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Alternative Oxidase Involvement in Cold Stress Response of *Arabidopsis thaliana fad2* and *FAD3+* Cell Suspensions Altered in Membrane Lipid Composition

Ana Rita Matos [1, 2, *], Cécile Hourton-Cabassa [1], Dominique Ciçek [1], Nathalie Rezé [1], Joao Daniel Arrabaça [2], Alain Zachowski [1] and François Moreau [1]

[1] *Université Pierre et Marie Curie-Paris 6, CNRS, UMR 7180 Physiologie Cellulaire et Moléculaire des Plantes, Le Raphaël, 3 Rue Galilée, F-94200 Ivry sur Seine, France*
[2] *Centro de Engenharia Biológica, Secção de Fisiologia e Bioquímica Vegetais, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal*

To investigate how the fatty acid composition of membrane lipids influences cell growth and mitochondrial respiration, in particular the expression and capacity of alternative oxidase (AOX), under cold stress, we used the *Arabidopsis thaliana fad2* knockout and *FAD3+* -overexpressing cultured cells lines affected in extrachloroplastic fatty acid desaturation activities. At 22°C, *fad2* mitochondria exhibited a low polyunsaturated fatty acid content and low protein to lipid ratio, while mitochondria from *FAD3+* were enriched in linolenic acid and in total membrane protein. As a consequence, both mutants showed a higher membrane microviscosity than the wild type. After exposure to 9°C, *FAD3+* mitochondria exhibited lower microviscosity and lower rigidification upon a temperature downshift than *fad2*. Furthermore, the extent of reduction of cell growth and respiratiory rates in the phosphorylating state was positively related to the cold sensitivity of each cell line, being more pronounced in *fad2* that in the wild type, whereas the stability of those parameters reflected the cold resistance of *FAD3+*. In contrast, an increase in AOX capacity was observed in the three cell lines at 9°C. These inductions were correlated to AOX protein amounts and seem to result from an accumulation of *AOX1c* transcripts in the three cell lines and of *AOX1a* transcripts in wild-type and *fad2* cells. The fact that there is no direct relationship between the degree of cold tolerance of each cell line and their ability to enhance their AOX capacity suggests that the participation of AOX in the response of Arabidopsis cells to cold stress does not necessarily favor cold tolerance.

Keywords: Alternative oxidase — *Arabidopsis thaliana* — Fatty acid desaturase — Low temperature — Mitochondria.

Abbreviations: AOX, alternative oxidase; BSA, bovine serum albumin; COX, cytochrome c oxidase; DBI, double bond index; DTT, dithiothreitol; FAD, fatty acid desaturase; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; RT–PCR, reverse transcription–PCR; WT, wild type.

Introduction

Low temperature is an environmental stress that adversely affects plant growth and crop production. Recently, microarray analyses have contributed to the identification of a great number of genes involved in the different steps of cold stress responses (see Vergnolle et al. 2005). However the mechanisms that allow plants to perceive and to withstand low temperatures are far from being elucidated.

One of the effects of low temperature in plants is the modification of their membrane lipid composition. The accumulation of polyunsaturated fatty acids (PUFAs) in polar lipids contributes to the preservation of membrane fluidity, a factor that affects the activity of membrane-bound proteins (Murata and Los 1997) such as complexes involved in mitochondrial respiratory function. Another effect of cold stress is the appearance of an oxidative burst due to formation of reactive oxygen species (ROS). Furthermore, the levels of oxidative stress and the ability to activate protective mechanisms, such as an increase in the activity of scavenging enzymes, differ according to the degree of tolerance of the plant (Prasad et al. 1995, De Santis et al. 1999). For example, a decrease in the expression and activity of cytochrome c oxidase (COX) followed by the accumulation of ROS was observed in maize seedlings (Prasad et al. 1994). Besides COX, the electron transport chain of plant mitochondria contains an alternative oxidase (AOX) which transfers electrons from the reduced ubiquinone to molecular oxygen, bypassing two proton-pumping sites (complexes III and IV) and thus reducing the yield of oxidative phosphorylation. AOX was initially identified in thermogenic *Araceae* flowers, but was further described in many other plant species and tissues, especially in response to biotic and abiotic stresses, including low temperature (see Finnegan et al. 2004). Several lines of evidence, in particular results obtained with transgenic plants of tobacco and Arabidopsis,

*Corresponding author: E-mail, armatos@fc.ul.pt; Fax, +351217500166.

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suggest that AOX is able to prevent ROS accumulation (Maxwell et al. 1999, Umbach et al. 2005).

In the past few years, the isolation of several *Arabidopsis thaliana* mutants with altered lipid composition allowed the identification at the molecular level of the enzymes that carry out lipid desaturation: the fatty acid desaturases (FADs) (see Wallis and Browse 2002). Those enzymes are located in either the chloroplast (FADs 4–8) or the endoplasmic reticulum (FAD2 and FAD3). FAD2 converts oleic acid (18:1) into linoleic acid (18:2) that is further desaturated by FAD3 to generate linolenic acid (18:3). Both enzymes are responsible for desaturation of fatty acids present in extraplastidial membranes, including mitochondria. Studies carried out on *fad2* plants, deficient in ω-6-oleate desaturase activity, have shown that they grow normally at 22°C but show reduced stem elongation at 12°C and are not able to survive at 6°C (Miquel et al. 1993). In a previous work using suspension-cultured cells grown at 22°C, we showed that the *fad2* mutant is affected in its mitochondrial dynamic and functional properties. Indeed, *fad2* mitochondria exhibited more rigid membranes, lower proton leaks and a higher metabolic control of the respiration linked to membrane proton leaks (Caiveau et al. 2001).

