Characterization of Mitochondrial Alternative NAD(P)H Dehydrogenases in Arabidopsis: Intraorganelle Location and Expression

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The intramitochondrial location of putative type II NAD(P)H dehydrogenases (NDs) in Arabidopsis was investigated by measuring the ability of isolated mitochondria to take up precursor proteins generated from cDNAs using an in vitro translation system. The mature proteins of NDA1, NDA2 and NDC1 were judged to be located on the inside of the inner membrane because they were protected from protease added after the mitochondrial outer membrane had been ruptured. In contrast, NDB1, NDB2 and NDB4 were not protected from protease digestion in mitochondria with ruptured outer membranes and were deemed to be located on the outside of the inner membrane. Expression of all ND genes was measured using quantitative reverse transcription–PCR (RT–PCR) to determine transcript abundance, and compared with expression of alternative oxidase, uncoupler proteins and selected components of the oxidative phosphorylation complexes. NDA1 and NDB2 were the most prominently expressed members in a variety of tissues, and were up-regulated in the early daytime in a diurnal manner. Analysis of array data suggested that NDA1 clustered closest to the gene encoding the P-subunit of glycine decarboxylase. Taken together with the diurnal regulation of NDA1 observed here and in other studies, this suggests that NDA1 plays a role in integrating metabolic activities of chloroplasts and mitochondria. NDA2, NDB2 and Aox1a were up-regulated in a coordinated manner under various treatments, potentially forming a complete respiratory chain capable of oxidizing matrix and cytosolic NAD(P)H. NDB1 and NDC1 were down-regulated under the same conditions and may be regarded as housekeeping genes.

Keywords: Alternative oxidase — Alternative NAD(P)H dehydrogenase(s) — Mitochondria — Protein import — Respiration.

Abbreviations: Aox, alternative oxidase; CI51, 51 kDa subunit of complex I; GFP, green fluorescent protein; ND, alternative NAD(P)H dehydrogenase; PGDC, P-subunit of glycine decarboxylase; PK, proteinase K; RPL19, cytosolic ribosomal protein of the large subunit; RPS1 and RPS13, nuclear-encoded mitochondrial ribosomal proteins of the small subunit; RT–PCR, reverse transcription–PCR; TIM, translocase of the inner mitochondrial membrane; Ucp, uncoupling proteins.

Introduction

Plant mitochondria contain a branched respiratory electron transport chain, with an ‘alternative’ pathway offering a non-energy-conserving route for electrons from NAD(P)H to O2. The components of the alternative chain [alternative oxidase (Aox) and alternative NAD(P)H dehydrogenases (NDs)], together with the uncoupling proteins (Ucps) have been more intensely studied than the five multisubunit protein complexes that comprise the energy-conserving cytochrome chain and the ATP synthase complex in plants. They are found in all plant species investigated to date and, while their exact roles remain unclear, all three components have been reported to be induced by various treatments or stresses. However, these studies used different plants, and apparently conflicting results have been reported in some instances (Finnegan et al. 1999, Sluse and Jarmuszczewicz 2004). As an example of the complexity of changes that occur with Aox, NDs and Ucps, a study examining potato leaves indicated that NDA1 transcript and protein decreased, Aox protein and activity remained unchanged and Ucp remained unchanged (Svensson et al. 2002). However, other studies have shown Aox transcript and/or protein and/or activity to be cold induced (Finnegan et al. 2004). Likewise, Ucp transcript and protein have been reported to be induced by chilling (Laloi et al. 1997, Maia et al. 1998, Ito 1999, Nantes et al. 1999), and notably both Aox and Ucp activity were reported to increase with cold stress of potato tubers (Calegario et al. 2003). However, none of these studies analysed all of the genes encoding the various alternative respiratory chain activities, and thus different findings may be due to different members of a gene family responding differently over time or between species (Thirkettle-Watts et al. 2003, Clifton et al. 2005).

