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Lack of cytochrome *c* in *Arabidopsis* decreases stability of Complex IV and modifies redox metabolism without affecting Complexes I and III

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ABSTRACT

We studied the role of cytochrome *c* (CYTc), which mediates electron transfer between Complexes III and IV, in cellular events related with mitochondrial respiration, plant development and redox homeostasis. We analyzed single and double homozygous mutants in both CYTc-encoding genes from *Arabidopsis*: *CYTC-1* and *CYTC-2*. While individual mutants were similar to wild-type, knock-out of both genes produced an arrest of embryo development, showing that CYTc function is essential at early stages of plant development. Mutants in which CYTc levels were extremely reduced respective to wild-type had smaller rosettes with a pronounced decrease in parenchymatic cell size and an overall delay in development. Mitochondria from these mutants had lower respiration rates and a relative increase in alternative respiration. Furthermore, the decrease in CYTc severely affected the activity and the amount of Complex IV, without affecting Complexes I and III. Reactive oxygen species levels were reduced in these mutants, which showed induction of genes encoding antioxidant enzymes. Ascorbic acid levels were not affected, suggesting that a small amount of CYTc is enough to support its normal synthesis. We postulate that, in addition to its role as an electron carrier between Complexes III and IV, CYTc influences Complex IV levels in plants, probably reflecting a role of this protein in Complex IV stability. This double function of CYTc most likely explains why it is essential for plant survival.

1. Introduction

Mitochondria are major sites of energy production within the cell. The events driven by mitochondria must be coordinated with other cellular compartments to carry out important metabolic and signaling processes such as lipid metabolism, amino acid biosynthesis, calcium signaling and the control of the redox status along the cellular cycle. In plants, communication of mitochondria and chloroplasts is essential because both organelles cooperate in energy metabolism [1,2]. In addition, strong evidence indicates the existence of retrograde regulatory signals originated inside mitochondria to adjust nuclear gene expression to whole cell metabolism [3–6].

The main activity occurring inside the mitochondria is the electron transport through the respiratory chain complexes located in the inner membrane to generate ATP in a process known as Oxidative Phosphorylation (OxPhos). The reducing equivalents generated from sugars and lipids are oxidized by the OxPhos machinery based on the sequential operation of four protein complexes termed Complex I (NADH dehydrogenase), Complex II (succinate dehydrogenase), Complex III (cytochrome *c* reductase) and Complex IV (cytochrome *c* oxidase) [7]. The electron transfer between these complexes is carried out by two connecting redox-active molecules: ubiquinone or Coenzyme Q (CoQ), embedded in the lipid bilayer, and the heme protein cytochrome *c* (CYTc), that faces the intermembrane space. In addition, the plant mitochondrial respiratory chain has unique features due to the presence of an alternative terminal oxidase and rotenone-insensitive NAD(P)H dehydrogenases that constitute nonphosphorylating bypasses activated under certain metabolic conditions [8,9]. Particularly, electron transfer through the alternative oxidase bypasses the action of Complex III, CYTc and Complex IV.

CYTc is a hydrophilic metalloprotein found in the mitochondrial intermembrane space or on the external surface of the inner membrane and contains a heme moiety covalently attached via thioether linkages between the cofactor and a CXXCH motif of the protein [10]. CYTc transfers electrons from cytochrome c_1 in Complex III to the COX2 subunit of Complex IV. There are two genes encoding CYTc in *Arabidopsis thaliana*: *CYTC-1* (At1g22840) and *CYTC-2* (At4g10040). They have a conserved exon–intron structure and have arisen from a recent duplication event [11]. Promoter analysis demonstrated a partial divergence in expression patterns, with overlapping mainly in anthers, and similar responses to carbohydrates [12]. Regarding the possible function and regulation of both proteins in *Arabidopsis*, *CYTC-1* expression is governed by *site II* elements, which coordinately regulate other nuclear genes encoding respiratory chain components [12–14], while *CYTC-2* expression is dependent on

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a G-box and an ACGT motif and is induced under several stress conditions [15].

In addition to its role as electron carrier in the mitochondrial respiratory chain, CYTc has been linked to other important cellular processes, triggering programmed cell death in mammals [16] and participating in the redox-mediated import of proteins to the intermembrane space in yeast [17]. Mutant analyses in yeast and mammals revealed that CYTc is structurally required for the assembly and stability of Complexes I, III and IV [18–20]. A binding site for CYTc within the structure of respiratory supercomplexes was proposed in yeast [21] suggesting that this supramolecular configuration could be the basis for efficient electron transfer from Complex III to Complex IV [22]. In plant mitochondria, it has been shown that CYTc participates in other metabolic pathways like the synthesis of ascorbate [23,24] and the conversion of D-lactate to pyruvate catalyzed by the enzyme D-lactate dehydrogenase [25].

In this study, we analyzed the role of CYTc in plants through the analysis of *Arabidopsis* insertional mutants in *CYTC-1* and *CYTC-2*. Knock-out of both genes is lethal to plants and produces an arrest of embryo development, showing that CYTc has an essential function. We also obtained and characterized plants with very low amounts of CYTc respective to wild-type. These plants showed a delay in development, altered expression of stress responsive genes and reduced levels and activity of Complex IV, suggesting that CYTc is particularly required for the stability of Complex IV in plant mitochondria.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana Heyhn. ecotype Columbia (Col-0) was purchased from Lehle Seeds (Tucson, AZ). Insertion lines were obtained from the Arabidopsis Biological Resource Center, Ohio State University [26] and from GABI-Kat [27]. The identification numbers of mutants are: GK586B10 (*CYTC-1* mutant *a*), SALK_143142 (*CYTC-1* mutant *b*), SALK_037790 (*CYTC-2* mutant *a*) and SALK_029663 (*CYTC-2* mutant *b*). The location of the insertions was determined using PCR with specific primers on genomic DNA prepared according to Edwards et al. [28]. The presence of the wild-type allele was assessed using gene specific primers flanking the insertion site (see oligonucleotide Table S1). To determine the exact location of the insertion, the products were cloned into vector pCR 2.1-TOPO (Invitrogen) and sequenced. Plants were grown in soil at 22–24 °C under long-day photoperiod (16 h light/8 h dark) at an intensity of approximately 100 μmol m^{-2} s^{-1} and 22–25 °C, unless otherwise indicated. Alternatively, *Arabidopsis* seeds were surface sterilized in a solution containing 70% (v/v) ethanol and 0.1% (w/v) SDS for 5 min, washed in distilled water and sown in Petri dishes containing 0.5× Murashige and Skoog (MS) medium and 1% (w/v) agar. Plates were cold stratified at 4 °C for 2 days and transferred to a growth chamber under long-day photoperiod. Root length and morphology analyses were made in plates incubated vertically.

2.2. Cell suspension cultures and isolation of mitochondria

Cell cultures of *Arabidopsis thaliana* wild-type (Col-0) and the *1*b*2*a mutant were established as described by May and Leaver [29]. Cells were cultivated in a suspension culture maintained as outlined previously [30]. Mitochondria were prepared from cell suspension cultures as described by Werhahn et al. [31].