Since the cold-sensitive mutant *fad2* was affected in its mitochondrial properties at normal growth temperature, we decided to investigate how the fatty acid modifications of membrane lipids influence mitochondrial function under cold stress. In addition, we have also used the transgenic plant *FAD3+* that overexpresses the *FAD3* gene from *Brassica napus* under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Arondel et al. 1992). *FAD3+* plants have increased amounts of PUFAs in their extrachloroplastic lipids, leading to altered mitochondrial membranes and increased cold tolerance compared with the wild type (WT) and *fad2*.

In the present study, we have investigated cell growth and membrane lipid composition in addition to biophysical parameters related to membrane dynamics (including microviscosity and lateral diffusion), in mitochondria isolated from WT, *fad2* and *FAD3+* suspension-cultured cells, grown at 22°C or exposed to low temperature (9°C). We have also compared the changes occurring under this cold stress in oxidation rates of various energetic states, AOX capacity, and protein contents and expression patterns of *AOX* and *COXII* genes in the three genotypes. The results show that cell growth, phosphorylating oxidative rates and *COXII* expression are inhibited by low temperatures in WT and *fad2* cells, but not in *FAD3+*, while AOX capacity, protein content and transcript amount increase in the three cell lines.

Results

Cell growth

When subcultured at weekly intervals, fresh weights of cell suspensions increased, as shown in Fig. 1. In WT cells, the exponential phase lasted only 2 or 3 d until day 5. This period was followed by a deceleration of the growth phase leading to a maximum fresh weigh on days 7–8, corresponding to about a 6-fold increase in the amount of cells. The general growth pattern of *fad2* or *FAD3+* cells in batch culture was comparable with that of the WT. However, the growth rate of *fad2* cells was lower, compared with

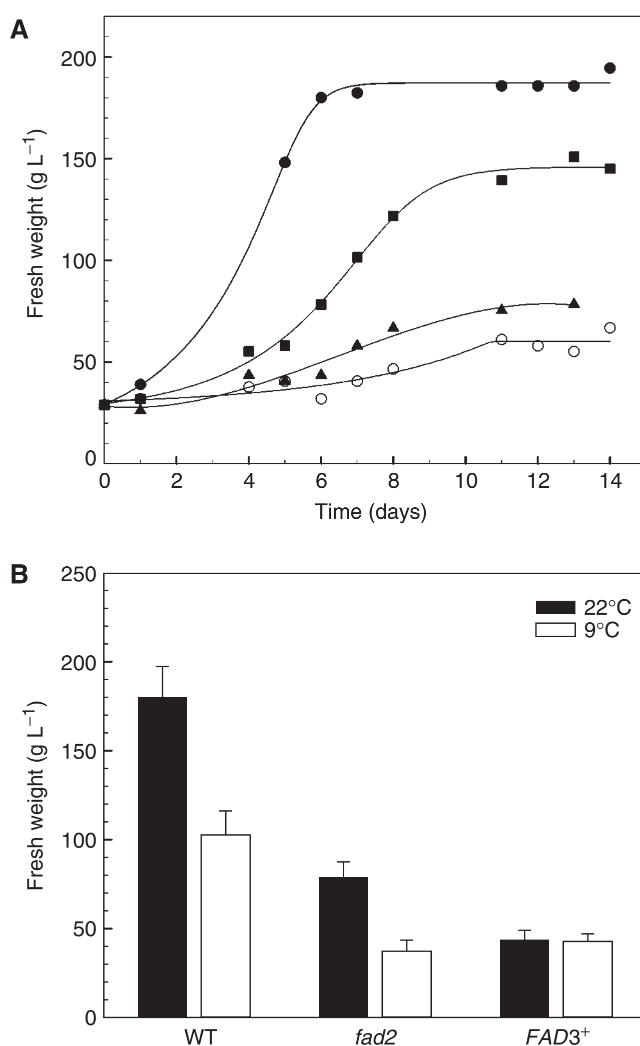

Fig. 1 (A) Growth characteristics of WT (filled circles), *fad2* (squares) and *FAD3+* (triangles) cells grown in batch culture at 22°C. Open circles represent WT cells grown at 9°C. Growth was expressed in fresh weigh (FW) in g l^{-1}. (B) Cell fresh weights after 6 d of culture at 22°C or grown for 3 d at 22°C then at 9°C for the last 3 d. Data are the average ± SD of three or more independent measurements.

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WT cells. The exponential phase lasted until day 6 and the maximum fresh weight was only 5-fold increased. For *FAD3+* cells, the growth rate evolution was still slower, since an approximately 2-fold increase of the fresh weight was obtained after 10 d of cell culture.

When continuously exposed to 9°C, the WT cells exhibited a longer lag phase and a very low growth rate, leading to a small amount of cells in suspension (<80 g in 2 l of medium), even after 12–15 d of culture (Fig. 1A). Because such amounts of cells are insufficient to purify mitochondria from either the WT or fad cells (data not shown), the cell suspensions were grown at 22°C for 3 d after subculture, then half of the suspensions were placed in a growth chamber at 9°C for 3 d (cold-treated), while the remaining suspensions were left at 22°C for a similar time period (control). Under these conditions of cold stress, the relative decrease in cell growth at 9°C vs 22°C was 43% for WT, 52% for *fad2* and unchanged for *FAD3+* (Fig. 1B). Finally, mitochondria were purified simultaneously from both groups of cells at day 6 after subculture, to analyse the effect of cold stress on membrane lipid composition, lipid dynamics and functional properties, including AOX protein content and capacity.