Aox is the most comprehensively studied alternative respiratory chain component. Our current understanding of Aox is that it is encoded in a small multigene family that...
results of all seven genes encoding NDs in various organs and over a 24 h time period to gain an overview of their expression. We have previously used this approach for NDA1 (At1g07180) to define an internal location (Moore et al. 2003). This result was confirmed here, where a precursor protein with an apparent molecular mass of 60 kDa was imported and processed to a protease-resistant product of 56 kDa in a membrane fraction obtained from a GFP targeting approach if proteins are cytosolic yet associated with the outer mitochondrial membrane, as shown for the enzymes of glycolysis (Giege et al. 2003). We carried out in vitro protein import assays into intact isolated mitochondria, followed by protease treatments to determine transport across the outer membrane. To determine if the imported proteins were located on the inter-membrane space side of the inner membrane (external NDs) or on the matrix side (internal NDs), the outer membrane was selectively ruptured after protein uptake but prior to addition of protease. This allows added protease access to components exposed to the inter-membrane space (Lister et al. 2002, Murcha et al. 2003, Murcha et al. 2004).

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indicates that this protein is located on the external face of the inner membrane. A similar pattern was observed for NDB2 (At4g05020) and NDB4 (At2g20800); proteins with apparent molecular masses of 63 and 65 kDa, respectively, were imported in a membrane potential-dependent manner. However, no processing was evident and no protected product was observed when the outer membrane was ruptured, demonstrating that NDB2 and NDB4 are also located on the inter-membrane space side of the inner membrane.

NDC1 (At5g08740) differs from the other putative ND proteins because its intra-mitochondrial location cannot be predicted from its primary sequence (it has been proposed to have evolved from a gene encoding a chloroplast protein, i.e. the gene is predicted to have a cyanobacterial ancestry; Michalecka et al. 2003). For this protein, a precursor with an apparent molecular mass of 70 kDa was imported to yield a processed product of 60 kDa, which was protected in outer membrane-ruptured mitochondria, indicating that it was imported to the matrix side of the inner membrane and thus is also designated as an internal ND. Consequently, of the seven putative ND proteins, we have shown three to be located on the external face of the inner membrane and another three to be internal.

We carried out additional protein uptake assays but increased the amount of protease to ensure that protease protection was not due to the NDs being resistant to the amounts of protease used in protein uptake assays. Usually, proteinase K (PK) is added to a final concentration of 0.4 mg ml⁻¹ to digest externally located precursor protein. We increased the amount of PK up to a final concentration of 10 mg ml⁻¹ (Fig. 2). With intact mitochondria, this higher level of protease did result in at least some digestion of inner membrane proteins as evidenced by the fact that some of imported translocase 23 of the inner mitochondrial membrane (TIM23) was converted to the proteolytic product of 16 kDa with intact mitochondria (Fig. 2, panel vii).

Fig. 1 Import of alternative NAD(P)H dehydrogenases into isolated mitochondria. In vitro ³⁵S-labelled precursors (lane 1) were incubated with mitochondria. Import into mitochondria was assessed by the generation of a protease-protected product that was dependent on the presence of a membrane potential (lanes 2–5). A product in lane 3 indicates that the protein has been taken across the mitochondrial outer membrane as it is protected from externally added protease. No such products were evident in lane 5 where valinomycin was added to dissipate the membrane potential and inhibit import. This indicated import into or across the inner mitochondrial membrane. The intraorganelle location of the imported protein was assessed by rupturing the mitochondrial outer membrane after the import reaction (lanes 6–9), designated Mit*OM. Note that import was carried out into intact mitochondria and, after this, but prior to protease treatment, the outer membrane was ruptured. The presence of a protease-protected product in lane 7 indicates that the imported protein is on the inside of the inner membrane as it remains protected even when protease has access to the outside of the inner membrane. As a control, the inner membrane protein TIM23 was used (panel vii). It yields a characteristic inner membrane-protected fragment when the outer membrane is ruptured, indicating the efficacy of the outer membrane rupture.