2.3. Respiration measurements

Oxygen consumption in cell suspension cultures and mitochondria was measured at 25 °C using Clark-type oxygen electrodes (Hansatech, Norfolk, England and Oroboros, Innsbruck, Austria). Mitochondrial respiration was measured in incubation medium (300 mM mannitol, 10 mM potassium phosphate pH 7.2, 10 mM KCl, 5 mM $MgCl_2$, and 150 μM ATP). Succinate (4 mM) was added as a substrate, and ADP (1 mM) was used to establish state 3 respiration, which was subsequently inhibited by KCN (1 mM).

2.4. Gel electrophoresis

Mitochondrial membrane proteins were separated by 2D Blue-native/SDS-PAGE according to the protocol described by Wittig et al. [32]. Digitonin (5 g per g protein) was used as a detergent for protein solubilization. Proteins were either visualized by Coomassie Brilliant Blue colloidal staining [33], Complex III activity staining [34] or CYTc oxidase activity staining [35]. After Complex III staining the gel strips were incubated in fixing solution (40% methanol, 10% acetic acid) to remove the Coomassie and better visualize the blue precipitate indicating activity. Gels were scanned and analyzed using Delta 2D 4.2 software (Decodon, Greifswald, Germany) as previously described [36].

2.5. Enzyme activity assays

The specific activities of respiratory chain complexes and of L-galactono-1,4-lactone dehydrogenase (L-GalLDH) were measured at room temperature in a total volume of 300 μl using an Epoch microplate spectrophotometer (BioTek Instruments, Winooski, USA). Mitochondria isolated from cell suspension cultures were permeabilized by freezing and thawing in reaction buffer (25 mM potassium phosphate pH 7.4 for respiratory chain complexes and 50 mM Tris pH 8.4 for L-GalLDH). Standard protocols were used for Complex I [37], II and IV [38] activity tests. L-GalLDH activity was assayed as described in Ostergaard et al. [39], but potassium cyanide (1 mM) was added to inhibit re-oxidation of CYTc by CYTc oxidase. In gel detection of SOD activity was carried out as described previously [40].

2.6. RNA isolation and analysis

RNA samples were prepared with TRIzol reagent (Invitrogen). For northern blots, specific amounts of total RNA were electrophoresed and hybridized as described by Welchen et al. [11]. RT-PCR analysis was performed according to O'Connell [41]. First strand cDNA synthesis was performed using the oligo dTv primer and MMLV reverse transcriptase (Promega) under standard conditions. PCR was performed on an aliquot of the cDNA synthesis reaction with primers specific for the *CYTC-1*, *CYTC-2*, *APX1*, *ASO*, *CAT3*, *AOX1a*, *AOX2*, *BCS1*, *PRXIIF* or actin (*ACT2* and *ACT8*) genes. Quantitative PCR was carried out using an MJ Research Chromo4 apparatus in 20 μl final volume containing 1 μl SYBR Green, 10 pmol of primers, 3 mM $MgCl_2$, dilutions of the reverse transcription reaction, and 0.2 μl platinum *Taq* polymerase (Invitrogen). Fluorescence was measured at 72 °C during 40 cycles. Relative transcript levels were calculated by a comparative C_t method. Expression values were normalized using *ACT2* and *ACT8* transcript levels as standard [42]. The sequences of all oligonucleotides used are shown in Table S1.

2.7. Western blot analysis

Standard protocols were used for Western blot analysis of protein extracts. Mitochondrial enriched protein extracts (50 μg) prepared from leaves or flowers were separated on 15% SDS-PAGE and transferred to Hybond-ECL (GE Healthcare). Blots were probed with a monoclonal anti-pigeon CYTc antibody (7H8.2C12, Pharmingen, San Diego, California, USA) or polyclonal rabbit antibodies against *Arabidopsis* COX2 [43] and gamma-carbonic anhydrase (kindly provided by Dr. Eduardo Zabaleta, IIB, Mar del Plata, Argentina), at dilutions of 1:1000, 1:2000 and 1:1000 respectively, and developed with anti-mouse or

anti-rabbit immunoglobin conjugated with horseradish peroxidase using the SuperSignal® West Pico Chemiluminescent Substrate (PIERCE).

2.8. Chlorophyll and anthocyanin content

Chlorophyll content was spectrophotometrically measured according to Porra [44]. Rosette leaves (100 mg) were placed in a tube containing 2 ml of 80% acetone and incubated overnight at 4 °C. After incubation, samples were centrifuged at 5000 ×*g* during 3 min and a dilution of supernatant corresponding to each sample was measured. The results were expressed as percentage of the chlorophyll content of wild-type leaves in five biological replicates for each genotype and in three different experiments. Total anthocyanins were estimated following Hodges et al. [45]. Leaves (100 mg) were homogenized in 2 ml of methanol–1% HCl. Samples were centrifuged at 5000 ×*g* during 5 min to remove cell debris and 1 ml of the supernatant, appropriately diluted in methanol–1% HCl, was used for the analysis. Total anthocyanins were determined spectrophotometrically as the difference between absorbance at 536 and 600 nm (to correct for phaeophytin absorbance). Results were expressed as mg/l of cyanidin 3-glucoside using an extinction coefficient of 26,900 $M^{-1}cm^{-1}$ at 536 nm. The results represent the mean ± SE of five independent samples.

2.9. Ascorbic acid content

Ascorbic acid content was measured in full expanded leaves of 21-day-old plants using a microplate-adapted colorimetric assay described by Gillespie and Ainsworth [46].

2.10. Determination of TBARS

Malondialdehyde content was measured according to Hodges et al. [45]. Plant tissue samples were homogenized with mortar and pestle in 25 ml/(g FW) of 80:20 (v:v) ethanol:water, followed by centrifugation at 3000 ×*g* for 10 min. A 1-ml aliquot of appropriately diluted sample was added to a test tube with 1 ml of either (i) a solution comprised of 20% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene, or (ii) a solution containing the above plus 0.65% thiobarbituric acid (TBA). Samples were then mixed vigorously, heated at 95 °C for 25 min, cooled, and centrifuged at 3000 ×*g* for 10 min. Absorbances were spectrophotometrically measured at 440 nm, 532 nm and 600 nm.

2.11. Electrolyte leakage

Electrolyte leakage was measured by placing entire rosettes in tubes containing 25 ml of double-distilled water. The first reading was done after 2 h of incubation at room temperature with gentle agitation, and afterwards the rosettes were exposed to 70 °C during 5 h to destroy cells. After that, the second reading was taken. Electrolyte leakage data are presented as percentage of the total amount of electrolytes present in the tissue.

2.12. Detection of superoxide and H_2O_2 in leaves

Histochemical detection of H_2O_2 was performed by incubating rosette leaves in a solution containing 1 mg ml^{-1} of 3,3′diaminobenzidine (DAB) as described by Thordal-Christensen et al. [47]. For superoxide detection, rosette leaves were incubated for 5 h in a superoxide staining solution (0.1 mg ml^{-1} NBT in 25 mM HEPES, pH 7.6) at room temperature. After incubation, chlorophyll was removed from leaves with 80% ethanol for 2 h at 70 °C. Superoxide anions were visualized as a dark precipitate and H_2O_2 produced a reddish-brown coloration.

2.13. Statistical analysis

Significant differences between means were evaluated by Student's *t*-tests using Excel software (Microsoft) and a statistical software package (SigmaPlot 11.0, Systat Software, Erkrath, Germany).