Membrane composition

As shown in Fig. 2, linoleic (18:2) and linolenic (18:3) acids are the major fatty acids of mitochondria in the WT cells. They account for 36 and 31%, respectively, of the total fatty acids, while the monounsaturated oleic acid (18:1) only accounts for 6%. The double bond index (DBI) of WT mitochondria, calculated accordingly to the formula: $DBI = 2\ [(18{:}1) + (2 \times 18{:}2) + (3 \times 18{:}3)]/100$, is 3.6. *fad2* and *FAD3+* mitochondria exhibited a completely different fatty acid composition compared with the WT. In *fad2*, the 18:1 content exceeded 50% of the total fatty acids, whereas the PUFA content was very low (<20%) and the DBI was

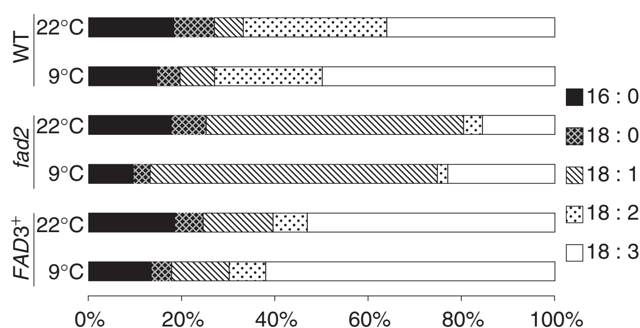

Fig. 2 Fatty acid composition of mitochondria isolated from WT, *fad2* and *FAD3+* cells cultured at 22°C or submitted to 9°C for 3 d. Data correspond to the mean of values obtained from two independent preparations. Differences between values of every duplicate were <10%.

2.2. In contrast, *FAD3+* mitochondria showed a very high level of 18:3, leading to a high DBI value (3.8). Both fad mutant lines share the characteristic of having significantly reduced amounts of 18:2.

In order to evaluate the impact of cold stress on membrane lipid composition, cells were exposed to 9°C for 3 d (Fig. 2). As expected, such a stress triggered an increase in the degree of unsaturation of mitochondrial lipids in the three cell lines, but to a much lower extent in both mutants. As a consequence, *fad2* mitochondria remained less unsaturated (DBI = 2.6) than WT and *FAD3+* mitochondria (DBI = 4.0 and 4.2, respectively).

Membrane lipid dynamics

Because we wanted to know whether the changes in membrane lipid composition were accompanied by modification of the temperature dependence of the membrane physical state, we probed the lateral diffusion of lipids within the bilayer of mitochondrial membranes using a pyrene-labeled fatty acid at different temperatures between 35 and 5°C, in three independent preparations of mitochondria for each genotype (Table 1). As expected, lipid lateral diffusion slowed down exponentially as the temperature decreased. The activation energy (*Ea*) of the motion, which directly governs its dependence upon the temperature, can be obtained by re-plotting the data in an Arrhenius plot. Data reported in Table 1 show that lateral diffusion in *fad2* mitochondria was more dependent on temperature than in WT mitochondria and in *FAD3+* membranes. These data clearly suggest that the rigidification upon a temperature downshift was more pronounced in *fad2* mitochondria and less important in *FAD3+*, when compared with WT mitochondria.

In this work, we also assayed a second biophysical parameter related to membrane fluidity of mitochondrial membranes, i.e. local microviscosity (Cantrel et al. 2000, Caiveau et al. 2001) determined at one specific depth of the membrane using the fluorescence anisotropy of the anthroyloxy fatty acid labeled at the 12th position (Table 1). The results showed that fluorescence anisotropy was higher in both *fad2* and *FAD3+* mitochondria isolated from cells cultured at 22°C. However, after exposure of the cells to cold, microviscosity remained almost unchanged in the WT whereas it was considerably enhanced in *fad2* mitochondria and, to a lesser extent, in *FAD3+*. As a consequence, the membrane microviscosity of mitochondria isolated from cells grown under cold temperature was lower in *FAD3+* than in *fad2*. Because membrane microviscosity results from both modification of the [18:3 + 16:0] content and membrane protein density, as previously suggested (Caiveau et al. 2001), these features of *fad2* and *FAD3+* cells reported here can probably be related to the low level of unsaturation in *fad2* and to the high protein to lipid ratio in *FAD3+*.

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Table 1 Biophysical and biochemical properties of mitochondrial membranes isolated from WT, *fad2* and *FAD3+* cells grown at 22°C or submitted to 9°C for 3 d

	Ea LD	Microviscosity[a]		[18:3 + 16:0]		Protein/lipid ratio	
Genotype	22°C	22°C	9°C	22°C	9°C	22°C	9°C
WT	18 ± 4	0.104	0.095	53.5	64.5	1.92	2.27
fad2	33 ± 3	0.143	0.284	32.7	32.3	1.41	2.38
FAD3+	11 ± 2	0.147	0.191	72.1	80.7	2.27	2.22

The activation energy of lipid lateral diffusion (*Ea* LD; expressed in kJ mol^{-1}) and protein (mg)/lipid (mg) ratio were determined as described in Materials and Methods. The [18:3 + 16:0] contents (%) were calculated from data presented in Fig. 2.
[a] Fluorescence anisotropy.

Table 2 Cytochromic respiration parameters of mitochondria isolated from WT, *fad2* and *FAD3+* suspension-cultured cells grown at 22°C or submitted to 9°C for 3 d

	State 3		State 4		RCR	
Genotype	22°C	9°C	22°C	9°C	22°C	9°C
WT	178 ± 7	143 ± 16	113 ± 5	119 ± 7	1.6	1.2
fad2	162 ± 11	116 ± 15	62 ± 6	53 ± 24	2.6	2.2
FAD3+	147 ± 15	144 ± 15	81 ± 12	95 ± 28	1.8	1.5

Oxidation rates were measured in state 3 and state 4 using a combination of succinate plus NADH as respiratory substrate, in the presence of ATP, and the respiratory control ratio (RCR) was calculated from these values. Data are the average ± SD of three or more independent mitochondrial preparations.

