO$_3$)$_2$, 23.1 μM H$_3$BO$_3$, 0.38 μM ZnSO$_4$, 0.29 μM Na$_2$MoO$_4$, 0.16 μM CuSO$_4$, 4.85 μM MnSO$_4$, 19.14 μM Fe-EDDHA (pH 5.8) (with or without 1 mM NH$_4$H$_2$PO$_4$). Plants were grown under greenhouse conditions at the Universitat de les Illes Balears or at the University of Western Australia, Perth for 4 to 5 weeks. The nutrient solution was renewed once a week.

**Determination of internal carboxylate concentrations**

Carboxylates were extracted from freeze-dried root segments based on Keerthisinghe *et al.* (1998) by grinding in a pre-chilled mortar and pestle with 1.0 mL ice-cold 5% (w/v) perchloric acid. The homogenates were clarified by centrifugation at 15,000 g for 30 min at 4 °C, and the supernatants neutralized with 5 M potassium carbonate. Carboxylate concentrations in the extracts were determined by HPLC according to Cawthray (2003).

**Immunodetection of AOX**

Crude cellular membranes were prepared (Chai *et al.* 2010) from samples of root tissue collected from plants grown in hydroponics. Membranes were resuspended in a small volume 300 mM sucrose, 10 mM TES, 1 mM glycine, pH 7.5 and the protein content determined (Peterson 1977). Approx. 20 μg of crude membrane protein was resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Protran®, Schleicher and Schuell, Dassel, Germany) using a semidry blotting apparatus (Trans-Blot® Semi-Dry electrophoretic transfer cell, Bio-Rad, City, Country). Blots were probed with monoclonal antibodies against AOX (AOA, Elthon, Nickels & McIntosh 1989) or porin
(GTMA, Lincoln NE, USA). The AOA anti-AOX antibody recognizes a conserved amino acid motif found in all AOX isoforms (Finnegan, Wooding & Day 1999).

Secondary antibody (ECL™ Anti-mouse IgG, horseradish peroxidase-linked whole antibody from sheep, Amersham Biosciences, Buckinghamshire, UK) was used for chemiluminescence detection using a commercial kit (ECL™ Western Blotting Detection Reagents, Amersham Biosciences, Buckinghamshire, UK) on films (Hyperfilm™, Amersham Biosciences, Buckinghamshire, UK). Signal intensities on the immunoblots were quantified by densitometry (ImageJ v.1.36, http://rsb.info.nih.gov/ij; software downloaded on 23 July 2010).

**Respiration and oxygen-isotope fractionation measurements**

Root samples were harvested, weighed and placed in a 3 mL stainless-steel closed cuvette that was maintained at 25°C using a copper plate and a copper coil connected to a temperature-controlled water bath. The fresh weight of the root samples did not change by more than 5% during the measurement of respiration (data not shown).

Respiration and oxygen-isotope fractionation measurements were done as described in Florez-Sarasa et al. (2007) with the following modifications: each measurement took approximately 45 minutes; values of the isotope ratios between m/z 34/32 \((^{18}\text{O}_2/^{16}\text{O}_2)\) and m/z 32/28 \((^{16}\text{O}_2/^{28}\text{N}_2)\) were obtained from a standard and the sample air via dual-inlet analysis with four replicate cycles. Calculations of the oxygen-isotope fractionation were made as described in Ribas-Carbo et al. (2005), and the electron partitioning between the two pathways in the absence of inhibitors was calculated as described by Guy et al. (1989). The \(r^2\) values of all unconstrained linear regressions between –ln \(f\) and ln \((R/R_0)\), with a minimum of five data points, were at least 0.995, the minimal acceptable level (Ribas-Carbo et al. 1997).
The electron partitioning to the alternative pathway ($\tau_a$) was calculated as follows:

$$\tau_a = \Delta_n - \Delta_c / \Delta_a - \Delta_c$$

where $\Delta_n$, $\Delta_c$, $\Delta_a$ are the oxygen-isotope fractionation in the absence of inhibitors, in the presence of SHAM, and in the presence of KCN, respectively. For KCN inhibitor treatments, root samples were incubated for 30 min by sandwiching between medical wipes soaked with a solution of 10 mM KCN. In addition, a piece of medical wipe wetted with 10 mM KCN was placed in the cuvette. $\Delta_a$ value obtained from four replicates was 28.0 ± 0.4‰. For SHAM inhibitor treatments, root samples were incubated for 45 minutes in a 25 mM SHAM solution. The $\Delta_c$ value obtained from four replicates was 18.5 ± 0.3‰. All stock solutions were freshly prepared before use.

The individual activities of the COP ($\nu_{cys}$) and AOP ($\nu_{alt}$) were obtained by multiplying the total respiration rate ($V_t$) and the partitioning to each pathway as follows:

$$\nu_{cys} = V_t \times (1 - \tau_a)$$

$$\nu_{alt} = V_t \times \tau_a$$

Several root segments of the same plant were pooled and used for each respiration measurement. Seven to 14 replicates were assayed for each type of root segment.

**Statistical analyses**

All the data presented were from at least three experiments with different groups of plants grown on separate dates under similar greenhouse conditions. A one-way analysis of variance (ANOVA) with a level of significance of P-value < 0.05 was performed. For the respiration and AOX densitometry data, statistical analysis was
performed with SPSS for Windows 17.0. The Duncan post-hoc test was used when statistically significant differences were obtained. For carboxylate analyses, ANOVA was followed by an LSD test using Statistix 8.1 (Analytical Software, USA).

RESULTS

White lupin (Lupinus albus) plants were grown hydroponically in a nutrient solution with (+P) or without (-P) phosphorus supply. No visual differences were observed in the aerial parts between +P and -P grown plants (Fig. 1A). However, only P-deprived plants developed cluster roots (Fig. 1B). Roots of +P and -P grown plants were divided into different segments that were studied separately (Fig. 1B). In +P grown plants, the root segments studied were root tips (Tip +P) and mature root sections (MR). In -P grown plants, root tips were also studied (Tip -P) together with cluster roots harvested at an early stage of development, before senescence, and subsequently divided into juvenile (JC) and mature (MC) segments.

Internal carboxylate concentrations

The internal concentration of malate, citrate, malonate, fumarate and cis-aconitate in the different root segments was determined by HPLC. Malate and citrate concentrations were consistently higher than those of all other carboxylates measured across all root segments (Table 1). Carboxylate concentrations were significantly higher in roots of -P grown plants than in roots of +P grown plants (Table 1). Among the different root segments of -P grown plants, malate concentrations were higher in root tips and MC contained twice as much citrate as the younger sections of these roots, and thus had the highest overall concentrations of the carboxylates analyzed (Table 1). Only traces of
malonate, fumarate and cis-aconitate were detected, and the concentrations were higher in root segments from –P grown plants.

Respiration and electron partitioning to the AOX pathway

Total respiration (V_t) and electron partitioning to the AOP (τ_a) were determined (Fig. 2) using the oxygen-isotope-fractionation technique. The total respiration rate (V_t) of mature roots (MR) from plants grown under +P conditions was almost half the rate in all other roots sections from both P treatments. In contrast, V_t of the juvenile segments of cluster roots (JC) was significantly higher than that of all other root sections in both treatments. The V_t was similar and intermediate in root tips of plants from both P treatments and in the mature segments of cluster roots (MC). MR showed the highest τ_a, mainly due to the lowest COP activity (v_cyt), whilst the AOP activity (v_alt) was similar to that in root tips of both P treatments. The τ_a was also high in JC and MC, but in contrast to the situation in MR, this was due to both cluster-root segments showing a significantly higher AOP flux than the other root sections did. The COP activity was highest in the younger root sections and decreased from the root tips to the mature sections of the +P treatment and from JC to MC.