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Both NDA1 and NDA2 were still completely protected in intact mitochondria, although at this concentration NDC1 was digested. Also, at 10 mg ml$^{-1}$ PK, the NDBs displayed degradation: NDB1 was partially degraded, NDB2 was almost completely digested and NDB4 was digested completely even at a concentration of 5 mg ml$^{-1}$ PK (Fig. 2, lanes 1–7). Even in outer membrane-ruptured mitochondria, NDA1, NDA2 and NDC1 products were protected from 5 mg ml$^{-1}$ PK, although NDC1 was degraded at a concentration of 10 mg ml$^{-1}$ PK. The proteins designated as external NDs were all susceptible to the lowest amount of PK, consistent with their location on the outside of the inner membrane.

Expression of alternative NAD(P)H dehydrogenases

We examined the expression profile of all seven ND genes in combination with Aox1a (At3g22370) and Aox1c (At3g27620), two of the five Aox genes in Arabidopsis that we have previously shown to be expressed at significant levels (Thirkettle-Watts et al. 2003). We also analysed the expression

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**Fig. 3** Expression profile using quantitative RT–PCR of alternative NAD(P)H dehydrogenases, alternative oxidase and other respiratory chain components in a variety of organs. The amount of cDNA resulting from reverse transcribing 1 µg of total RNA is shown in fmol. D = day, W = week, Bud = flower bud, Flower = whole floral organ. Error bars are SEs where $n = 3$. 

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of two genes designated to encode Ucps, Ucp1 (At3g54110) and Ucp2 (At5g58970). These encompass all the known genes encoding so-called alternative respiratory chain components in Arabidopsis. As controls, we measured the expression of the F1β subunit of ATP synthase (At5g08670), the 51 kDa subunit of complex I (CI51; At5g08530) and a mitochondrially encoded gene, the F1α subunit of ATP synthase (AtMg01190). As it has been previously reported that some of the ND genes are regulated in a diurnal manner, we also measured the expression of the P-subunit of glycine decarboxylase (PGDC; At4g33010), a nuclear gene encoding a mitochondrial protein known to be light regulated (Turner et al. 1992, Vauclare et al. 1996). We measured the expression of these genes in 4-, 6- and 10-day-old roots, 4-, 12- and 20-day-old cotyledons, 2-, 3-, 4- and 5-week-old second rosette leaves and in floral buds and flowers. In view of the reported light regulation, we measured expression in 5-week-old second rosette leaves at five time points in a 24 h day/night cycle in order to observe any diurnal expression pattern. We also transferred plants from normal growth conditions to darkness for 4 d, as previously described, in order to test/confirm the light-regulated nature of these genes (Michalecka et al. 2003). We also compared the expression profiles we obtained with expression data from previously published microarray experiments, using Genevestigator to extract and display the expression profiles (Zimmermann et al. 2004).

In order to assess the amount of each ND gene transcript relative to each other, we developed a quantitative reverse transcription–PCR (RT–PCR) assay for each gene (Clifton et al. 2005). NDA1 was expressed at the highest level, displaying approximately a 20-fold range in a 24 h day/night growth period (Fig. 3, 4A, Table 1). NDB3 was expressed at the lowest levels, displaying a 5-fold range over a 24 h growth period (Table 1). NDA1 was almost absent from roots, while NDB2 and NDB3 were primarily expressed in roots and to a lesser extent in buds and flowers. The predominance of NDA1 in aboveground organs was confirmed by microarray data, as was the restricted expression pattern of NDB2 and NDB3 (data not shown).

