Cold stress decreases the capacity for respiratory NADH oxidation in potato leaves

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Abstract Cold stress effects on the expression of genes for respiratory chain enzymes were investigated in potato (*Solanum tuberosum* L., cv. Desiree) leaves. The *nda*1 and *ndb*1 genes, homologues to genes encoding the non-proton-pumping respiratory chain NADH dehydrogenases of *Escherichia coli* and yeast, were compared to genes encoding catalytic subunits of the proton-pumping NADH dehydrogenase (complex I). Using a real-time PCR system, we demonstrate a specific and gradual decrease of the NDA1 transcript after exposing the plants to 5°C. After 6 days of cold treatment the NDA1 transcript abundance is 10% of the original level. This decrease is accompanied by specific decreases of immunodetected NDA protein and internal rotenone-insensitive NADH oxidation in mitochondria isolated from cold-treated plants. The alternative oxidase is not cold-induced neither at the protein nor at the activity level. The results are discussed in relation to the recent finding that the *nda*1 gene expression is completely light-dependent.

Key words: NADH dehydrogenase; Alternative oxidase; Cold treatment; Leaf mitochondrion; mRNA quantification; Real-time PCR

1. Introduction

In addition to the rotenone-sensitive NADH dehydrogenase, complex I, which is present in almost all eukaryotes, the respiratory chain of plant mitochondria contains four extra non-proton-pumping and rotenone-insensitive NAD(P)H dehydrogenases [1]. On the external side of the inner membrane separate dehydrogenases oxidize cytoplasmic NADH and NADPH [2]. Also on the internal side, there are separate rotenone-insensitive dehydrogenases present for oxidation of matrix NADH and NADPH [3–5].

Our knowledge about the physiological roles of these dehydrogenases is still very incomplete. Oxidation of NAD(P)H by additional dehydrogenases bypasses the proton-pumping complex I, leading to lower respiratory energy conservation. It seems likely that these enzymes, as was suggested for the alternative oxidase [6], are present to allow a flexible tuning of the redox balance in the cytosol and matrix, and to adjust to the changing need for ATP synthesis. Abiotic and biotic factors often change suddenly in nature, and for short-term acclimation it may be crucial for the plant cell to be able to up- or down-regulate the expression of the different NAD(P)H dehydrogenases rapidly. External mitochondrial NADH oxidation is induced in red beetroots upon slicing and aging of root tissue [7], and it has been proposed that the additional NAD(P)H dehydrogenases generally may be up-regulated under stress conditions [8].

Access to the plant homologues (*nda*1 and *ndb*1) to rotenone-insensitive NADH dehydrogenases of yeast mitochondria and *Escherichia coli* now allows us to study the expression of these genes under different conditions. The gene products, NDA1 and NDB1, are localized at the internal and external sides of the inner mitochondrial membrane, respectively [9]. The *nda*1 gene was recently shown to be completely dependent on light for expression, suggesting a function for the NDA1 enzyme in close association with photosynthetic metabolism [10]. To further survey the expression of the *nda*1 and *ndb*1 genes, we here investigate how these genes are regulated following cold treatment of potato plants. Cold stress has previously been reported to induce enzymatic changes in the plant respiratory chain, especially in energy-dissipating pathways [11,12]. We demonstrate that the *nda*1 gene is specifically down-regulated by cold treatment, and that this decrease in expression is accompanied by a decrease in detected NDA protein and lower activity of internal rotenone-insensitive NADH oxidation.

2. Materials and methods

2.1. Plant material and preparation of mitochondria

Mature leaves of 6–8 week old potato plants (*Solanum tuberosum* L., cv. Desiree) were used for all experiments. Plants were grown in soil in climate chambers at 20°C, 60–80% relative humidity, with a photoperiod of 16 h light of 300 μmol m^{-2} s^{-1}. The same light and humidity conditions were maintained during the cold treatment at 5°C. Mitochondria were purified from leaves according to [13].

2.2. RNA preparation and cDNA synthesis

RNA was isolated from 50 mg of potato leaves using the RNeasy Plant Mini kit (Qiagen). 1 μg of total leaf RNA was added into each cDNA synthesis reaction using the SuperScript II RT preamplification system (Invitrogen). Oligo$(dT)_{12-18}$ primers were used in all cDNA synthesis reactions.