AOX protein levels

The relative AOX and porin protein levels in total cellular membrane preparations were determined in the different root segments by immunoblot analysis. After normalization using the porin signals, the highest relative levels of AOX were detected in root tips from +P plants, followed by the mature cluster roots from -P plants (Fig. 3). The remaining three root segments had lower, but similar, relative AOX protein levels, (Fig. 3).
DISCUSSION

The induction of cluster-root formation under P deficiency is well documented and characterized in white lupin plants (Gardner, Parbery & Barber 1981; Johnson, Allan & Vance 1994; Massonneau et al. 2001; Lambers, Clements & Nelson 2013). In the present study, cluster-root formation was induced by growing white lupin plants in a P-deprived solution, and we studied how respiratory processes were affected in roots as a function of developmental stage in plants with and without cluster roots. In a previous study, the accumulation and release of carboxylates was shown to be influenced most by the presence of P during growth, but also changed depending on the developmental stage of the cluster roots (Neumann et al. 1999). Malate was shown to accumulate in juvenile clusters, while citrate increasingly accumulates as cluster roots mature (Neumann et al. 1999). Accordingly, higher concentrations of citrate were detected in all root segments studied from P-deprived plants compared to P-supplied plants, and the highest citrate concentration was detected in mature segments of the cluster roots. Malate concentration also increased in the absence of P, but, interestingly, its concentration was higher in the root tips than in the cluster-root segments of P-deprived plants.

The production of large amounts of malate and citrate in cluster roots is driven by a modified carbon metabolism (Johnson, Allan & Vance 1994; Massonneau et al. 2001). This metabolic modification is thought to involve a high respiratory flux, driven by the production of citrate, under conditions where mature cluster roots do not require ATP for growth and a low phosphate concentration may limit COP activity (Rychter & Mikulska 1990; Juszczuk et al. 2001; Kania et al. 2003; Shane et al. 2004). The present results on the in vivo activity of COP support this hypothesis, because it was lower in
mature segments of root clusters than in juvenile ones. Juvenile segments of cluster roots had similar *in vivo* COP activity to the actively growing root tips of the same plants, presumably due to the metabolic demand associated with rapid growth. A similar decrease in COP activity from young, rapidly growing root tips to mature sections was also observed in the non-cluster roots of the +P treatment. Consistent with the high metabolic activity associated with cell division and elongation (Wanner 1950), root tips of both P treatments showed similar and high COP activity. A decrease of COP activity together with an increase in the partitioning to AOP was previously observed to be correlated with a decrease of relative growth rate in soybean roots (Millar *et al.* 1998) and *Arabidopsis* leaves (Florez-Sarasa *et al.* 2007). Developmental changes in soybean roots were also associated with a decrease on COP capacity due to decreases in succinate dehydrogenase capacity and COX I protein (Millar *et al.* 1998). These developmental changes may have also influenced COP *in vivo* activity besides adenylate control. Together with the results presented here, these observations show that the *in vivo* COP activity is tightly modulated by the growth rate and/or the developmental stage of the tissue, while AOP activity is not. The relationship between plant growth and AOP activity is complex (Vanlerberghe 2013). Although the AOP is not an energy-conserving pathway (Moore & Siedow 1999), it has beneficial roles in plant development and survival that may become more important during periods of stress (Juszczyk, Malusà & Rychter 2001; Florez-Sarasa *et al.* 2011; Vanlerberghe 2013).

Under conditions where COP activity is restricted (i.e. by low ATP demand), the AOP may allow the oxidation of NADH and continuation of TCA cycle reactions to support various biosynthetic reactions (Lambers, Robinson & Ribas-Carbo 2005). Specifically, in this context, high AOP activity has been hypothesized to be necessary to oxidize
NADH formed during citrate production (Shane et al. 2004). Consistent with this idea, the *in vivo* activity of AOP in our study was higher in both juvenile and mature segments of cluster roots in comparison with all the other segments studied. Therefore, the hypothesis presented above is confirmed for mature segments of clusters where AOP activity was kept at high levels regardless of the decrease in COP activity and where citrate accumulation is higher compared to that in the other root segments.