It had been reported previously that NDA1 was light regulated in potato and Arabidopsis (Michalecka et al. 2003, Svensson and Rasmusson 2001), and that NDC1 was light regulated in Arabidopsis (Escobar et al. 2004). In order to assess light regulation, we analysed transcript abundance at five time points in a 24 h growth period. To determine diurnal regulation, we followed two approaches. In accordance with

### Table 1: Statistical analysis of the diurnal quantitative RT–PCR assays for all the genes investigated in this study

| Gene | ANOVA | Diurnal | Dark transfer |
|------|-------|---------|---------------|
|      | P-value (<0.05) | Fold change (0 versus 1 h) | Students t-test | P-value (<0.05) | Fold change (control versus 4 d dark) | Students t-test | P-value (<0.05) |
| **Internal** | | | | | | |
| NDA1 | Yes | 11.5 | Yes | 7.37 | Yes | |
| NDA2 | No | 2.9 | No | 1.17 | No | |
| NDC1 | No | 2.3 | No | 1.94 | Yes | |
| NDB1 | No | 1.9 | No | 0.84 | No | |
| NDB2 | No | 4.6 | Yes | 1.23 | No | |
| NDB3 | No | 1.5 | No | 0.70 | No | |
| NDB4 | No | 4.0 | No | 3.05 | No | |
| **External** | | | | | | |
| Aox1a | No | 2.7 | No | N/A | N/A | |
| Aox1c | No | 2.0 | No | N/A | N/A | |
| UCP1 | No | 1.0 | No | N/A | N/A | |
| UCP2 | No | 1.7 | No | N/A | N/A | |
| FGα | No | 1.8 | No | N/A | N/A | |
| FGβ | No | 14.2 | Yes | N/A | N/A | |
| PGDC | No | 3.8 | No | 16.38 | Yes | |
| **Complex I subunit** | | | | | | |
| RPS1 | Yes | 3.1 | No | N/A | N/A | |
| RPS13 | No | 2.6 | No | N/A | N/A | |
| RPL19 | No | 3.1 | No | N/A | N/A | |

ANOVA was used to test the significance of the differences in expression observed over the time course of the experiment; a P-value below 0.05 is indicated. Student’s t-test was used to test the difference between transcript abundance at 0 h compared with 1 h; a P-value below 0.05 is indicated. Fold changes between 0 and 1 h are shown. The fold changes on transfer to dark and the P-value below 0.05 are indicated. N/A indicates not applicable as not measured.
previous publications to define genes up-regulated in the early
day in a diurnal manner, the average transcript abundance is
calculated from all sampling points and then each value is
expressed relative to this value (Smith et al. 2004). This means
that the judgement is based on differences from the mean rather
than a comparison between individual time points, and analy-
sis of variance (ANOVA) was carried out to indicate signifi-
cance (Table 1). We also compared the transcript abundance at

Fig. 4 Expression profile using quantitative RT–PCR of alternative NAD(P)H dehydrogenases, alternative oxidase and other respiratory chain
components at five time points in a 24 h growth cycle (A) and on transfer to darkness for 4 d (B). For the diurnal profile, transcript abundance is
shown relative to the mean of all five time points that was set to 100%. For the transfer to darkness, the highest value was set to 1 and other values
were expressed in a relative manner. Error bars are SEs where n = 3.
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0–1 h into the light using a Student t-test to calculate significance. Both analyses indicated that the NDA1 transcript was up-regulated in a diurnal manner (Fig. 4, Table 1). The comparison between 0 and 1 h into the light also suggested that the NDB2 transcript was up-regulated in a diurnal manner.