2.3. Primer design and real-time PCR

PCR reactions, for relative quantification of individual cDNA molecules, were run in a Rotor-Gene 2072 Real-Time Cycler (Corbett Research). The following specific primer pairs were designed using the Wisconsin Package Version 10.1 (Genetics Computer Group, Madison, WI, USA), NDA1 (EMBL sequence database accession: AJ245861): 5′-GCC CTT CAC CTT TTG TCA AC-3′; 5′-TTC CTC CAG CTC ACT ACA CG-3′, NDB1 (AJ245862): 5′-AGA

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GCG AAG GAC CAA CCT CC-3′; 5′-ACA ACC CTT CAC TCG CAA CC-3′, 76 kDa subunit (X85808): 5′-GCA GGA AAG CTG TCT GAC GC-3′; 5′-ACA CAC CAG CAC CGA CGA TG-3′, 28.5 kDa subunit (X84319; X84320): 5′-TCG TCA GCT TGT TTT GGC AG-3′; 5′-GGC TTC TTG GCA GAA TCC AC-3′. A standard series of four-fold serial dilutions of linearized plasmid template, containing cDNA for the gene to be investigated, were used in each PCR run (Fig. 1). Real-time PCR with *Taq* DNA Polymerase (Promega) and SYBR Green (1:50 000, Sigma) was carried out for 45 cycles, each consisting of 45 s at 95°C, 45 s at 60°C (NDA1, NDB1, and 76 kDa) or 64°C (28.5 kDa), and 45 s at 74°C. In each PCR run, a minus reverse transcriptase control was included to check that possible contamination by genomic DNA in the RNA preparations did not interfere with the quantification of cDNA templates. PCR products were routinely controlled for having the correct melting point. Finally, representative reactions were analyzed on agarose gels to check the specificity of the PCR reactions. General molecular cloning techniques were used according to [14].

2.4. Activity measurements

External oxidation of NADH or NADPH (1 mM) and alternative oxidase activities were measured on intact mitochondria in a Clark Oxygen Electrode (Rank Brothers, Cambridge, UK) at 25°C in assay medium (0.3 M sucrose, 10 mM MOPS, pH 7.2, 2.5 mM $MgCl_2$, 0.1 mM EGTA and 0.4 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone) supplemented with 1 mM $CaCl_2$. Alternative oxidase capacity was estimated as the part of external NAD(P)H oxidation that was inhibited by SHAM (1 mM) in the presence of antimycin A (0.4 μM) and pyruvate (10 mM). Internal NADH oxidation was measured spectrophotometrically as described in [10].

2.5. Western analysis

Western blotting was carried out as in [15] after SDS–PAGE separation of mitochondrial proteins according to [16]. Antibody recognition was visualized with the ECL system (Amersham). NDA and NDB proteins were recognized with antibodies against peptides of NDA1 and NDB1, respectively [10,17]. The 76 kDa subunit of complex I was detected by antibodies against the 78 kDa subunit of *Neurospora* [18], the alternative oxidase by monoclonal antibodies against the maize protein [19], and the T-protein of the glycine decarboxylase complex and the mitochondrial serine hydroxymethyl transferase as described in [20]. For detection of the uncoupling protein, antibodies against the *At*PUMP1 [21] were used.

3. Results

3.1. The nda1 transcript is specifically down-regulated in cold-treated plants

For quantification of transcript levels, a real-time PCR system was developed, showing a linear response to the logarithm of template concentration over the sensitivity range used (Fig. 1). Leaf transcripts for the NDA1, NDB1, and the 28.5 and 76 kDa subunits of complex I were investigated after cold treatment for 2, 4 and 6 days. The level of NDA1 transcript decreases substantially after 2 days at 5°C. After 6 days of cold treatment the NDA1 transcript is further down-regulated to about 10% of the level at the start of the experiment and the 20°C control (Figs. 1 and 2A). For the NDB1 transcript, no clear difference from the control levels could be seen upon cold treatment (Fig. 2B). The abundance of transcripts for complex I subunits appears to be up-regulated during cold stress (Fig. 2C,D). The most distinct increase (more than two-fold) is seen after 4 days for the 76 kDa subunit (Fig. 2C).