3. Results

3.1. Arabidopsis CYTC genes show functional redundancy

Arabidopsis contains two CYTc-encoding genes, *CYTC-1* (At1g22840) and *CYTC-2* (At4g10040) [11]. We have characterized two insertional mutants for each of these genes, with T-DNA insertions within the transcribed regions. The *1*a mutant contains a T-DNA insertion near the end of the first intron, 669 bp downstream of the start codon of *CYTC-1* (Fig. 1A). Plants homozygous for this insertion were identified by PCR with specific oligonucleotides (Table S1) and are assumed to be knock-out because the corresponding transcript was not detected by RT-PCR (Fig. 1B). The second mutant in the *CYTC-1* gene (*1*b) contains a T-DNA within the 3′-UTR, 25 bp downstream of the stop codon (Fig. 1A). Homozygous mutant plants originate an amplification product in RT-PCR reactions when oligonucleotides located upstream of the insertion site are used (Fig. 1C), suggesting that they produce an RNA that comprises the entire *CYTC-1* coding region. Transcription of *CYTC-1* was analyzed in this mutant by qRT-PCR and northern blot using RNA extracted from 2-week-old plants grown under normal conditions. These experiments indicated that *CYTC-1* transcript levels in these plants are less than 20% compared to wild-type (Fig. 1B). *CYTC-2* mutants *2*a and *2*b contain insertions within exons 1 and 3, respectively (Fig. 1A). No amplification product was detected in homozygous plants from these mutants either by qRT-PCR (Fig. 1B) or end-point RT-PCR (not shown). All homozygous insertional single mutants described above have similar levels of CYTc protein, that were reduced about 50% respective to wild-type, as indicated by Western blot analysis (Fig. 1D). These plants showed growth characteristics roughly similar to those of wild-type plants under normal growth conditions, suggesting the existence of functional redundancy between both genes. Particularly, *CYTC-1* mutants showed a slight delay in the emergence of the first flower bud (Fig. 2A; stage 5.1 according to Boyes et al. [48]). Moreover, these mutants had shorter primary roots (Fig. 2B) and delayed inflorescence stem elongation (Fig. 2C) respective to wild-type and *CYTC-2* mutants. These results are in agreement with previous observations that *CYTC-1* expression is higher in the meristematic regions of the plant [12].

3.2. CYTc is essential for embryo development and influences plant growth

To obtain plants with mutations in both *CYTC* genes, reciprocal crosses of single knock-out (*1*a, *2*a, *2*b) or knock-down (*1*b) lines were performed. For the *1*a mutant, it was not possible to obtain double-homozygous mutant plants in crosses with either the *2*a or *2*b mutants. Plants with three T-DNA insertions (i.e. homozygous for one gene and heterozygous for the other) grew normally but showed defective seeds with arrested embryos, most likely due to lethality caused by the lack of CYTc (Fig. S1). The proportion of normal to abnormal seeds and the distribution of the wild-type and mutant alleles in the progeny are in agreement with this observation. We conclude that CYTc function is absolutely required at early stages of embryo development.

In the case of the *1*b mutant, double homozygous insertional lines with *2*a and *2*b were obtained. Western blots of leaf and flower extracts with antibodies against CYTc indicated that these plants contain very low amounts of CYTc, since only a faint reactive band could be observed (Fig. 3A). On the contrary, the levels of COX2, a mitochondrial-encoded protein that forms part of Complex IV, and of gamma-carbonic

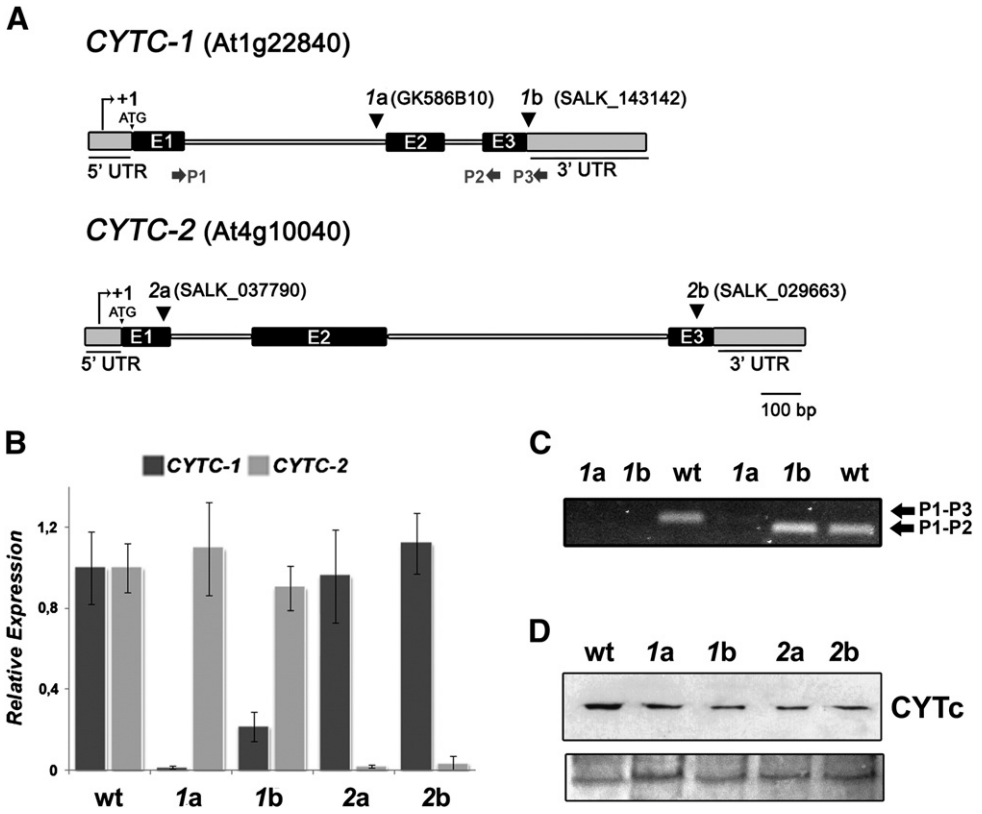

Fig. 1. Identification of *CYTC-1* and *CYTC-2* mutants. (A) Schematic representation of T-DNA insertion sites (arrowheads) in *CYTC-1* and *CYTC-2* genes. Boxes indicate exons and lines are introns. P1, P2 and P3 refer to primers used in RT-PCR assays. (B) Analysis of *CYTC* transcript levels in homozygous mutants by qRT-PCR. (C) Analysis of *CYTC-1* transcripts by RT-PCR in *1*a and *1*b homozygous single mutants. Arrows indicate the position of PCR products according to the primers used: P1–P2, 360 bp; P1–P3, 390 bp. See Table S1 for oligonucleotide sequences. (D) Western blot of leaf protein extracts from 4-week-old-single mutants and wild-type plants. Antibodies against CYTc were used. The lower image shows a Ponceau S staining of the membrane to ensure equal protein loading.

anhydrase, a constituent of Complex I encoded in the nucleus, were not significantly altered. These double mutants had smaller rosettes than wild-type (Fig. 3B). Microscopic observations indicated that decreased leaf growth is probably caused by a pronounced decrease in the size of parenchymatic cells (Fig. 3C), suggesting that reduced CYTc levels originate an impairment in cell growth.

