The Plant Journal (2001) **26**(2), 171–180

The male sterile *G* cytoplasm of wild beet displays modified mitochondrial respiratory complexes

Eric Ducos[1,2], Pascal Touzet[1,3] and Marc Boutry[2]
[1]*Laboratoire de Génétique et Evolution des Populations Végétales, UPRESA-CNRS 8016, FR CNRS 1818, Université de Lille I, F-59655 Villeneuve d'Ascq cedex, France,*
[2]*Unité de Biochimie Physiologique, Université Catholique de Louvain, B-1348 Louvain la Neuve, Belgium, and*
[3]*Institut Agro-Alimentaire de Lille (IAAL) Université de Lille I, F-59655 Villeneuve d'Ascq cedex, France*

Received 13 October 2000; revised 5 February 2001; accepted 9 February 2001.
*For correspondence (fax +33 3 20 43 69 79; e-mail pascal.touzet@univ-lille1.fr).

Summary

Cytoplasmic male sterility (CMS) in higher plants has been mainly studied in cultivated species. In most cases, pollen abortion is linked to the presence of an additional mitochondrial polypeptide leading to organelle dysfunction in reproductive tissues. In wild beet, both CMS and hermaphrodite plants coexist in natural populations. The *G* cytoplasm is widely distributed along the Western European coast, and previous genetic studies have demonstrated that this cytoplasm confers male sterility in beet. In the present study, we have identified two mutations of *G* mitochondrial genes, each of which results in the production of a respiratory chain complex subunit with an altered molecular weight; the NAD9 subunit has a C-terminal extension while the COX2 subunit has a truncated C-terminus. NADH dehydrogenase activity was unchanged in leaves, but cytochrome *c* oxidase activity was reduced by 50%. Moreover, Western blot analyses revealed that alternative oxidase was more abundant in male sterile *G* plants than in a fertile control (*Nv*), suggesting that this alternative pathway might compensate for the cytochrome *c* oxidase deficiency. Implications of respiratory chain changes and a putative link with CMS are discussed.

Keywords: Cytoplasmic male sterility, respiratory chain, mitochondria, wild beet, cytochrome c oxidase, NADH:ubiquinone oxidoreductase.

Introduction

The main function of mitochondria in eukaryotic organisms is the production of energy through oxidative phosphorylation. The electrochemical gradient produced by proton translocation through the mitochondrial inner membrane is used for ATP production by the mitochondrial ATP synthase. As oxidative phosphorylation supplies most of the cellular ATP, changes in the respiratory chain are very deleterious to the organism. Plant mitochondria have several characteristics. For instance, they contain additional NAD(P)H dehydrogenases and a cyanide-resistant pathway in the respiratory chain. The plant mitochondrial genome is specific in that it contains large non-coding regions and several open reading frames, and has high recombinational activity.

Cytoplasmic male sterility (CMS), a particular mitochondrial dysfunction linked to a mutation of the mitochondrial genome, has been seen in over 150 plant species (Kaul, 1988). It is a maternally inherited trait that leads to abortion of pollen development without affecting the vegetative developmental stages of the plant. Fertility can be phenotypically restored by nuclear restorer genes. Because of its agronomic interest, the molecular mechanism of CMS has been identified and characterized in several cultivated species, such as maize (Dewey *et al.*, 1987), petunia (Nivison and Hanson, 1989), sunflower (Horn *et al.*, 1991), common bean (Johns *et al.*, 1992) and *Brassica* (Grelon *et al.*, 1994; Singh and Brown, 1993). In most cases, male sterility is linked to a mitochondrial dysfunction associated with the presence of an additional chimeric gene coded by the mitochondrial genome (Schnable and Wise, 1998). For instance, the chimeric T-*urf13* gene of maize male-sterile T cytoplasm encodes a 13-kDa pore-forming protein which is assembled as an oligomer in the mitochondrial inner membrane (Levings, 1993) and leads to organelle breakdown in tapetum cells and thus to male gametogenesis abortion.

In a few species, the mitochondrial dysfunction leading to male sterility can be linked more directly to alterations in the expression of mitochondrial genes implicated in respiratory chain. For example, in sorghum CMS, subunit 6 of the mitochondrial ATP synthase (*atp6*) seems to be involved in male sterility (Howad and Kempken, 1997). Whereas editing of CMS-*atp6* transcripts in the anther is strongly reduced compared to that in fertile plants, RNA editing of these transcripts increases following fertility restoration (Pring *et al.*, 1999). In rice, an additional copy of *atp6* (B-*atp6*) in CMS cytoplasm seems to be involved in the expression of the male sterile phenotype (Akagi *et al.*, 1994). Restoration of male fertility is associated with increased editing and processing of B-*atp6* transcripts (Iwabuchi *et al.*, 1993). Although there is no definitive evidence linking pollen disruption to the respiratory chain dysfunctions, the involvement of these modified genes in male sterility is strongly suggested, as their expression is altered in a nuclear restorer context.