Respiration and AOX capacity

To evaluate the effects of the modifications of membrane lipid composition on mitochondrial respiration, we measured the oxidation rates in the phosphorylating state (state 3) and in the non-phosphorylating state (state 4) using succinate plus external NADH as respiratory substrates, which, in combination, allowed the highest rates of respiration in *Arabidopsis* mitochondria. As shown in Table 2, state 4 oxidation rates of mitochondria isolated from cells grown at 22°C were reduced in both *fad2* and *FAD3+* cells compared with the WT, whereas state 3 oxidation rates were not significantly different in the three cell lines. This is particularly evident for *fad2* mitochondria, resulting in a higher respiratory control ratio (RCR) in this genotype. It has to be mentioned that, in mitochondria of the three cell lines, the state 3 respiration and the maximal rates of the cytochromic pathway measured in the presence of propyl gallate to inhibit the alternative pathway were not significantly different, suggesting that the AOX was not activated under these conditions (data not shown). The exposure of cells to low temperature induced a decrease in the respiratory rates under phosphorylating conditions of WT and *fad2* mitochondria, but not in *FAD3+*. In any case, state 4 respiration rates remained unchanged.

The AOX capacity was determined with the same respiratory substrates (succinate plus external NADH) in the presence of KCN to inhibit the cytochrome pathway, whereas dithiothreitol (DTT) and pyruvate were added to activate AOX fully (Fig. 3A). In cells grown at 22°C, the AOX capacity in WT mitochondria was two (*FAD3+*) to four (*fad2*) times higher than in the two mutants. When cells were transferred to 9°C, AOX capacity changes were important in both mutants (increased by a factor of two in *FAD3+* and a factor of three in *fad2*) but moderate in WT cells (increased by about 25%). In any conditions, the maximal AOX capacity of both fad genotypes remained lower than that of the WT.

AOX expression

To check if the increase in the AOX capacity resulted from an accumulation of AOX protein, we undertook its immunodetection with antibodies raised against the *Sauromatum guttatum* AOX (Elthon et al. 1989). Using β-mercaptoethanol to reduce AOX fully, a single band of about 35 kDa was detected by the antibodies in isolated mitochondria. In cells grown at 22°C, AOX could only be detected in WT mitochondria, but exposure to low temperature resulted in an important increase of AOX protein, both in the WT and in the two fad lines (Fig. 3B). This result was consistent with the AOX capacity changes detailed above. However, although mitochondria from *fad2* and *FAD3+* cells grown at 22°C displayed measurable AOX

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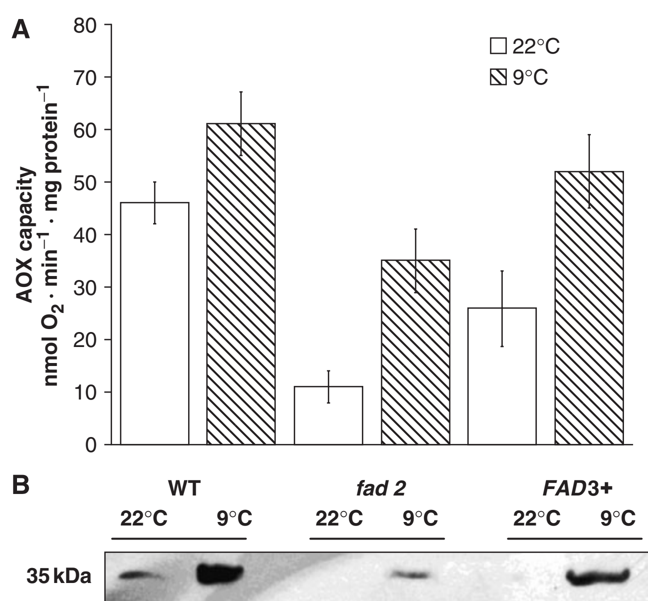

Fig. 3 Alternative pathway capacity and immunodetection of AOX proteins in mitochondria isolated from WT, *fad2* and *FAD3+* cells cultured at 22°C or submitted to 9°C for 3 d. (A) AOX capacities were determined as described in Materials and Methods, and are the average of three or more independent mitochondrial preparations. Control cells (white), cold-treated cells (gray). (B) Immunodetection of AOX proteins in isolated mitochondria. In each lane, 40 µg of total mitochondrial proteins were separated by SDS–PAGE, transferred to a membrane and hybridized with anti-AOX-specific antibodies.

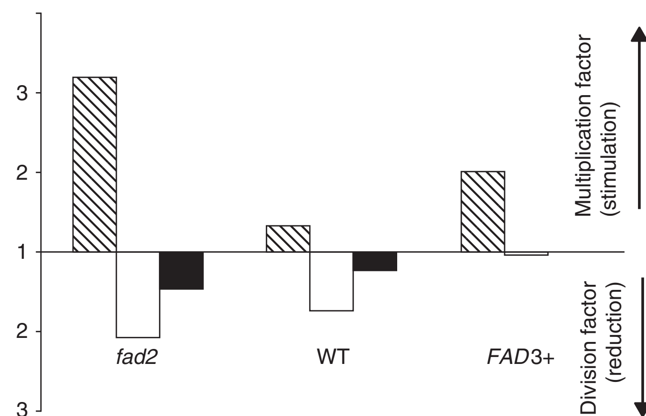

Fig. 4 Relative changes in AOX capacity (gray columns), cell growth (white columns) and state 3 respiration rate (black columns) between control (22°C) and cold-treated (9°C) WT, *fad2* and *FAD3+* cells. The basal value measured at 22°C in each case is represented by the number 1. Numbers above or below this basal value signify induction (multiplication factor) or reduction (division factor), respectively.

activities, we failed to detect the protein by immunoblots, indicating that the amounts of AOX were below the detection limit in these genotypes.