The mutants also showed decreased root growth (Fig S2) and delayed flowering, that can be ascribed to lower CYTc-1 levels, as suggested by the analysis of single mutants, and a general delay in growth rate, according to the *Arabidopsis* growth stages established by Boyes et al. [48] (Fig. S2). Photosynthetic parameters and chlorophyll content were similar to those of wild-type plants of the same developmental stage until the onset of senescence in wild-type plants (not shown). After that stage, differences in chlorophyll content, anthocyanin accumulation and membrane permeability (measured as ion leakage from cells) were observed (Fig. S3) indicating that the delay in development in plants with decreased CYTc extends to the entire plant life cycle.

3.3. A decrease in CYTc affects the amount and activity of Complex IV

Due to its role as electron carrier shuttling electrons between cytochrome c_1 in Complex III and the COX2 Cu_A center in Complex IV, we analyzed the impact of lower CYTc levels on mitochondrial respiration. A cell culture of the *1*b*2*a line was established and used to isolate mitochondria. Oxygen consumption rates of cells from the mutant line were similar to wild-type (Fig. 4A). In contrast, isolated mitochondria from the mutant line had 50% decreased respiration rates (Fig. 4B). In both systems, a relative increase in cyanide-resistant respiration was evident in the mutant line, suggesting that lower CYTc levels affect the flow of electrons through the cyanide-sensitive pathway.

We also analyzed the activity of respiratory complexes in mitochondrial extracts isolated from suspension cells. The results indicated that the activity of Complex IV decreased to about 25% in mutants compared to wild-type (Fig. 5A). Under the same conditions, the activities of Complexes I and II did not show significant differences respective to wild-type. A significant decrease in Complex IV activity was also observed by in-gel activity staining of Blue-native gels (Fig. 5B), while Complex III was not affected. Since Complex IV activity assays are performed with the addition of external CYTc, the results imply that either the amount or the specific activity of Complex IV is affected in CYTc deficient plants. To establish the amount and composition of respiratory complexes in *CYTC* mutants, we performed comparative two-dimensional Blue-native/SDS-PAGE using mitochondrial extracts from wild-type and mutant cell suspension cultures. Statistical analysis of the gels showed a significant decrease in the abundance of the individual subunits of Complex IV by 50–90% in the mutant (Fig. 6, Table S2). These results indicate that lower CYTc levels cause a decrease in the amount of assembled Complex IV. Analysis of the spot volumes additionally revealed a 1.5-fold increase in abundance for the subunits of succinate dehydrogenase (Fig. 6, Table S2), although the enzyme activity was not changed (Fig. 5A). The abundance of the other OxPhos complexes and supercomplexes was identical in mitochondria from the wild-type and mutant lines.

3.4. A decrease in CYTc produces the induction of antioxidant genes with no increase in ROS levels or cell damage

It is well established that a close relationship exists between plant mitochondrial respiratory activity and the maintenance of whole cell

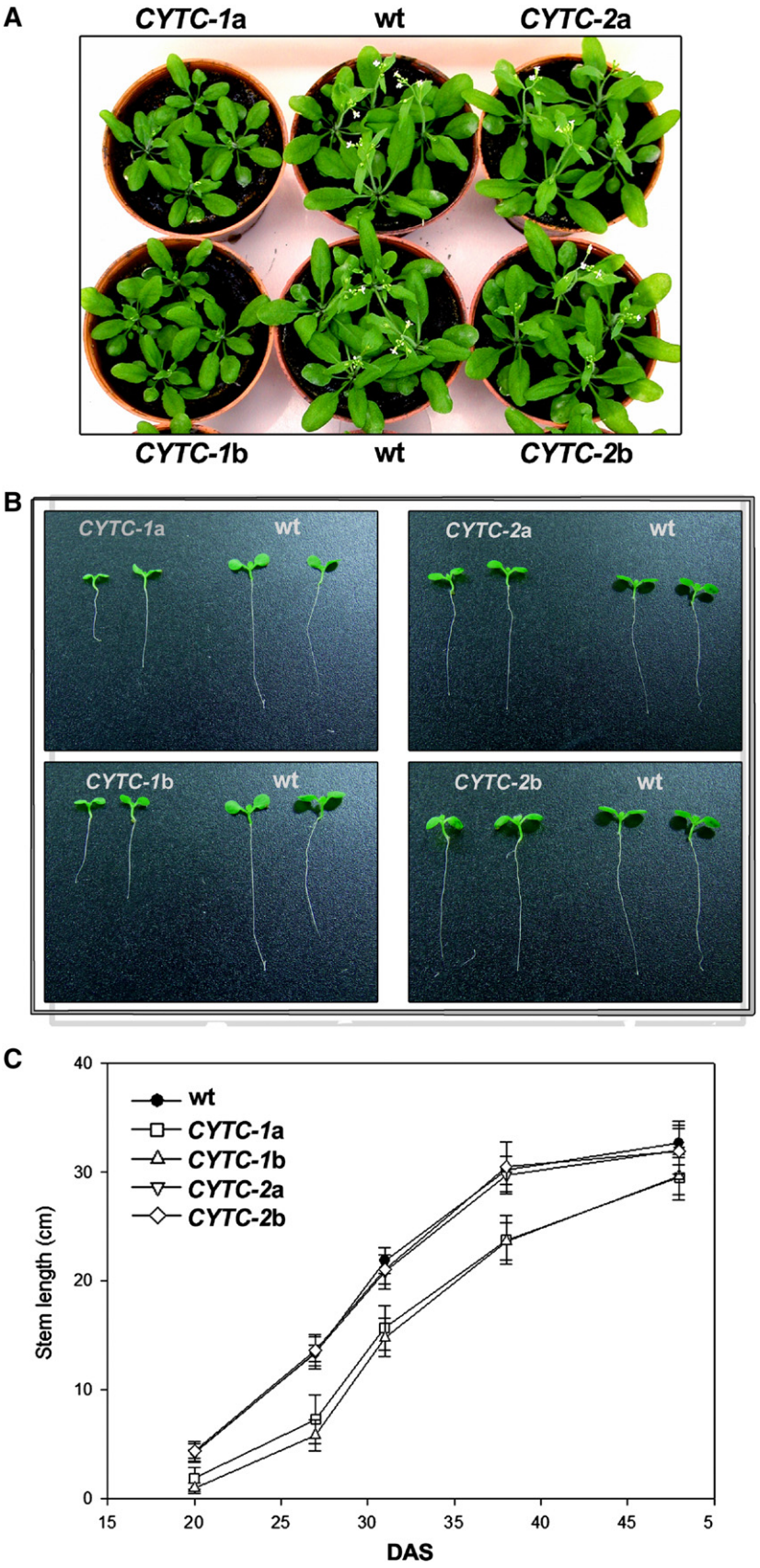

Fig. 2. Phenotype of single *CYTC* mutants. (A) Phenotype of wild-type (wt) and single homozygous 28-day-old mutant plants grown in soil. (B) Root length in 10-day-old seedlings grown vertically on plates containing 0.5× MS medium. (C) Stem length vs. time (DAS: days after sowing) in wild-type and single mutant plants.