To investigate further the diurnal regulation of the various ND genes, we analysed array data that had been previously determined to detect diurnal regulation of genes (Smith et al. 2004). This independent study offers some advantages. First, it was carried out in another laboratory and, although growth conditions differed, the experimental design was completely independent. Secondly, data from several genes present on these arrays have been defined as diurnally regulated and thus act as controls. The results obtained were similar to those we obtained in our expression analysis (Fig. 5). NDA1 displayed a distinct diurnal pattern, very similar to the positive controls, CCA1 and LHY, MYB transcription factors and central components of the circadian clock. The only other gene that displayed an appreciable increase on transfer to the light at 12 h was NDB2, again as we observed in our analysis where a 4-fold increase was observed (Table 1). This differed from the diurnal patterns observed with CCA1 and LHY in that it did not display an increase in expression until 12 h compared with 8 h, and the increase was not as dramatic. However, this was similar to CAB4 (another positive control) and thus in agreement with the quantitative RT–PCR data showing that the NDB2 transcript appears to be up-regulated in the early daytime in a diurnal manner. Previous analysis indicated that NDC1 was regulated in a diurnal manner (Escobar et al. 2004), but this was not observed in this study or other studies (Smith et al. 2004). Transferring 5-week-old plants into darkness for 4 d as previously described (Michalecka et al. 2003) did result in a large decrease in transcript levels of NDA1 and NDB2 (Fig. 4B, Table 1). In the case of NDC1, a <2-fold change was observed upon transfer to darkness, which although significant cannot be taken as evidence of diurnal regulation (Fig. 4B, Table 1). Placing plants in continued darkness for several days is likely to induce changes in transcript abundance not due to light regulation, and such treatments need to be interpreted with caution (Weaver and Amasino 2001, Lin and Wu 2004).

We used Genevestigator (Zimmermann et al. 2004) to compare the expression of ND with Aox and Ucp, as well as F1β and α CI51 and PGDC (Fig. 6). Data presented are a compilation from several arrays and thus treatments are not directly comparable, but the analysis provides an overview of up- or down-regulation of transcript abundance. All the data for the genes examined are presented but routinely only 2-fold increases or 50% decreases are generally considered to be reliable from such data. However, these data do allow a view of co-expression patterns. Expression of NDA2 and NDB2 appeared to be particularly coordinated, both increasing in abundance in 10 different treatments using a 2-fold change in transcript abundance as a cut-off (Fig. 6). Expression of Aox1a appeared to be regulated in concert with these two ND genes, with tran-

![Fig. 5](https://academic.oup.com/pcp/article-abstract/47/1/43/1867495)

**Fig. 5** Analysis of the diurnal expression pattern of ND genes analysed from microarray data. The expression levels of the genes analysed in this study over a 24 h period were plotted relative to the mean signal from all time points. Two independent array sets were performed in this study and the average values are shown. Additionally, the patterns of transcript abundance for the genes previously shown to be diurnally controlled, CCA1, LHY and CAB4, are included for comparison.

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Table 2  Summary of the data presented in this study

| Gene   | Locus  | Location | Predicted | Actual | Mature | Tissue | Diurnal | Smith et al. (2004) | L–D |
|--------|--------|----------|-----------|--------|--------|--------|---------|---------------------|-----|
| NDA1   | At1g07180 | Internal | 57 kDa    | 60 kDa | 56 kDa | C, L, B, F | +,+       | +                    | +   |
| NDA2   | At2g29990 | Internal | 57 kDa    | 59 kDa | 55 kDa | R, C, L, B, F | -,-      | -                    | -   |
| NDC1   | At5g08740 | Internal | 57 kDa    | 70 kDa | 60 kDa | R, C, L, B, F | -,-      | -                    | +   |
| NDB1   | At4g28220 | External | 63 kDa    | 60 kDa | 60 kDa | R, C, L, B, F | -,-      | -                    | -   |
| NDB2   | At4g05020 | External | 65 kDa    | 63 kDa | 63 kDa | R, B, F | -,-      | +                    | -   |
| NDB3   | At4g21490 | External | 64 kDa    | N/A    | N/A    | R, B, F | -,-      | -                    | -   |
| NDB4   | At2g20800 | External | 65 kDa    | 65 kDa | 65 kDa | R, C, L, B, F | -,-      | -                    | -   |

The abbreviation, locus and intramitochondrial location are shown in the first three columns. The predicted mass, the apparent mass of precursor and mature proteins are indicated. The organs where expression is detected are indicated C = cotyledon, L = leaf, B = floral bud, F = flower, R = root. A summary of the diurnal expression data is given in the last three columns. The ‘Diurnal’ and ‘L–D’ columns refer to the data presented in Fig. 4A and B, respectively, with statistically significant differences (P < 0.05) indicated by +. ‘A’ and ‘T’ in the ‘Diurnal’ column refer to the ANOVA and t-tests performed on the quantitative RT–PCR data. An indication of which NDs were diurnally regulated based on data from Smith et al. (2004) is also given.