Citrate has been reported to be a potent inducer of AOX protein synthesis (Vanlerberghe & McIntosh 1996; Gupta et al. 2012). This is consistent with higher levels of AOX protein in mature cluster segments than in the root tips and juvenile cluster segments of the same plants, which had lower citrate levels than in mature clusters. Recently, it was shown that the accumulation of citrate can be induced by inhibition of aconitase by nitric oxide (NO) produced inside tissues. This situation led to enhanced AOX protein synthesis and capacity (Gupta et al. 2012). In relation to this, citrate exudation decreases in mature cluster roots of white lupin after inhibition of NO synthesis (Wang et al. 2010), and recently, it was suggested that NO can enhance citrate exudation in cluster roots once initiated by P deficiency (Meng et al. 2012). Nitric oxide also inhibits COP activity, but not AOP activity (Millar & Day 1996). Together, these observations suggest a regulatory mechanism by which NO levels, which are modulated during cluster-root development (Wang et al. 2010), may regulate TCA cycle and COP activities, facilitating citrate production and AOX expression, but further experiments are needed to confirm this hypothesis in cluster roots.

The levels of AOX protein were previously reported to be higher in juvenile than in mature cluster roots (Kania et al. 2003). In our study, the two types of cluster-root segments that were investigated (JC and MC) were dissected from cluster roots of a single cluster that would have been considered juvenile in the former study (Kania et al.
Our work, then, confirms and extends the results of the earlier study indicating that each cluster follows a similar developmental program, and that the stage of that program is determined by the age of the rootlets. This differs from the developmental pattern in *Hakea prostrata*, where all rootlets of a single cluster are at the same developmental stage Shane *et al.* (2004).

Citrate accumulation, AOX protein induction and *in vivo* activation of the AOP were well coordinated in the mature segments of cluster roots. However, the fact that juvenile cluster-root segments, with lower AOX protein levels and higher COP activity, also have high AOP activity suggests a specific activation of the AOX pathway in cluster roots, independent of the COP restriction and AOX protein increase. In higher plants, AOX exists as a dimer and can be redox-regulated by the formation of disulfide bridges between conserved cysteine residues of each monomer (Umbach & Siedow, 1993; Vanlerberghe 2013). While the redox regulation of AOX has been well documented *in vitro*, its impact on the *in vivo* activity of AOP remains controversial (Millar *et al.* 1998; Vanlerberghe, Yip & Parsons, 1999; Millenaar & Lambers, 2003; Noguchi *et al.* 2005). In soybean roots, Millar *et al.* (1998) reported developmental changes on the redox state of AOX together with increases on AOP *in vivo* activity. However, other reports found that AOX protein was always in its reduced state in roots of different species regardless of their *in vivo* AOP activity (reviewed in Millenaar & Lambers, 2003). These controversial results can be partly explained by the technical difficulties to keep the redox state of AOX during the protein extraction or mitochondrial isolation procedures (Umbach & Siedow, 1997; Vanlerberghe, Yip & Parsons, 1999). In the present study, the AOX protein was quantified in cell membrane fractions under reducing conditions and therefore the redox state was not analyzed. On the other hand, some keto acids activate AOX activity through an allosteric interaction...
As noted above, citrate and malate were detected at high levels in cluster roots, but neither of these directly activate AOX (Millar et al. 1996). Nevertheless, a possible activation of AOX by pyruvate formed by the conversion of malate to pyruvate via the malic enzyme-dependent reaction cannot be ruled out (Millar et al. 1996). Although recent evidence suggests an important role for pyruvate in the activation of AOX (Oliver et al. 2008; Dinakar, Raghavendra & Padmasree 2010), the impact of pyruvate on AOP activity in vivo has not been confirmed using stable isotope analysis and is still a matter of debate (Millenaar et al. 1998; Gaston et al. 2003).