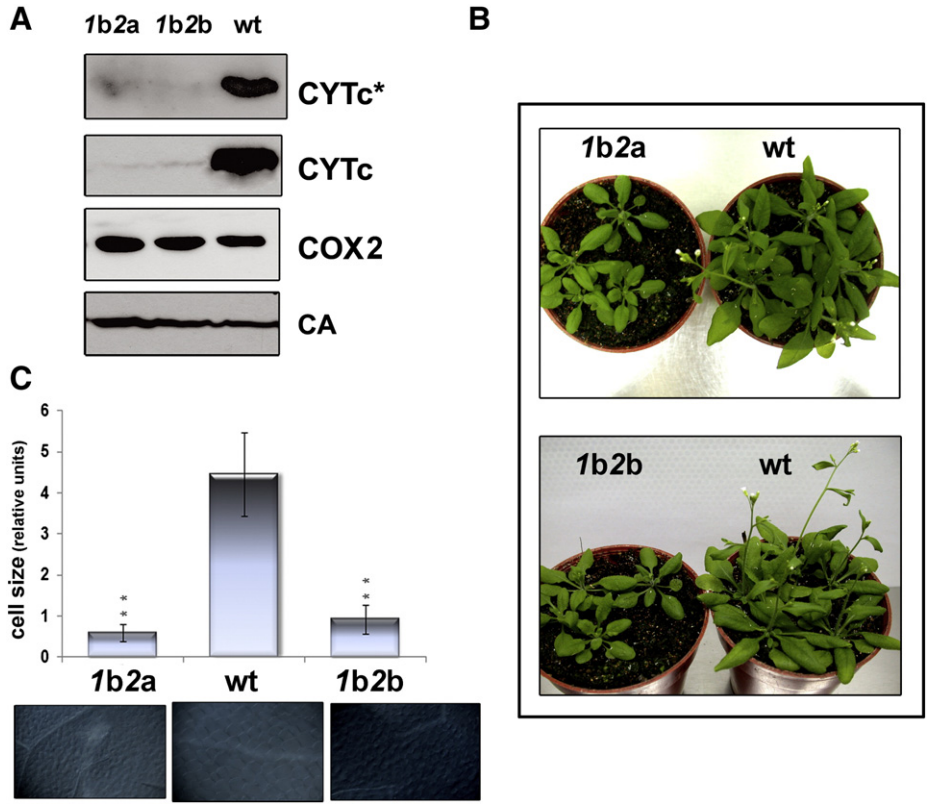

Fig. 3. Characterization of double homozygous (*1*b2a, *1*b2b) *CYTC* mutant plants. (A) Western blot of leaf and flower (*) protein extracts from 4-week-old double mutants and wild-type plants. Antibodies against CYTc, COX2 and gamma-carbonic anhydrase (CA) were used. (B) Phenotype of wild-type (wt) and double mutants grown in soil under long day photoperiod (16 h light/8 h dark). (C) Size of parenchymatic cells in leaves from 21-day-old plants previously cleared with chloral hydrate. Relative cell area was measured in pixels from micrographs using the program ImageJ (http://rsbweb.nih.gov/ij/).

redox homeostasis [1,49–51]. We therefore analyzed the impact on redox parameters of the decrease in CYTc levels, which may result in a block of the electron transport chain and the generation of reactive oxygen species (ROS) due to overreduction of upstream components, as observed in animal systems [52]. We explored ROS levels by histochemical detection using NBT and DAB. Double homozygous mutants showed lower ROS levels than wild-type when NBT, which detects mainly superoxide, was used (Fig. 7A). Less consistent results, suggesting the presence of either reduced or similar ROS levels, were observed when DAB was used (not shown). In addition, we measured the amount of TBA-reactive substances (TBARS) to estimate lipid peroxidation in membranes as a consequence of ROS production. Lipid peroxidation levels were similar in 3-week-old mutant and wild-type plants under normal growth conditions (Fig. 7B). Accordingly, ion leakage values were also similar in both types of plants (not shown). The results suggest that reduced CYTc levels do not originate an increase in ROS production, perhaps due to the flow of electrons through the alternative pathway.

We analyzed the expression of genes that respond to oxidative stress in mitochondria and other cellular compartments by qRT-PCR. Transcript levels of *AOX1a*, a stress responsive gene that encodes an isoform of the alternative oxidase, were increased 3- to 6-fold in

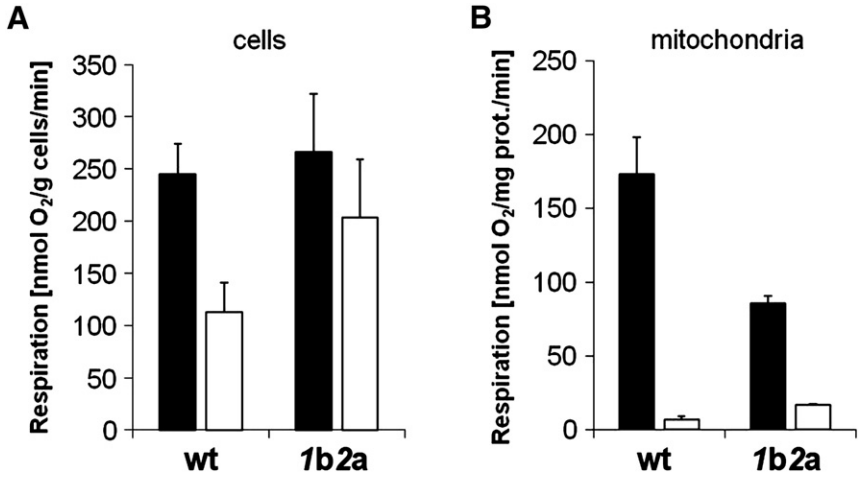

Fig. 4. Increased rates of alternative respiration in CYTc deficient cultured cells and mitochondria. Oxygen consumption was measured before (filled bars) and after (open bars) the addition of potassium cyanide (1 mM). (A) Respiration rates of wild-type (wt) and mutant (*1*b2a) cell suspensions. (B) State 3 respiration rates of mitochondria isolated from wild-type (wt) and mutant (*1*b2a) cells with succinate (4 mM) added as a substrate.

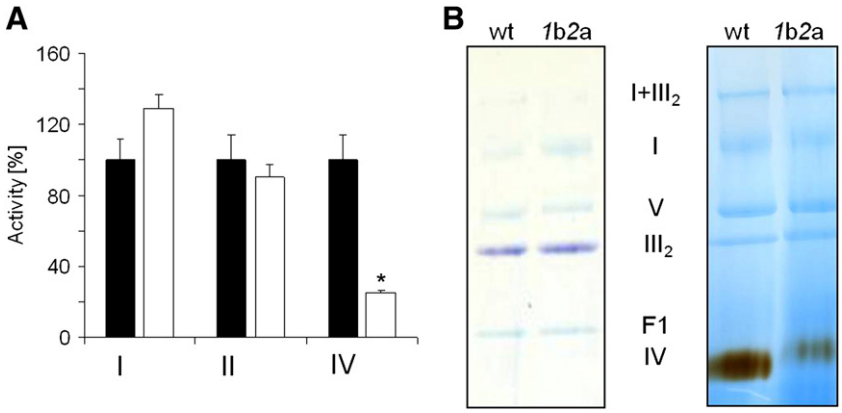

Fig. 5. CYTc deficiency leads to a decrease in Complex IV activity. (A) Relative activities of respiratory Complexes I, II and IV (respective to wild-type) in mutant (open bars) and wild-type (filled bars) mitochondria. *Significantly different from wild-type ($P<0.05$). (B) Separation of mitochondrial protein complexes by Blue-native PAGE and staining for Complex III (left) and CYTc oxidase (right) activity. $I+III_2$, supercomplex containing Complex I and dimeric Complex III; I, Complex I; V, ATP synthase complex; III_2, dimeric Complex III; F1, F1 subcomplex of ATP synthase; IV, Complex IV.