In order to analyse the relationships between the changes of mitochondrial respiration in response to low temperature, the lipid composition of mitochondria and the degree of cold tolerance of WT, *fad2* and *FAD3+* cells, we determined the relative changes of three parameters in control and cold-treated cells (Fig. 4): cell growth, mitochondrial state 3 respiration and AOX capacity. Under the conditions used in this study, *FAD3+* cell growth was not significantly affected by cold, indicating that *FAD3+* cells were actually more tolerant to cold stress than the wild-type cells and that *fad2* cells were highly sensitive to low temperature. Interestingly, the changes in phosphorylating oxidation rates in response to cold temperature were much more important in *fad2* mitochondria and, to a lesser extent, in WT than in *FAD3+*. This result suggests that the extent of reduction of the respiration rate in the phosphorylating state is positively related to the cold sensitivity of cells. In contrast, greater changes in AOX capacity were found in both mutants compared with WT mitochondria, indicating that there are no direct relationships between the cold resistance of cells and their ability to enhance their AOX capacity.

In order to define which *AOX* gene was expressed in Arabidopsis cell suspensions and to investigate if the increase in AOX protein content observed was regulated at the transcriptional level, gene expression was analysed by semi-quantitative reverse transcription–PCR (RT–PCR) using specific pairs of primers for each gene as described in Materials and Methods. In *Arabidopsis*, AOX proteins are encoded by five genes subdivided into two groups: *AOX1a–AOX1d* and *AOX2* (Saisho et al. 1997). The recently identified *AOX1d* member (Thirkettle-Watts et al. 2003) was not considered in our experiments. Since oxygen consumption was shown to decrease in response to low temperature, we also checked the expression of a gene specific to the cytochrome pathway, the *COXII* gene that encodes subunit II of the cytochrome c oxidase which, in Arabidopsis, is present in the mitochondrial genome. The expression of a constitutive gene coding for a ribosomal protein (*S24*) was also used as internal standard for RT–PCRs.

The expression of the four *AOX* (*AOX1a*, *AOX1b*, *AOX1c* and *AOX2*) and *COXII* genes was first analyzed in 3-day-old WT, *fad2* and *FAD3+* cells grown at 22°C (at time zero before the beginning of the cold treatment). Fig. 5A shows that among the four *AOX* genes tested, only *AOX1a* and *AOX1c* are expressed in Arabidopsis cells, under our experimental conditions. *AOX1a* transcripts were present in WT cells, almost absent in *fad2* and undetected in *FAD3+*, whereas *AOX1c* is expressed in the three cell lines

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but at higher levels in WT cells. The expression of the remaining *AOX* genes, *AOX1b* and *AOX2*, was not detected. The steady-state level of *COXII* transcripts was similar in the three cell lines.

In order to investigate how the expression of each *AOX* and *COXII* gene was modulated during the 3 d for which the low temperature treatment lasted, we prepared RNA from cells exposed for 1, 2 or 3 d days at 9°C, as well as from the corresponding controls left at 22°C (Fig. 5B). In WT cells, the expression levels of *AOX1a* and *AOX1c* at normal growth temperature (22°C) decreased throughout the time course of the cell culture. Under these conditions, only *AOX1c* transcripts were detected in both *fad2* and *FAD3+* cells, and the expression of this gene also decreased with cell age at 22°C in both mutants as in the WT. Cold treatment caused a significant increase in the amounts of all the *AOX* transcripts in the three cell lines. Since control and cold-treated mitochondria were extracted from cells at day 3, the transcript levels detected at those time points (inside rectangles in Fig. 5B) should be compared with the AOX protein contents and capacity previously presented. As in control cells, the expression of *AOX1b* and *AOX2* was not detected whatever the cell line grown at 9°C. In contrast to *AOX*, the expression of *COXII* remained relatively unchanged throughout the culture period for the three genotypes, but was significantly reduced by low temperature in WT and *fad2* cells. Interestingly, in *FAD3+* cells, the expression of this gene was not affected by cold. The constitutive expression of the housekeeping gene *S24* shows that equal amounts of RNA were used as template for RT–PCRs.

Discussion

In the present work, we used the *fad2* and *FAD3+* cell lines of *A. thaliana* as a genetic model to investigate the role of fatty acid composition in plant response to cold treatment, namely in cell growth and in mitochondrial respiration properties, including AOX involvement, which has long been proposed to be an important factor allowing plants to tolerate chilling-induced damage (Purvis and Shewfelt 1993).

As mitochondrial lipids are mainly dependent on the eukaryotic pathway of lipid biosynthesis, including the fatty acid desaturases FAD2 and FAD3 associated with the endoplasmic reticulum, mitochondria isolated from *fad2* knockout and *FAD3+*-overexpressing cell lines exhibited completely opposite membrane lipid composition, as expected. Data reported in this paper show that, whereas *fad2* mitochondria have an extremely low content in PUFAs, as previously reported (Caiveau et al. 2001), mitochondria from transgenic cells overexpressing linoleate desaturase (*FAD3+*) were highly unsaturated, especially in