3.2. The amount of NDA protein is decreased after 6 days of cold treatment

To investigate how protein abundance is affected by cold stress, leaf mitochondria were purified from potato plants,

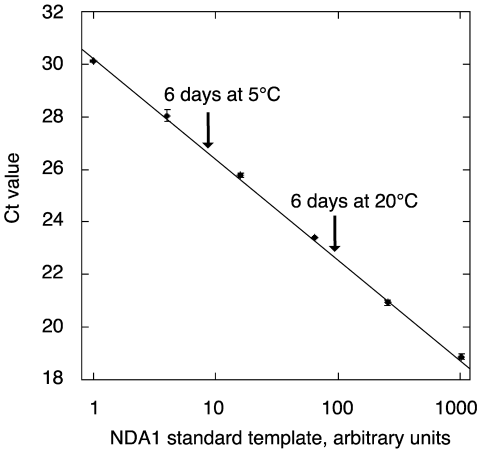

Fig. 1. Real-time PCR standard curve for consecutive four-fold dilutions of added NDA1 plasmid template. Ct value stands for the fractional cycle number at which the fluorescence passes the fixed threshold. Error bars show standard deviation for two separate PCR reactions. The arrows denote the average NDA1 Ct values for potato leaves after 6 days at 5°C and 20°C.

which had been cold-treated for 2 and 6 days. After 2 days of cold treatment, there was no clear difference in immunodetected protein levels for NDA, NDB, alternative oxidase, the 76 kDa subunit of complex I, the T-protein of the glycine decarboxylase complex, and the serine hydroxymethyl transferase (Fig. 3A). However, after 6 days of cold treatment, a specifically decreased level of immunodetected NDA protein is evident (Fig. 3B). This is in agreement with the low level of *nda*1 expression observed at cold stress (Figs. 1 and 2A). The other investigated proteins showed no significant change after 6 days of cold treatment (Fig. 3B).

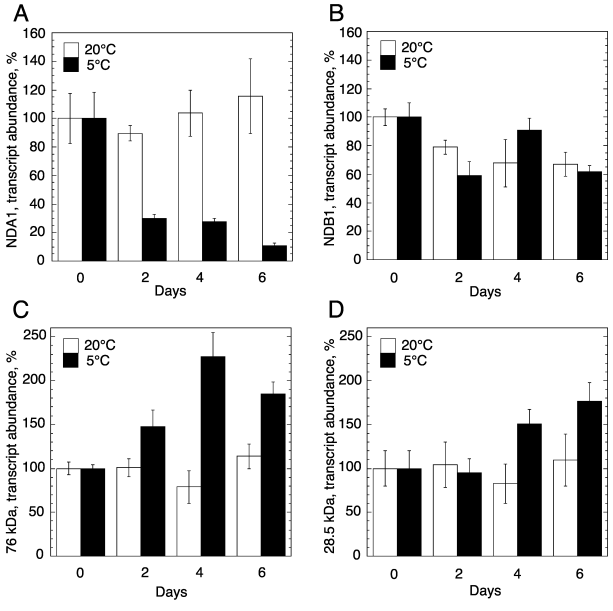

Fig. 2. Effects of cold treatment with time on transcript levels for NAD(P)H dehydrogenase genes in potato leaves. Transcript levels are shown for *nda*1 (A), *ndb*1 (B), and complex I subunit genes (C and D) as percentage of the level at the start of the experiment. Error bars show standard errors for three separate preparation series.