In conclusion, the activity of AOP was induced in vivo in cluster roots of white lupin. The increased in vivo activity of AOP was not simply related to changes in AOX protein levels or COP activity, suggesting an activation of AOX via post-translational regulation. This higher AOP activity coincided with an increased concentration of malate and citrate in cluster roots. Therefore, these results support the hypothesis that the increased activity of AOP mediates the oxidation of NADH associated with the accumulation of citrate to high levels in cluster roots of white lupin.

ACKNOWLEDGEMENTS

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immunoblot experiments. Also we are very grateful to Sharon Robinson for her critical comments on the manuscript.

REFERENCES


**TABLE I.** Internal carboxylate concentrations. Concentrations (μmol g\(^{-1}\) DW) of malate, citrate, malonate, fumarate and \textit{cis}-aconitate in root segments of P-supplied and P-deprived plants (see legend to Fig. 1 for details on root segments). Data are means ± SE of 1 to 4 replicates; nd, not detected. Different letters indicate statistically different concentrations for the carboxylate according to a LSD at a significance level of P < 0.05.

<table>
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<tr>
<th></th>
<th>Tip +P</th>
<th>MR</th>
<th>Tip -P</th>
<th>JC</th>
<th>MC</th>
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<tr>
<td><strong>Malate</strong></td>
<td>61 ± 6c</td>
<td>69 ± 7c</td>
<td>373 ± 21a</td>
<td>290 ± 28b</td>
<td>303 ± 18b</td>
</tr>
<tr>
<td><strong>Citrate</strong></td>
<td>44.1</td>
<td>29.2 ± 7.2c</td>
<td>103 ± 41.0b</td>
<td>120 ± 14.3b</td>
<td>237 ± 16.9a</td>
</tr>
<tr>
<td><strong>Malonate</strong></td>
<td>nd</td>
<td>nd</td>
<td>12.7 ± 3.5a</td>
<td>5.4 ± 1.8a</td>
<td>5.8a</td>
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<tr>
<td><strong>Fumarate</strong></td>
<td>0.6ab</td>
<td>0.4 ± 0.1b</td>
<td>1.1 ± 0.1a</td>
<td>1.1 ± 0.1a</td>
<td>1.1 ± 0.1a</td>
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<tr>
<td><strong>cis-aconitate</strong></td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>0.01a</td>
<td>0.01 ± 0.001a</td>
</tr>
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583 TABLES
584
585 **Table I.** Internal carboxylate concentrations. Concentrations (μmol g\(^{-1}\) DW) of malate, citrate, malonate, fumarate and \textit{cis}-aconitate in root segments of P-supplied and P-deprived plants (see legend to Fig. 1 for details on root segments). Data are means ± SE of 1 to 4 replicates; nd, not detected. Different letters indicate statistically different concentrations for the carboxylate according to a LSD at a significance level of P < 0.05.
FIGURE LEGENDS

**Figure 1.** *Lupinus albus* plants grown with (+P) or without (-P) phosphate. Photographs of shoots (A) and roots (B) of plants grown with and without P. The indicated root segments are: root tips of P-supplied (Tip +P) or P-deprived (Tip –P) plants; mature root segment of P-supplied plants (MR); and juvenile segment of cluster roots (JC) and mature segment of cluster roots (MC) of P-deprived plants.

**Figure 2.** Respiration and mitochondrial electron partitioning. Total respiration ($V_t$), electron partitioning to the alternative pathway ($\tau_a$), cytochrome pathway activity ($v_{cyst}$) and alternative pathway activity ($v_{alt}$) in root segments of P-supplied and P-deprived plants (see Fig. 1B for details on root segments). Data are means ± SE of 7 to 14 replicates. Significant differences ($P < 0.05$) are denoted by different letters.

**Figure 3.** Alternative oxidase (AOX) protein levels. AOX protein and porin were detected by immunoblotting in root segments of P-supplied and P-deprived plants (see Fig. 1B for details on root segments). The intensities of the signals from AOX were normalised to those from porin and expressed relative to the highest value obtained. Means ± SE ($n = 4$) are shown.