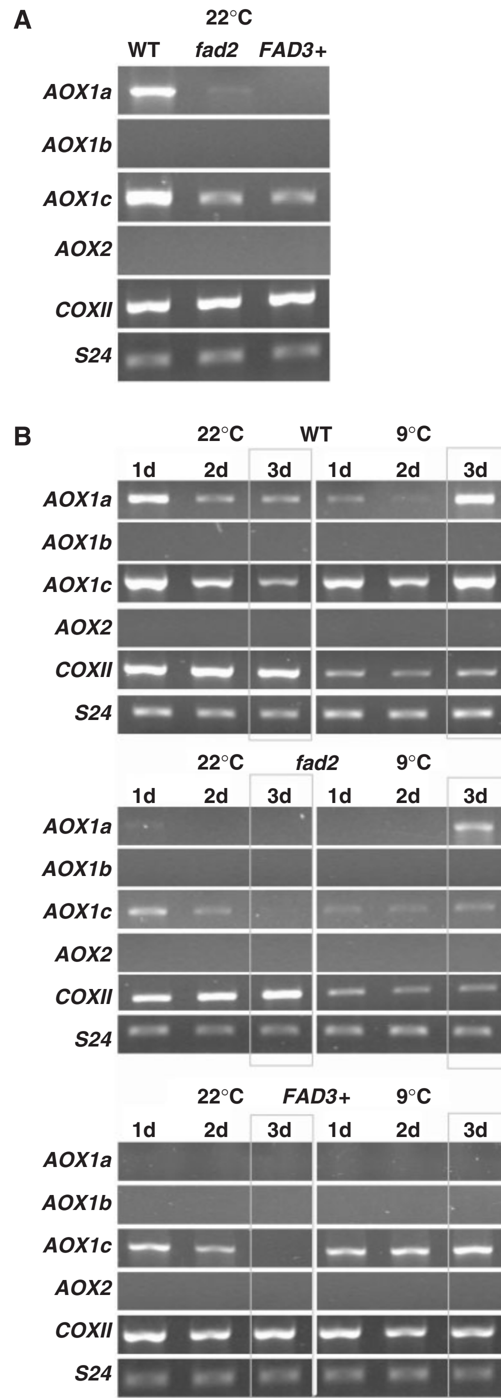

Fig. 5 Gene expression in WT, *fad2* and *FAD3+* suspension-cultured cells. *AOX1c*, *COXII* and *S24* were amplified by 30 cycles of PCR using 100 ng of DNase-digested RNA as template, and *AOX1a*, *AOX1b* and *AOX2* were amplified by 40 cycles using 200 ng of RNA as template. (A) Comparison of gene expression between 3-day-old WT, *fad2* and *FAD3+* cells grown at 22°C. (B) Comparison of gene expression between cells grown at 22°C or submitted to 9°C for 3 d. Control and cold-treated cells were harvested 1 (1d), 2 (2d) or 3 (3d) days after the beginning of the treatments for RNA extractions.

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terms of linolenic acid content, thus having a high DBI. When cells were exposed to 9°C for 3 d during their growth cycle, the most important changes in membrane fatty acid composition were observed in WT mitochondria which showed an increase in the PUFA content (18:2 and 18:3) associated with a reduction in palmitic acid. To a much lesser extent, comparable changes were observed in both *fad2* and *FAD3+* cells. Indeed, despite the lack of the ω-6-oleate desaturase in *fad2* cells, significant amounts of 18:3 could be detected in purified mitochondria from this mutant, reinforcing previous observations made by Miquel and Browse (1992) which suggest that considerable two-way exchanges of lipids occur between the chloroplast and the extrachloroplastic compartment in *A. thaliana*. Equally interesting is the fact that the level of 18:3 in mitochondria isolated from *FAD3+* was further enhanced after cold exposure.

The drastic changes in membrane lipid composition were accompanied by modifications of the temperature dependence of the membrane physical state. Based on the determination of the activation energy of lipid lateral diffusion, it appears that *fad2* mitochondria exhibit a more important rigidification upon a temperature downshift than WT mitochondria, whereas rigidification is less important in *FAD3+* mitochondria. It is interesting to note that comparable data were obtained with the plasma membrane isolated from the same Arabidopsis cell lines (Vaultier et al. 2006). These results indicate that the activation energy of lateral diffusion that characterizes the biophysical properties of the membrane in *FAD3+*-overexpressing cells is a good indicator of their resistance to cold temperature. In contrast, the opposite variations of microviscosity (fluorescent anisotropy parameter) in WT and *FAD3+* mitochondria suggests that factors other than the 18:3 + 16:0 content and protein to lipid ratio may influence this parameter. Possible explanations for apparent higher microviscosity in *FAD3+* mitochondria could be a tight packing of membrane lipids or the presence of an endogenous quencher leading to changes in fluorescence anisotropy in this genotype. Taken together, the present data on both membrane microviscosity and lipid lateral diffusion can probably be related to the higher cold resistance or sensitivity of the cell growth after a 3 d exposure, in *FAD3+* and *fad2*, respectively.

In Arabidopsis, as in the other plants studied so far, AOX proteins are encoded by a small gene family (Considine et al. 2002), displaying distinct expression patterns in different organs (Tirkettle-Watts et al. 2003) and in response to stresses (Lacomme and Roby 1999, Huang et al. 2002, Clifton et al. 2005). Our results indicate that *AOX1a* and *AOX1c* are the two isoforms expressed in Arabidopsis cell suspensions, which is in good agreement with a recent paper describing 'Genevestigator' analysis of all the AOX genes in such a material (Clifton et al. 2006). In addition, those microarray-based studies indicated that *AOX1a* was the most stress responsive gene, while *AOX1c* was unresponsive in suspension cells. Interestingly, we show that both *AOX1a* and, for the first time, *AOX1c* are stimulated by cold in cultured Arabidopsis cells. Both the level of *AOX* transcripts and AOX protein were much lower in *fad2* and *FAD3+* mitochondria isolated from cells grown at 22°C, clearly indicating transcriptional (or transcript stability) regulation, although changes in protein translation level or integration into the mitochondria inner membrane cannot be excluded. In contrast, we showed that the expression of the *COXII* gene, encoded by the mitochondrial genome, was similar in the three genotypes. Thus, the low level of AOX expression in mutants appears to be an indirect consequence of mutation (*fad2*) or insertion (*FAD3+*) of desaturase genes, thus indirectly related to the different fatty acid composition of the membranes, and not necessarily connected to the resulting cold tolerance or cold sensitivity of mutants, as characterized by large differences in cell growth rates at low temperature.