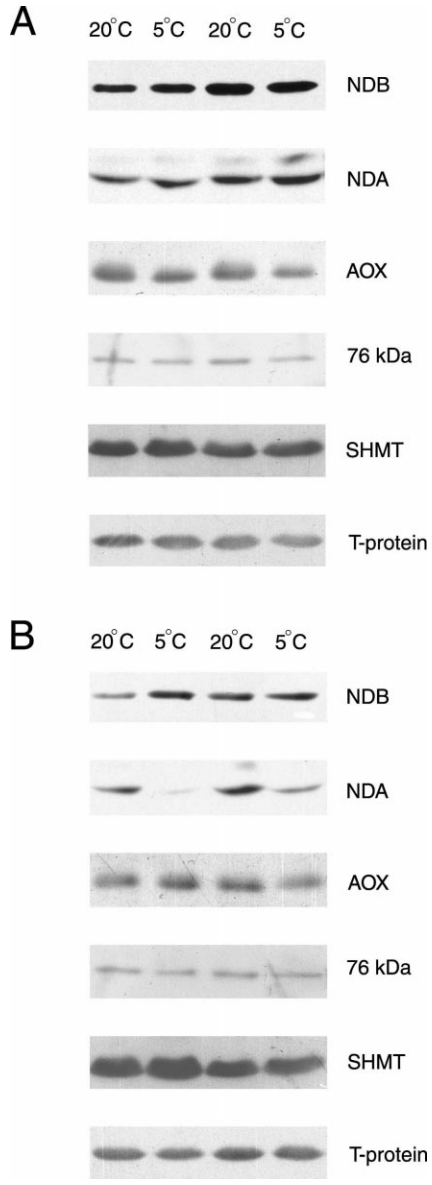

Fig. 3. Western analysis of respiratory chain proteins in potato leaf mitochondria after cold treatment of potato plants. Mitochondria were purified after 2 days (A) and 6 days (B) of cold treatment. The figure shows duplicate data sets from separate preparations. The proteins analyzed are denoted to the right of the Western blots, where AOX stands for the alternative oxidase, SHMT the mitochondrial serine hydroxymethyl transferase, and T-protein the T subunit of the glycine decarboxylase complex.

3.3. Rotenone-insensitive oxidation of matrix NADH is decreased by cold treatment

Activity measurements were carried out on the mitochondrial preparations used for Western blot analyses. After 2 days of cold stress, a slightly lower level of internal rotenone-insensitive NADH oxidation is observed compared to the control activity (Fig. 4A). After 6 days of cold stress, this activity is approximately 40% lower than the control (Fig. 4C). In the same mitochondrial preparations, no significant difference is seen for the rotenone-sensitive (via complex I), and external NADH oxidation (Fig. 4). In contrast, a lower level of external NADPH oxidation is observed after both 2 and 6 days of cold treatment (Fig. 4B,D). For the alternative oxidase, no significant difference in capacity was seen after 6 days of cold stress (Fig. 4D).

4. Discussion

In potato leaves, cold stress gives a dramatically lower NDA1 transcript level (Figs. 1 and 2A), accompanied by a specific decrease in immunodetected NDA protein (Fig. 3B) and a significantly lower capacity of internal rotenone-insensitive NADH oxidation (Fig. 4C) in the leaf mitochondria. These results suggest that a decreased matrix NADH oxidation may take place in the leaf in response to cold treatment, and further support previous evidence [9,10] that the NDA1 protein is responsible for at least a substantial part of the rotenone-insensitive oxidation of matrix NADH in potato leaf mitochondria.

The potato family contains both cold-tolerant and cold-sensitive species. *S. tuberosum* belongs to the latter group, where cold treatment will not induce frost hardiness [22]. Indeed, the potato plants used in the present study showed stress in the form of antocyanin formation in some of the leaves after 6 days of cold treatment (not shown).

Cold treatment has been shown to modify the respiratory chain by increasing the amount and activity of alternative oxidase in *Nicotiana tabacum* cells [11]. The phenomenon is however species-dependent. For example, protein level and activity of alternative oxidase are elevated by cold treatment in mung bean hypocotyls but not in soybean cotyledons [23]. Purvis and Shewfelt [24] suggested that alternative oxidase may ameliorate cold stress by decreasing the production of reactive oxygen species, which may be generated by a reduced ubiquinone pool when the activity of the dehydrogenases exceeds the capacity of oxidases in the respiratory chain. That elevated expression of alternative oxidase may have this func-