Discussion

A critical step in identifying gene function is the determination of the subcellular location of the encoded proteins. We have carried out this analysis with six of the seven genes for putative NDs in Arabidopsis. Three genes encoded internal proteins, NDA1, NDA2 and NDC1, whereas NDB1, NDB2 and NDB4 encoded external proteins. From the protease digestion experiments, it was also evident that both the external and internal NDs are likely to have different topologies on the inner membrane. For the internal NDs, NDA1 and NDA2 were essentially completely protected in both mitochondria and outer membrane-ruptured mitochondria, at the highest amount of protease used. In contrast, NDC1 displayed greater sensitivity to protease, being completely digested by 10 mg ml⁻¹ of protease in intact mitochondria. The use of TIM23 as a control indicates that at this concentration the protease does have access to the inner membrane, suggesting that NDC1 has domains exposed on the intermembrane space side of the inner membrane. This is supported by the fact that NDC1 can also be digested with a higher concentration of protease in outer membrane-ruptured mitochondria. Secondary structure prediction of NDC1 with a variety of programs indicated four regions between residues 220 and 350 with the potential to form α-helices and displayed moderate hydrophobicity, suggesting that it could be transmembranous. In the case of the external NDs, a similar situation was observed. NDB4 was completely digested by 10 mg ml⁻¹ of protease, and a distinct protease-generated fragment with an apparent molecular mass of 55 kDa was observed with 5 mg ml⁻¹ protease. NDB2 was almost completely digested by 10 mg ml⁻¹ protease and noticeably there was a significant decrease of intensity of the protected product with treatment by 1.2 mg ml⁻¹ protease or greater compared with NDB1. With NDB2, a protease-generated fragment was evident at 58 kDa at all protease concentrations. These protease fragments produced with intact mitochondria may indicate that some parts of NDB2 and NDB4 are located in the outer membrane.

The organ expression analysis reported here provides the first comprehensive and quantitative view of the expression of all NDs together with other alternative respiratory chain components (Table 2). The patterns obtained are in general agreement with a previous study carried out examining the organ expression profiles of NDs; however, data presented in this study provide quantitative analysis compared with non-quantitative analysis carried out previously (Michalecka et al. 2003). Although protein levels and activity cannot be directly inferred from mRNA abundance, these patterns of expression do allow a gene-specific designation of the organ and developmental signals that dictate expression. Overall, NDA1 represented the most highly expressed genes encoding an internal ND, and NDB2 represented the most highly expressed gene encoding an external ND (Table 1, Fig. 3). We were also able to detect the expression of NDB3, albeit at low levels, which has not been reported previously. NDB3 was expressed in all organs analysed but was most prominent in roots.