mutant plants (Fig. 7C). Mutants also showed increased transcript levels of *ASO*, *APX1* and *CAT3*, genes encoding cytosolic ascorbate oxidase and peroxidase and peroxisomal catalase, respectively (Fig. 7C). On the contrary, the expression levels of genes encoding the mitochondrial proteins AOX2 (alternative oxidase isoform related to stress inside the chloroplast), BCS1 (a stress-responsive protein putatively involved in Complex III biogenesis), PRXIIF (mitochondrial peroxiredoxin) and ACO3 (mitochondrial aconitase 3) were similar to wild-type (Fig. S4). The levels of MnSOD, FeSOD and Cu/ZnSOD, measured by enzymatic in-gel activity, were also similar to wild-type (Fig. S4). The results indicate that reduced CYTc levels originate signals that cause an increase in the expression of several genes related to the response of plants to stress.

3.5. CYTC mutants have decreased L-GalLDH activity but normal ascorbic acid content

CYTc participates as electron acceptor in the last step of ascorbic acid (AsA) synthesis. This reaction is catalyzed by the enzyme L-galactono-1,4-lactone dehydrogenase (L-GalLDH), which catalyzes the conversion of L-galactonolactone to AsA [23,24,53]. We observed that L-GalLDH activity was decreased to 60% of wild-type levels in *CYTC* mutants (Fig. 8A). In spite of this, AsA levels were normal in *CYTC* mutant plants (Fig. 8B). To evaluate the capacity of *CYTC* mutants for increased AsA synthesis, we also measured AsA levels after exposing plants to cycles of high light ($700\ \mu mol\ m^{-2}\ s^{-1}$), conditions that result in an increase in the AsA content of plants [54–57]. As observed in Fig. 8B, AsA levels increased about 3-fold both in wild-type and mutant plants under these conditions. This indicates that *CYTC* mutants are able to respond to an increased requirement for AsA synthesis even if they possess reduced levels of L-GalLDH and CYTc.

4. Discussion

4.1. CYTc is essential at early stages of plant development

We have performed a functional analysis of plant CYTc through the study of insertional mutants in both *Arabidopsis* genes encoding this protein. The fact that single knock-out mutants exhibit characteristics similar to those of wild-type plants indicates the existence of a high degree of functional redundancy, a common phenomenon observed in *Arabidopsis* [58]. Nonetheless, plants with very low *CYTC-1* expression levels show decreased growth of roots and the inflorescence stem, suggesting that this particular gene is required for these processes. The results are consistent with studies of the respective promoter regions showing that *CYTC-1* is preferentially expressed in root and shoot apical meristems [12]. Thus, it is likely that the observed growth defects reflect these expression patterns rather than functional differences of the encoded proteins.

Knock-out of both *CYTC* genes was lethal and produced an arrest of embryo development. This shows that CYTc function is essential at early stages of plant development, probably through its role in cyanide-sensitive respiration. Related to this, previous genetic approaches in mice demonstrated that CYTc deficiency causes embryonic lethality by midgestation. While heterozygous animals were viable and fertile and exhibited no obvious phenotypic abnormalities, homozygous null ($Cytc^{-/-}$) animals were not obtained [59]. In this sense, plants and animals share an absolute requirement for CYTc dependent respiration at early stages of development, showing that plant alternative respiration cannot replace a block of the electron chain between Complexes III and IV at this stage, probably because the amount of ATP generated under these conditions is too low to allow cell survival. Plants defective in the assembly of mitochondrial *c*-type cytochromes or in accessory proteins for Complex IV biogenesis also show embryo lethality [43,60,61]. Conversely, there are several examples of plant mutants lacking components of Complex I that are viable and have minor phenotypic alterations [1,62–67], indicating that Complex I can be dispensable for plant survival.

4.2. Is CYTc in excess in plant cells?

Even if CYTc is absolutely required for plant survival, our results show that plants with extremely reduced CYTc levels develop rather normally, although with a delay in their life cycle. This contrasts with the situation in mammals, in which small alterations in components of the mitochondrial respiratory chain produce severe disease and cell death [59,68]. This may be in part related to the presence of alternative respiratory pathways that allow plants to modify their respiratory efficiency and better cope with changes due to decreased OxPhos function [69]. Accordingly, CYTc deficient cells and mitochondria show increased alternative respiration.

An additional possibility is that there is an excess of CYTc in plant cells respective to what is needed for its normal respiratory functions. A similar observation was made in yeast, where the knock-out of *cyc1*, one of the two CYTc-encoding genes present in this organism, causes a 95% decrease in protein levels but only mild effects in respiration rates on non-fermentable carbon sources and Complex IV assembly [19]. The "excess" CYTc may be required in plants to optimize respiration under certain conditions or to perform other non-essential functions, like AsA synthesis [23,24] or D-lactate oxidation [25]. Studies in yeast have shown a kinetic compartmentalization