Are the changes in AOX expression and activity strictly correlated to the development of cold tolerance, as evidenced by changes in membrane fatty acid composition and 'fluidity', or are these changes independent events? Desaturase mutants provide an interesting tool to answer such a question since they exhibit limited capacities to adjust their membrane composition. Our results show that *FAD3+* cells were not affected by temperature in their growth yield and in state 3 oxidation rates, whereas *fad2* and WT cells displayed lower fresh weights and lower state 3 oxidation rates after exposure to 9°C for 3 d, this effect being more pronounced in *fad2* cells. Accordingly, *FAD3+* cells exhibited cold resistance whereas *fad2* and WT cells appear sensitive and tolerant to cold, respectively. Data on *FAD3+* differ from published data concerning *Nicotiana tabacum* plants overexpressing the *FAD3* gene (Hamada et al. 1998) that did not display significantly increased tolerance to cold. On the other hand, our results on *fad2* cells which showed the most important decrease in fresh weight and in state 3 oxidation rate were in accordance with another work (Miquel et al. 1993) in which an increased susceptibility to low temperature of *A. thaliana fad2* plants was demonstrated. The *fad2* phenotype can be related to the high microviscosity of the membrane and the high dependency of lipid movements upon a downshift of temperature found in its mitochondria. These typical features could be responsible, at least in part, for the reduction of the respiratory activities (cf. Tables 1 and 2). On the other hand, the resistance to cold of *FAD3+*-overexpressing cells can be related to the activation energy of lateral lipid diffusion found in their membranes.

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It has been previously observed that chilling temperatures impair the respiratory activity of the cytochrome pathway and that the cytochrome c oxidase activity and the amount of COXII protein are reduced by cold (Prasad et al. 1994, Davy De Virville et al. 2002). In the present work, the expression of the *COXII* gene, encoded by the mitochondrial genome, was very stable throughout the culture period studied whatever the genotype. However, an important decrease was observed in cold-treated WT and *fad2* cells, while the transcript levels in *FAD3+* remained stable. Such expression patterns were well correlated with the cytochrome pathway capacities measured in isolated mitochondria, suggesting that this complex IV subunit could be the limiting element regulating the cytochrome pathway and might be one of the determinant features in the reduction of the state 3 oxidation rates observed in *fad2* and WT mitochondria after cold exposure.

Besides growth and respiratory rate changes in response to cold treatment, we checked how AOX capacity responded to low temperature in fad mutants. We observed an increased AOX capacity, well correlated with protein accumulation, after 3 d of exposure to 9°C in the three cell lines. This result is reported here for the first time in *A. thaliana* cell cultures. Actually, AOX activity induction, as well as protein or transcript accumulation, has often been reported after cold treatments in several plant species (Elthon et al. 1986, Mc Nulty and Cummins 1987, Stewart et al. 1990, Vanlerberghe et al. 1992, Ito et al. 1997, Gonzalez-Meler et al. 1999, Kreps et al. 2002, Takumi et al. 2002), while in other plants authors failed to detect such induction (Gonzalez-Meler et al. 1999, Svensson et al. 2002, Taylor et al. 2005). Thus the importance of AOX involvement in the response of plants to cold is questionable. In the particular case of Arabidopsis, in a study carried out recently with *AOX1a* antisense transgenic plants, AOX was shown to play a role in acclimation of shoot growth at low temperature (Fiorani et al. 2005). In the present work, our results showed a higher maximal AOX capacity in WT cells when cultured at 22 or 9°C than in the chilling-resistant *FAD3+* cells and the chilling-sensitive *fad2* cells. On the other hand, the *fad2* genotype exhibited the most important AOX capacity induction by cold compared with WT and *FAD3+* cells. In *fad2* cells, our results clearly indicate that there is a positive relationship between cold sensitivity and AOX capacity induction, whereas the lower AOX capacity induction observed in *FAD3+* cells suggested that AOX induction is not crucial in developing cold tolerance in Arabidopsis cells. In accordance with our results, other authors found that the electron partitioning to the alternative pathway in response to low temperature was significantly increased in a stressed chilling-sensitive maize cultivar compared with a tolerant cultivar, suggesting again that the increased activity of AOX might be related mostly to chilling susceptibility of plants rather than their tolerance (Ribas-Carbo et al. 2000).

In conclusion, we have shown that modifications in the PUFA content of mitochondrial membranes affected functional properties of this organelle when *A. thaliana* cells are grown at 22°C. Interestingly, as shown by the analysis of the fresh weight and the respiratory rates of *FAD3+* cells, increased amounts of 18:3 associated with low thermal dependency of the lipid lateral diffusion favored cold resistance, whereas both the drastic reduction of PUFAs (18:2 + 18:3) in *fad2* cells, and the inability of this genotype to synthesize 18:3 in response to cold stress, increased both membrane rigidity and cold sensitivity. Moreover, AOX capacity was shown to be cold stimulated in the three cell lines, independently of the mitochondrial membrane lipid composition, changes in lipid dynamics or the cytochrome pathway rate variation. The increase in AOX capacity seemed to result from an increase in the steady-state levels of AOX proteins, mainly driven by *AOX1a* and *AOX1c* transcript accumulation in cells, and might function to prevent the eventual mitochondrial damage induced by low temperature, especially in cold-sensitive cells.