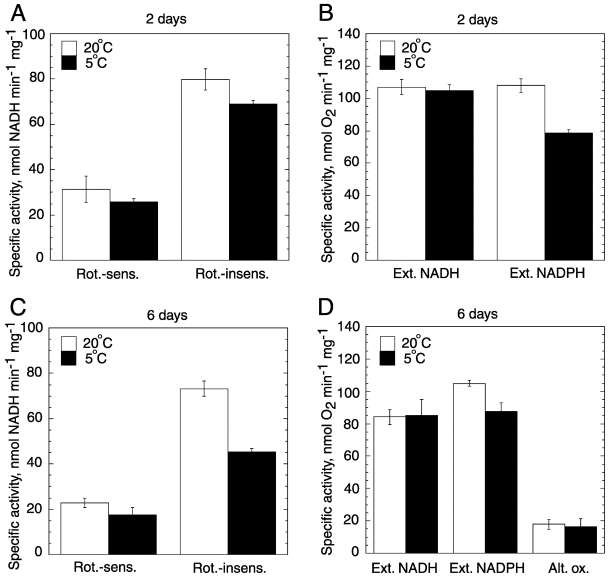

Fig. 4. Cold stress effects on respiratory chain activities of potato leaf mitochondria. Activity measurements were carried out on mitochondria purified after 2 days (A and B) and 6 days (C and D) of cold treatment. Rot.-sens., internal rotenone-sensitive NADH oxidation (via complex I); Rot.-insens., internal rotenone-insensitive NADH oxidation; Ext. NAD(P)H, external NAD(P)H oxidation; Alt. ox., alternative oxidase. Error bars display standard errors for three separate mitochondrial preparations.

tion has since been supported by several lines of evidence as reviewed in [1]. In potato, the alternative oxidase is not induced by cold treatment, neither at the protein (Fig. 3) nor at the activity (Fig. 4D) levels. However, in line with the original hypothesis [24], a down-regulation of internal NADH dehydrogenase capacity during cold treatment (Fig. 4) may lead to a decreased production of reactive oxygen species by limiting the reduction of ubiquinone. In other plants, an induced alternative oxidase would achieve the same effect by elevating the capacity for ubiquinol oxidation. Further comparison of NADH dehydrogenase and alternative oxidase capacities in response to different plant treatments is needed to test this hypothesis.

The *StUCP* gene, encoding a potato uncoupling protein, has been demonstrated to be strongly induced at the RNA level after cold stress of potato leaves [12]. To the best of our knowledge, an induction has not been shown at the protein level. In the present investigation, no induction of *St*UCP protein was detectable in the cold-treated plants (not shown). The antibodies used were directed against the homologous *At*PUMP1 protein, which has previously been shown to be cold-induced at the RNA level in *Arabidopsis* seedlings [25]. However, in potato leaves there may be UCP isoforms that are not recognized by these antibodies.

We previously showed that the expression of the *nda*1 gene is light-dependent, increases with development from young to mature leaves, and displays a diurnal rhythm. These results strongly suggest a function for the *nda*1 gene linked to the photosynthetic metabolism [10]. A possible physiological explanation for the results presented here relates to the effects of temperature on photorespiration. The ratio of photorespiratory flux to photosynthesis decreases at lower temperature [26]. Since the photorespiratory glycine oxidation is a major generator of matrix NADH in illuminated leaves, a decrease in photorespiration may decrease the demand for reoxidation of matrix NADH by the respiratory chain. The down-regulation of the internal rotenone-insensitive NADH oxidation and the *nda*1 gene in the present investigation would be consistent with this. In our study the abundance of mitochondrial photorespiratory proteins did not decrease in the cold-treated plants (Fig. 3). However, since the photorespiratory enzymes may have a slower turnover than the NDA1 protein, we cannot at present rule out that the decrease in NDA1 is caused in response to a change in photorespiration.

Two days of cold stress causes a dramatic decrease in the photosynthetic efficiency of *S. tuberosum* leaves [27]. A decrease in photosynthetic activity and associated carbohydrate status in the cold-stressed potato leaves may be an alternative reason for the down-regulation of the rotenone-insensitive NADH oxidation shown here (Fig. 4). Numerous studies have shown that illumination of chilling-sensitive plants at low temperatures increases the photosynthetic generation of reactive oxygen species that can overwhelm the protective processes in the plant cell [28]. An elevated level of reactive oxygen species may mediate the repression of the *nda*1 gene under cold stress conditions, analogous to its capacity to induce alternative oxidase in other species. The precise nature of the signaling systems involved in the control of the *nda*1 gene is an issue for future investigations.

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