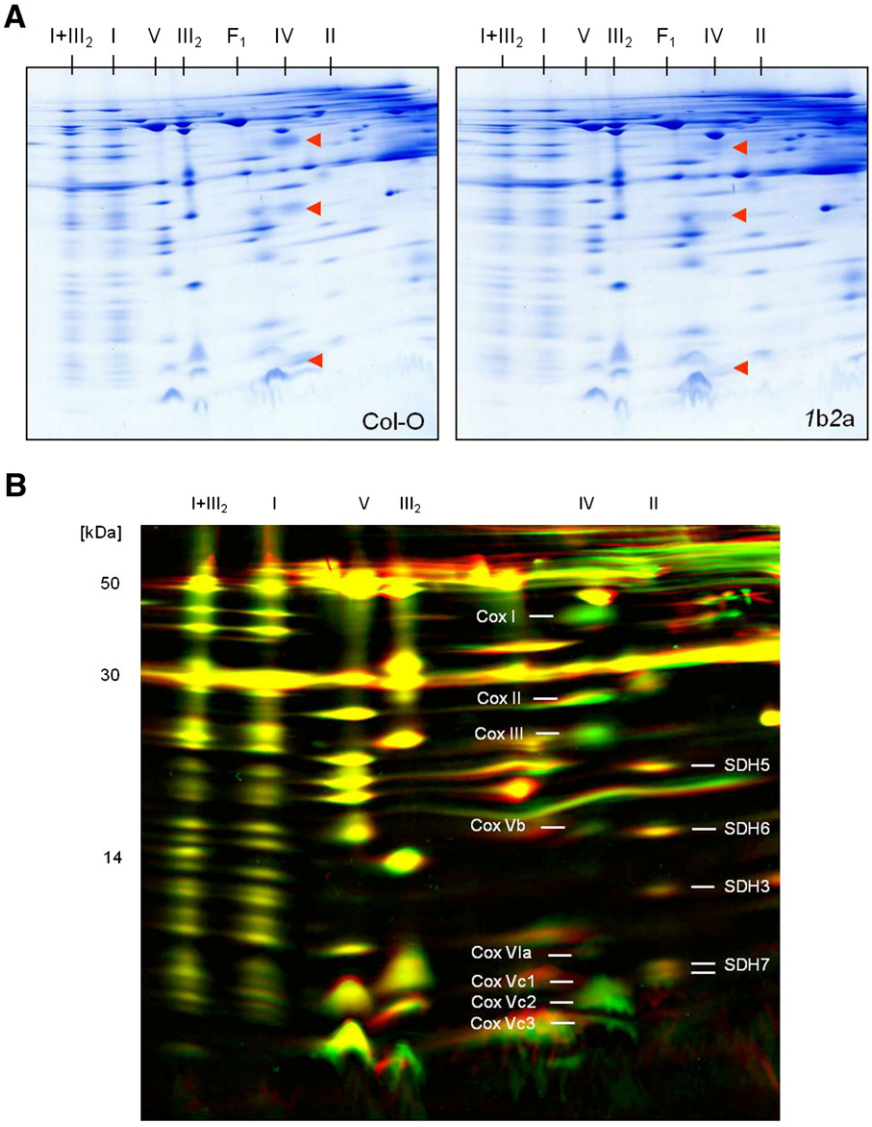

Fig. 6. CYTc deficiency leads to a decrease in Complex IV. Two-dimensional resolution of mitochondrial protein complexes by Blue-native/SDS-PAGE. (A) Coomassie stained gels of wild-type (Col-0, left) and mutant (*1b2a*, right) mitochondrial protein extracts. Red arrows indicate bands correspond to selected Complex IV subunits. (B) An overlay image was created from three wild-type and three mutant Coomassie stained gels using the software Delta2D. Yellow spots are equally abundant in wild-type and mutant samples; green spots are of lower abundance and red spots are of higher abundance in mutant mitochondria than in wild-type mitochondria, respectively. The positions of Complex IV and Complex II subunits are indicated. Molecular masses of standard proteins are given to the left and the identities of protein complexes are indicated above the gel. I + III_2, supercomplex containing Complex I and dimeric Complex III; I, Complex I; V, ATP synthase complex; III_2, dimeric Complex III; F1, F1 subcomplex of ATP synthase; IV, Complex IV; II, Complex II. For statistical evaluation of the results see Table S2.

with an active CYTc form bound to the membrane and a soluble form that contributes little to the electron transport rate [70] and can be recruited to maintain energy production levels when the respiratory chain is inhibited [71]. Furthermore, it has been reported that there are two different CYTc pools in mammalian mitochondria: a soluble or loosely membrane-bound pool, sensitive to electrostatic alterations, that represents about 90% of the total and a pool that remains bound to the inner membrane and is probably bound to respiratory complexes [19,72].

CYTc may be required for efficient cell growth since reduced levels of this protein produce a marked decrease in leaf parenchymatic cell size. A link between CYTc and cell growth has been postulated earlier on the basis of the presence in plant *CYTC* genes of regulatory elements named *site II*, that are also found in genes required for active biogenesis of cell components as, for example, ribosomal protein genes [13]. This effect may be due to the respiratory function of CYTc. Indeed, similar elements are also present in a majority of genes encoding respiratory chain components [13].

CYTc knock-down plants also exhibit a slower growth rate and increased longevity by delaying the onset of symptoms related to natural senescence. This growth retardation is a feature that has been observed in other characterized plant mutants with defects in respiratory chain components [43,62,63,65,69].

4.3. Plant CYTc influences Complex IV levels and activity

We observed that mutants with low amounts of CYTc have increased proportion of alternative respiration, probably to compensate the decrease in cyanide-sensitive respiration. It is interesting that overall respiratory rates were decreased in isolated mutant mitochondria, suggesting that a decrease in CYTc affects the maximal capacity of the respiratory chain. We also showed that CYTc deficiency leads to a significant decrease in the amount and activity of Complex IV. We detected a decrease in the abundance of all subunits that form part of Complex IV, with no effect on the abundance of the other respiratory complexes, suggesting that, as proposed previously in

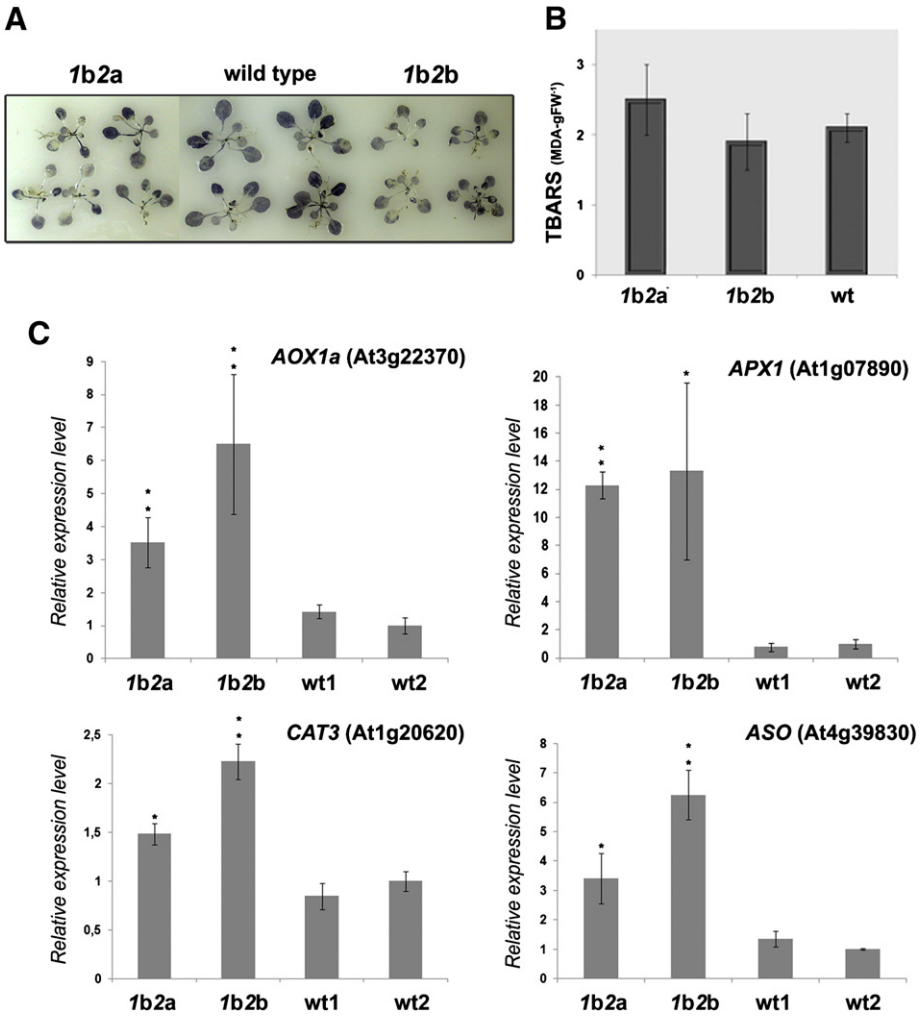

Fig. 7. ROS levels and the expression of stress responsive genes in *CYTC* mutant and wild-type plants. (A) ROS detection using NBT in 21-day-old plants grown in MS plates. Four plants from each line are shown as example. The staining was repeated several times with plants at different stages grown either in MS medium or soil with similar results. (B) Lipid peroxidation measured as malondialdehyde (MDA) recovered per gram of 21-day-old rosette leaves of plants grown under normal conditions. TBARS: TBA reactive substances. (C) Transcript levels of stress responsive genes (*AOX1a*: alternative oxidase; *ASO*: ascorbate oxidase; *APX1*: cytosolic ascorbate peroxidase; *CAT3*: peroxisomal catalase) were determined by qRT-PCR from total RNA extracted at the same stage of plants as in (B). Data are referred to wild-type transcript levels and are the mean (±SE) of three replicates. Asterisks indicate significant differences respective to wild-type, according to Student's *t* tests (*P<0.05, **P<0.01).