On the other hand, our analysis of the diurnal expression pattern of the ND genes differed from previous studies. Whereas we detected diurnal regulation of NDA1 and confirmed this using array data, where several positive controls were present, we did not observe a dramatic effect of light on NDC1 expression (Table 2). Likewise the independent array data do not reveal any distinct light-regulated pattern for NDC1. Originally only NDA1 was reported to be light regulated in Arabidopsis (Michalecka et al. 2003), with a later study...
Fig. 6 Summary of Genevestigator Response Viewer array data. The Response Viewer function shows the response of individual genes to a variety of stresses. Wild-type ATH1 22k array chips were used. Euclidean distance and complete linkage were used to cluster the data using TMeV. For the red–green scheme, red indicates that the signal intensity of the treatment is higher than the signal intensity of the corresponding control, and green means the opposite. The ratio is given in a linear scale. The colour coding is fixed for each scale type so as to visualize easily changes in signal intensity levels as shown in the key. If raw signal data are above 125 in a treated sample yet below 125 in the control sample, fold change data are indicated in bold in white. If raw signal data for treated and control samples are above a 125-fold change, data are indicated in white. If raw signal data are below 125 in treated samples yet above 125 in control samples, fold change data are indicated in white italic text. If raw signal is below 125 in control and treated samples, fold change is indicated in yellow italic text.
reporting light regulation of NDC1 (Escobar et al. 2004). The reason for this apparent discrepancy may be that under the different growth conditions, the genes are regulated by different factors. However, our data were consistent with a previous study examining the diurnal up-regulation of genes early in the day (Smith et al. 2004). Our study differed from previous studies; this study used external standards to quantify for linear and efficient amplification and differences were not based on threshold cycles that can vary depending on the means by which the latter is calculated (Clifton et al. 2005).

This study did uncover a diurnal pattern for NDB2 and this diurnal pattern was also evident in independent array experiments. Therefore, it appears that both the prominent internal (NDA1) and external (NDB2) NDs are under diurnal control. Along with Aox and Aox2 (see below) this has the potential to form a complete respiratory chain capable of oxidizing internal or external NAD(P)H. Light regulation of Aox has been reported previously in many species, initially in soybean at a protein level (Obenland et al. 1990), but subsequently in tobacco, potato and soybean (McCabe et al. 1998, Svensson and Rasmusson 2001, Dutilleul et al. 2003, Michalecka et al. 2003). Therefore, it appears that a complete alternative respiratory chain is regulated in a complementary manner with chloroplast function.

Analysis of array data using Genevestigator also allowed an overall picture of the response of all of the alternative respiratory chain components to various treatments to be obtained. From this analysis, it is clear that Aox1a, NDB2 and NDA2 respond similarly in many situations. NDC1 and NDA1 also behaved similarly but in the opposite direction from the other three genes, being down-regulated in more cases than up-regulated. Notably, the only treatments in which Aox and NDs displayed opposite trends in transcript abundance involved chemicals affecting growth. For example, uniconazole and paclobutrazole are both gibberellic acid biosynthesis inhibitors; naphthylphthalamic acid (NPA) is an inhibitor of polar auxin transport; isoxaben is a cellulose synthesis inhibitor; brz220 and brz91 are brassinosteroid synthesis inhibitors; and MG13 is a proteasome inhibitor. Although the latter’s main role has been described as cytosolic protein degradation, it is now emerging in mammalian systems to play a central role in the regulation of cell cycle, cell growth and differentiation (Dalton 2004).

Although protein levels and activity cannot be directly inferred from transcript abundance, previous studies examining Aox protein levels indicated that increases in transcript abundance resulted in increases in protein levels, and vice versa (Finnegan et al. 2004). Likewise, for studies with NDs in potato where transcript and protein levels have been examined, changes in transcript and proteins show the same trend (Svensson and Rasmusson 2001, Michalecka et al. 2004). First, it appears that Aox1a is co-expressed with NDA2 and NDB2, indicating that any proposed role needs to be discussed in terms of a biochemical pathway from the oxidation of NAD(P)H (either internal or external) to the reduction of O2 to H2O (Clifton et al. 2005). In this context, a clear role in maintaining redox balance becomes evident, as the induction of an internal and external ND together with a terminal oxidase allows the oxidation of matrix and cytosolic reducing equivalents. This could lead to an alteration of metabolism under conditions of macronutrient stress or inhibition of the cytochrome electron transport chain (Vanlerberghe and McIntosh 1997, Sieger et al. 2005). It may also decrease the production of reactive oxygen species by ‘burning off’ any excess reducing equivalents, allowing these components to act as a survival pathway (Robson and Vanlerberghe 2002). However, the complete picture is likely to be more complex, as NDB1 and NDC1 transcripts were largely unchanged by various treatments. Thus these less well-studied genes may be important links in coordinating energy metabolism but have been largely unrecognized because attention has focused on the large increases in transcript abundance of other gene family members in response to so-called ‘stress’ treatments. Furthermore, the close clustering of NDA1 and PGDC suggests that the former may be linked to chloroplast function via photorespiration. We have demonstrated previously that Aox2 in Arabidopsis is responsive to perturbation of chloroplast function (Clifton et al. 2005). Taken together, these results indicate that the various members of the gene families encoding ND and Aox have specialized and linked expression patterns. In Arabidopsis, Aox2 and NDA1 regulation appears to be linked to chloroplast function, whereas Aox1a, NDA2 and NDB2 appear to be primarily up-regulated in response to a wide variety of treatments. NDB1 and NDC1 could be regarded as housekeeping genes as they were largely unchanged by any treatment (Fig. 6).