In sugar beet (*Beta vulgaris* ssp. *vulgaris*), the best studied CMS is the *Owen* type. This cytoplasm has been used worldwide for decades in breeding programs as a unique source of CMS (Owen, 1945). Although a restoration effect has been described on *coxI* transcripts in bud flowers (Kubo *et al.*, 1999), the mechanism inducing pollen abortion remains unclear. New CMS sources can be obtained from natural populations of sea beet (*Beta vulgaris* ssp. *maritima*) (Cuguen *et al.*, 1994). The male-sterile *G* cytoplasm (also called *R*) was first identified in 1970 in the Canche estuary (northern France) and was introduced as an alternative CMS in sugar beet breeding programs (Saumitou-Laprade *et al.*, 1993). Population genetics studies have revealed that this male sterile cytoplasm is widely spread in wild beet populations; this has been documented on the French coast (Cuguen *et al.*, 1994), and, more recently, in the rest of Western Europe (Desplanque *et al.*, 2000).

In this study we present a molecular study of CMS *G* and show that it exhibits two modified respiratory chain complex subunits: NAD9 and COX2. While NADH dehydrogenase activity is unchanged, cytochrome *c* oxidase activity is reduced in sterile *G* plants. We believe that this peculiar cytoplasm is the first described plant mitochondrial mutant affecting the respiratory chain to be maintained in natural populations.

Results

In organello *labeling of mitochondrially encoded proteins*

Following *in organello* ^{35}S-labeling of isolated mitochondria, SDS-PAGE and autoradiography clearly revealed the presence of three variant labeled products in the *G* cytoplasm (Figure 1a). No additional or missing bands were found in the sterile cytoplasm. The molecular weights and intensities of the variant proteins suggested a relationship between these three *G* variants and three *Normal vulgaris (Nv)*-labeled products (respective apparent molecular weights: *Nv* 25, 32, and 34 kDa; *G* 27, 31, and 33 kDa). Pulse-chase labeling showed that, after chase, the intensity of the 34 kDa (*Nv*) and 33 kDa (*G*) bands decreased markedly, while that of the 32 kDa (*Nv*) and 31 kDa (*G*) bands increased (Figure 1b), suggesting that the 34 and 33 kDa bands could be precursors, respectively, of the 32 and 31 kDa proteins.

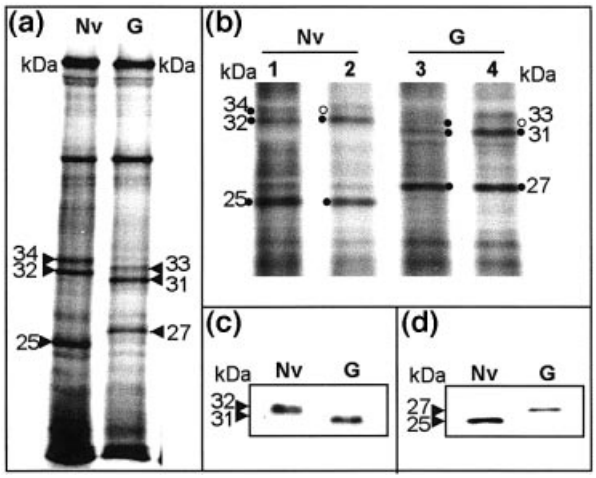

Figure 1. Analysis of variant mitochondrial products.
(a) Autoradiogram of *in organello* labeled polypeptides. Newly synthesized mitochondrial proteins, labeled *in organello* with [^{35}S]methionine were analyzed by 12–17% SDS-PAGE, electroblotted onto an Immobilon-P membrane, and autoradiographed. The arrowheads indicate size variants.
(b) Pulse-chase labeling of mitochondria. Mitochondria were labeled *in organello* for 7 min, then chased (lanes 2 and 4) or not chased (lanes 1 and 3) for 40 min with 2.5 mM cold methionine. The filled circles (●) indicate variant bands and the empty circles (○) bands the intensity of which decreased during chase.
(c) Western blot using anti-COX2 antiserum.
(d) Western blot using anti-NAD9 antiserum.