yeast [19,21] and mammals [22,73], plant CYTc would have an "extra" structural function to ensure the correct assembly of Complex IV subunits or to increase its stability. CYTc may remain in close contact with other respiratory complexes of the inner mitochondrial membrane and thus have a structural role as a constituent of supercomplexes. III + IV supercomplexes have been characterized for several plants, e.g. potato, spinach and asparagus [74] and are assumed to occur also in *Arabidopsis* in vivo. A difference

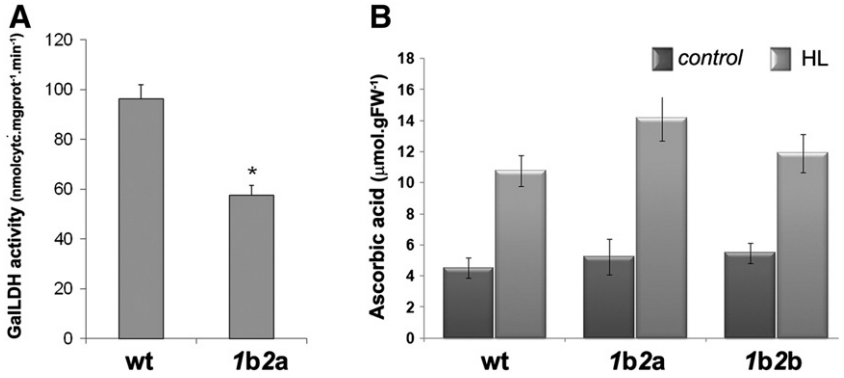

Fig. 8. Ascorbic acid metabolism in *CYTC* mutants. (A) L-GalLDH activity [nmol $CYTc_{red.}$ mg protein^{-1} min^{-1}] in mitochondria prepared from cell suspension culture. (B) AsA content in 21-day-old *Arabidopsis* rosette leaves of mutant and wild-type plants before (dark-gray bars) and after (light-gray bars) a 3-day exposure to high-light (700 µmol m^{-2} s^{-1}; 12 h light/12 h dark). Bars represent the mean (±SE) of three replicate experiments. Asterisks indicate significant differences respective to wild-type, according to Student's *t* tests (*P<0.05).

with the mammalian system, however, is that mitochondria from *cytc* knock-out mouse cells lack fully assembled Complexes I and IV and have reduced levels of Complex III [20], while only Complex IV seems to be affected in plants. We also detected a slight (1.5-fold) increase in Complex II subunits, which however did not lead to a higher enzyme activity. A possible explanation for this discrepancy could be that Complex II activation is impaired in the mutant due to lower levels of the allosteric activators, such as ATP [75]. In yeast, cells expressing only 5% of wild-type CYTc protein levels have 40% of Complex IV activity, while a complete lack of CYTc causes the loss of subunits I, II and III and a decrease in the amounts of the nuclear encoded subunits IV, V, VI and VIa [18,19].

Even if Complex IV levels are decreased, we did not detect significant changes in the total amount of subunit 2 in whole cell or mitochondrial extracts. This indicates the presence of unassembled COX2 subunit. This result is in agreement with the observation that respiratory complex assembly is driven by nuclear-encoded subunits, while subunits encoded in mitochondria, like COX2, are present in excess [76].

4.4. CYTc mutant plants show induced expression of antioxidant genes

The respiratory activity of mitochondria is responsible for the generation of molecules with enormous reactivity known as ROS, mainly as a consequence of Complex I and Complex III activities [49,77]. Studies with inhibitors of Complex III have shown that a decrease in the flow of electrons through the cyanide-sensitive respiratory pathway induces ROS production and the expression of alternative oxidase genes, mainly *AOX1a* in *Arabidopsis* [50]. It has been proposed that ROS generated in mitochondria due to inhibition of the respiratory chain mediate *AOX* gene induction. According to our results, CYTc deficient plants show higher expression levels of *AOX1a* and other genes that are usually induced under stress conditions, even if we did not detect a general increase in ROS levels. This may indicate that either *AOX1a* induction in *CYTC* mutants is the consequence of a different signaling pathway or ROS production is highly localized. A similar observation was made in tobacco plants that lack Complex I due to mutations in the mitochondrial *NAD7* gene [1]. It is also interesting that other genes encoding mitochondrial proteins that respond to stress, like *PRXIIF* or *BCS1*, are not induced in CYTc deficient plants, indicating that the response is specific. We also detected induction of genes that encode proteins localized outside mitochondria, like cytosolic ASO and APX1, and peroxisomal CAT3. These data show that the response to CYTc deficiency extends to other cellular compartments beyond mitochondria.

4.5. Low CYTc levels are enough to maintain normal content and capacity for AsA synthesis

CYTc has been related to AsA synthesis in plants [23]. Even if the activity of L-GalLDH, the enzyme that catalyzes the final step of AsA synthesis and uses CYTc as electron acceptor [24], is reduced in *CYTC* mutants, the AsA content of leaves was comparable to wild-type, showing that low CYTc levels are enough to maintain a normal capacity for AsA synthesis. Previously, no relationship between the amount of L-GalLDH and the rates of AsA synthesis or accumulation could be established, and the abundance of L-GalLDH could not be used as an indicator of the capacity for AsA accumulation in plants [78]. Alhagdow et al. [79] demonstrated that tomato plants with up to an 80% reduction in L-GalLDH activity had no significant changes in total AsA content. Furthermore, other routes of AsA synthesis have been proposed in plant cells. The most established one resembles the mammalian route and is represented by the precursor L-gulono-1,4-lactone [80], but homologues of the enzyme catalyzing its conversion to AsA have not been identified in plants. If normal AsA levels in *CYTC* mutants are maintained through these alternative pathways is a question to be elucidated in the future.

5. Conclusion

In conclusion, we studied the role of CYTc in plants and demonstrated that, even if it is an essential protein, plants can survive with very low CYTc levels respective to the amounts present in wild-type plants. Reduced CYTc levels affect mainly mitochondrial respiration and Complex IV assembly or stability, and produce a compensatory effect by increasing alternative respiration. Reduced CYTc levels also originate signals that influence the expression of a specific set of genes involved in responses to oxidative stress or changes in redox metabolism. Future experiments will be devoted to study in detail how the flow of electrons through the respiratory chain is remodeled and how this is connected with other cellular processes affected by a decrease in CYTc.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2012.04.008.

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