Materials and Methods

Plant material

The initial cell suspension culture of WT *A. thaliana* L. (Heynh) ecotype Columbia, cultured for >10 years in our laboratory, was provided by Dr. M. Axelos (CNRS, Toulouse). The *fad2* genotype was obtained from the lipid mutant library established by Browse and Sommerville (Lemieux et al. 1990), and the *FAD3+* genotype (line 4–2) was constructed by Arondel et al. (1992). Cell suspension cultures of *fad2* and *FAD3+* genotypes were established in the laboratory. Cells were subcultured weekly by transferring 25 ml aliquots of cell suspension into 500 ml Erlenmeyer flasks containing 200 ml of fresh medium. Cells were grown under continuous light (100 μmol $m^{-2} s^{-1}$) at 22°C in an orbital shaker (140 r.p.m.). The culture medium (Gamborg B5) was bought from Duchefa, France. The cell growth of the different genotypes was monitored by fresh weight measurements of cell suspensions cultured in a set of Erlenmeyer flasks containing 25 ml of medium. Weight was determined throughout the culture cycle, after eliminating the culture medium by centrifugation at low speed.

Low temperature treatment

Twenty Erlenmeyer flasks containing 200 ml of cell culture were kept at 22°C for 3 d after subculture. After this period, half of the flasks were placed in a grown chamber at 9°C for 3 d (cold-treated), with the same light and agitation conditions, while the remaining 10 flasks were left at 22°C for a similar time period (control). For RNA extractions, cells were harvested 1, 2 and 3 d after the beginning of the treatment from control and cold-treated groups. Mitochondria were extracted simultaneously from both groups of cells at day 6 after subculture. The effect of cold stress on cell growth was estimated by fresh weight determination of the cells

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pooled from the 10 flasks (2 liters) before extraction of mitochondria.

Preparation of mitochondria

Mitochondria from control or cold-treated cells were extracted simultaneously from 100–300 g of 6-day-old suspension cultures and purified on Percoll gradients according to Davy de Virville et al. (1998). Mitochondria were used immediately for functional measurements or kept at −20°C for further analysis. Based on galactolipid content (used as a marker of plastidial membranes), the purity of the mitochondrial fraction was higher than 95%. The outer membrane integrity of fresh mitochondria was higher than 90%, as deduced from cytochrome c oxidase activity measurements (Davy de Virville et al. 1998). Submitochondrial particles used to determine the protein to lipid ratios were prepared as previously detailed (Caiveau et al. 2001). Protein concentration was determined according to Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

Lipid analysis

Mitochondrial lipids were extracted according to Folch et al. (1957), re-suspended in ethanol : toluene (1 : 4, v/v) and stored under an N_2 atmosphere at −20°C until used. Methyl esters were prepared by transmethylation of fatty acids with methanol : sulfuric acid (97.5 : 2.5, v/v) at 70°C for 60 min and analyzed by gas chromatography (Varian chromatograph model 3300) at 210°C, on a fused-silica capillary column (50×0.3 mm) coated with Carbowax 20 M. Quantitative analysis of fatty acid methyl esters was carried out using methyl heptadecanoate (C 17:0) as an internal standard.

Lipid dynamics

The lateral diffusion of the excimer-forming probe, 1-pyrenedodecanoic acid (Molecular Probes), was determined at different temperatures (from 5 to 35°C) in purified mitochondrial membranes according to Cantrel et al. (2000). Aliquots of membranes (100 nmol phospholipids) were suspended in 1.2 ml of 20 mM MOPS, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM KCl (pH 7.2) medium. Successive additions of the probe were made to give a molar ratio (probe/lipids) varying from 0.001 to 0.13.

Microviscosity was assayed by measuring fluorescence depolarization of 12-anthroyloxy palmitic acid (Molecular Probes) embedded in mitochondrial membranes. The probe to phospholipid molar ratio was 0.02. The samples were incubated in the presence of probe at 20°C for 20 min before measurement.

Oxygen uptake

Oxygen consumption was measured at 20°C using a Clark-type oxygen electrode. Air-saturated electrode medium contained 0.3 M mannitol, 10 mM phosphate buffer pH 7.2, 10 mM KCl, 5 mM $MgCl_2$ and 1 g l^{-1} fatty acid-free BSA (Sigma). Mitochondria (0.1–0.4 mg protein ml^{-1}) were incubated with 200 μM ATP. Oxidation was triggered by addition of succinate (10 mM) plus NADH (1 mM) in the presence (state 3) or absence (state 4) of ADP (from 25 to 100 μM). AOX capacity was determined in the same mediun containing KCN (1 mM), using succinate plus NADH as respiratory substrates and in the presence of DTT and pyruvate (1 mM each) to activate AOX fully, which was completely inhibited by subsequent addition of propylgallate.

Western blot analysis

Mitochondria were suspended in Laemmli buffer containing freshly added β-mercaptoethanol to reduce AOX fully. Proteins were separated by SDS–PAGE in 11% (v/v) polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad) in a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) for 30 min at 135 mA using transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3). The anti-AOX monoclonal mouse antibody (Elthon et al. 1989) was used at a dilution of 1 : 200. The secondary antibody was an anti-mouse horseradish peroxidase conjugate. Protein bands were detected with a chemiluminescence detection kit (Renaissance, NEN, USA).

Gene expression analysis

Analysis of gene expression was performed by semi-quantitative RT–PCR. For RNA extractions, cells were filtered, immediately fixed in liquid nitrogen and stored at −80°C until use. RNA was extracted as described by Verwoerd et al. (1989). After DNase digestion (RNase-free DNase, Sigma), RNA concentration was determined with a spectrophotometer and adjusted to 20 ng μl^{-1}. RNA dilutions were further checked by electrophoresis using the ImageQuant software. RT–PCR was performed with the OneStep RT–PCR kit (Qiagen). The number of PCR cycles and amounts of template were adjusted for each gene in order to avoid saturation. Primer sequences used to amplify *AOX* genes were those previously used by Saisho et al. (1997). For *COXII* and *S24*, the following primers were used: COXIIfw, TGATTGTTCTAAAATGGTTATTCCTC; COXIIrev, ATTA ATTGATTGGATACCCGAGAAC; S24fw, TCCAGGAGC AGTTCGTTATTGATG; S24rev, TCACTTCTTCTTGGCAT CACCAG.

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