Identification of the labeled variant proteins

In beet, subunit 2 of the cytochrome *c* oxidase (COX2) is encoded by the mitochondrial genome (Senda *et al.*, 1991) and is translated as a precursor (preCOX2), which is cleaved at its N-terminus during assembly of the complex (Herrmann *et al.*, 1995). The molecular weights for preCOX2 and mature COX2 predicted from the DNA sequence are 29.5 and 27.8 kDa, respectively. The pulse-chase experiment described previously strongly suggested that the 34 and 33 kDa-labeled products are precursors of the 32 and 31 kDa proteins, respectively, leading us to think that the 32 kDa (*Nv*) and 31 kDa (*G*) polypeptides may

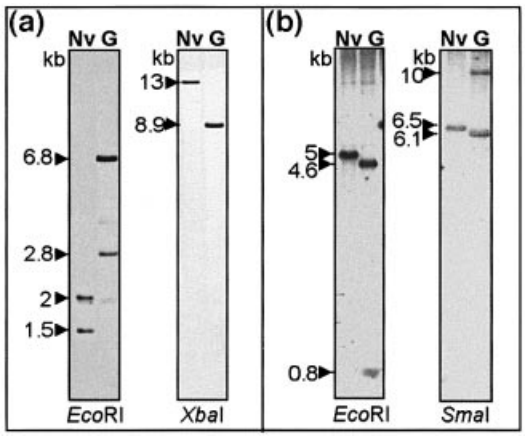

Figure 2. Southern blotting of total DNA from *Nv and G* plants using *cox2* or *nad9* probes.
Total DNA (20 µg) was digested with the indicated enzymes, electrophoresed, and transferred to a nylon membrane (Hybond N) for Southern blotting with a *cox2* (a) or *nad9* (b) probe. The molecular weights of the bands are indicated in kb.

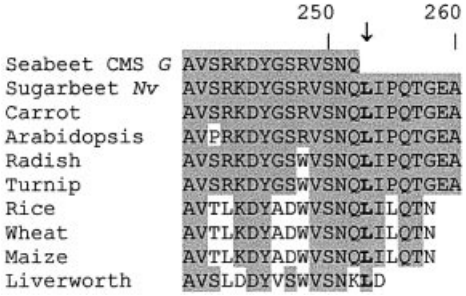

Figure 3. Amino acid sequence alignment of the predicted C-terminal sequence of COX2 from various species. Identical amino acids are shown in gray. The vertical arrow indicates the premature stop codon in *G* COX2. The highly conserved leucine[253] is shown in bold. The sequences are numbered from the first amino acid residue of beet COX2.

be COX2, a hypothesis confirmed by Western blotting using anti-COX2 antiserum (Figure 1c).

When probes for *nad6*, *cox3*, *atp6* and *nad9* mitochondrial genes encoding 20–30 kDa polypeptides were used in Southern blotting experiments, only the *nad9* probe gave a pattern, that differed between the two cytoplasms (data not shown). The predicted size of subunit 9 of NADH: ubiquinone oxidoreductase (NAD9) is 23 kDa (Kubo *et al.*, 1993), and, in the plant, NAD9 has an apparent molecular weight of about 25–30 kDa on SDS-PAGE (Lamatina *et al.*, 1993). Western blotting of a beet mitochondrial fraction using anti-NAD9 antiserum revealed that the 25 kDa (*Nv*) and 27 kDa (*G*) labeled products corresponded to NAD9 (Figure 1d).

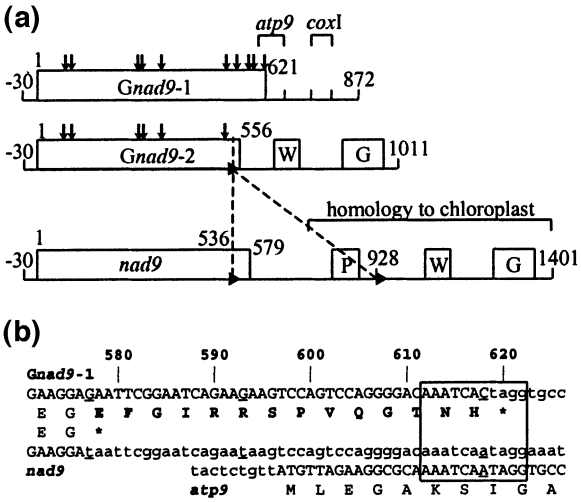

Figure 4. Genomic organization of the *Nv* and *G nad9* genes.
(a) Diagram showing the genomic clones. The nucleotides are numbered from the translation initiation codon of *nad9*. The vertical arrows indicate single point mutations in *G* compared with the *TK81-O* sequence (Kubo *et al.*, 1995). The horizontal brackets (⌐¬) indicate DNA segments homologous to *atp9, cox*1, or chloroplast DNA. The G*nad9*–2 deletion is indicated by the dotted lines, the horizontal arrowheads indicating 6 bp short repeats. The boxes labeled P, W and G represent the chloroplast *trnP-trnW-petG* gene cluster.
(b) Nucleotide and predicted amino acid sequences at the *nad9 and* G*nad9*–1 C-termini. Predicted translated nucleotides are shown in upper cases. Mutated nucleotides are underlined. The asterisk (*) indicates a stop codon. Additional amino acids in G*nad9*–1 are shown in bold. The open box represents 11 nucleotides common to G*nad9*–1, *nad9* and *atp9* genes.