Materials and Methods

Plant growth
Arabidopsis thaliana cv. Columbia was used in this study. Plants were grown at 22°C with a 16 h light (100 µmol m⁻² s⁻¹) and 8 h dark photoperiod.

Primers for quantitative RT–PCR
All primers except for PGDC used in this study have been previously described (Murcha et al. 2003, Thirkettle-Watts et al. 2003, Lister et al. 2004, Clifton et al. 2005).

The primers for PGDC were forward (5’-CCGTAACCATCA-TGGAGGAATCC-3’) and reverse (5’-CTGAGGAGTGACACAGTACT-AGC-3’)

Isolation of total RNA and cDNA synthesis for quantitative RT–PCR
Transcript abundance was measured using the iCycler™ and iQ SYBR Supermix (Bio-rad, Perth, Australia) as described previously (Thirkettle-Watts et al. 2003, Lister et al. 2004). Three independent cDNA preparations were analysed for every organ and each sample was analysed in duplicate. The standard error was calculated for every data point. Transcript levels were calculated from the standard curve of known DNA concentrations for every gene analysed. The data are normalized to show the expression of each gene relative to the maximum value, which is set to 1.
Mitochondrial alternative NAD(P)H dehydrogenases

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Genevestigator analysis

The Genevestigator Arabidopsis microarray database was used to analyse the response of the genes of interest in this study to a variety of stresses (Zimmermann et al. 2004). The Responser Viewer tool was utilized; ATH1 22k array wild-type only arrays were chosen and the genes of interest were selected. The data were visualized using a linear scale from a total of 1424 array experiments. The TMeV program utilized; ATH1 22k array wild-type only arrays were chosen and the data were visualized using a linear scale from a total of 1424 array experiments. The TMeV program was used to cluster the genes and stresses; Euclidean distance and complete linkage were chosen for the hierarchical clustering (Saeed et al. 2003).

Precursor proteins, mitochondrial isolation and outer membrane-ruptured mitochondria

Precursor proteins were produced in a coupled transcription–translation system (T-3T) programmed with cDNA according to the manufacturer’s instructions (Promega, Melbourne, Australia).

Percoll density gradient potato tuber (Solanum tuberosum cv Desiree) mitochondria were isolated according to Millar et al. (2001). The Genevestigator Arabidopsis microarray database was used to analyse the response of the genes of interest in this study to a variety of stresses (Zimmermann et al. 2004). The Responser Viewer tool was utilized; ATH1 22k array wild-type only arrays were chosen and the genes of interest were selected. The data were visualized using a linear scale from a total of 1424 array experiments. The TMeV program was used to cluster the genes and stresses; Euclidean distance and complete linkage were chosen for the hierarchical clustering (Saeed et al. 2003).

Protein import into mitochondria and outer membrane-ruptured mitochondria

Assays of import into potato mitochondria were carried out at as outlined previously (Lister et al. 2002, Murcha et al. 2003). A 200 µg aliquot of mitochondria was used in each import assay. Import assays were carried out at 23°C for 20 min as previously documented (Lister et al. 2002, Murcha et al. 2003).

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