These experiments show that the *G* COX2 subunit is smaller than the corresponding *Nv* product, while the converse holds for the NAD9 subunit.

Southern blot analysis of cox2 *and* nad9

Southern blotting of total DNA digested with *Eco*R1 using a *cox2* probe revealed two bands in both cytoplasms (Figure 2a, left). In the *Nv* cytoplasm, bands of 1.5 kb and 2 kb corresponded, respectively, to the 5′ and 3′ *cox2* extremities. This pattern has been previously described in several species and subspecies in the genus *Beta* (Senda *et al.*, 1995). CMS *G* shares the 6.8 kb fragment (*cox2* 5′ extremity) with the *Owen* CMS cytoplasm (Senda *et al.*, 1991), but the 2.8 kb fragment seen is specific to G*cox2 and* presumably corresponds to the 3′ extremity. These results suggest that both extremities of the G*cox2* gene are rearranged in the sterile *G* cytoplasm compared with *Nv*. *Xba*I digestion indicated that the *cox2* gene is present as a single copy in both cytoplasms (Figure 2a, right).

Southern blotting using an *nad9* probe indicated that, in the *G* cytoplasm, *nad9* is present as two copies (Figure 2b). This conclusion was confirmed by *Hind*III and *Xho*I digestions (data not shown). In all cases, one copy of *G*

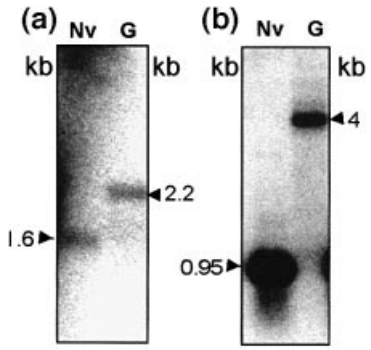

Figure 5. Northern blotting of *cox2* and *nad9* genes.
RNA (10 μg) was electrophoresed, transferred, and hybridized with the *cox2*-exon1 probe (a) or the *nad9* probe (b).

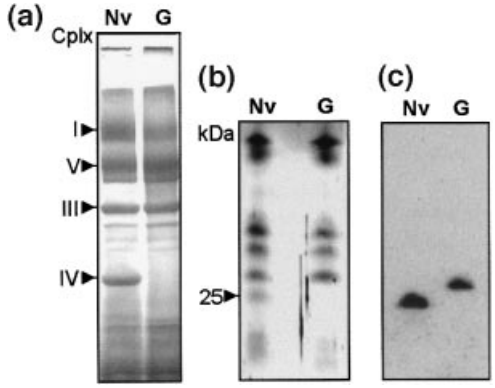

Figure 6. Analysis of mitochondrial protein complexes by blue native PAGE.
(a) Mitochondrial complexes were solubilized using *n*-dodecylmaltoside, electrophoresed on a 5–12% blue native polyacrylamide gel, and the proteins stained with Coomassie blue. The arrowheads indicate respiratory chain complexes.
(b) Subunits of complex I separated on the blue native gel in (a) were analyzed in a second dimension on SDS-PAGE, and silver stained. The arrowhead indicates the 25 kDa subunit.
(c) A gel similar to that shown in (b) analyzed by Western blotting using anti-NAD9 antiserum.

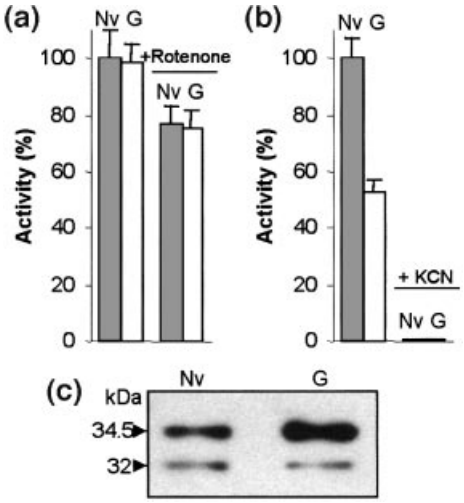

Figure 7. Enzymatic activity of mitochondrial complexes.
(a) NADH dehydrogenase activity was determined by recording cytochrome *c* reduction at 550 nm, as described in Experimental procedures, with NADH as substrate in the presence of KCN (100% value, 0.28 ± 0.036 μmol min^{-1} μg^{-1}). In the two right-hand lanes, 60 μM rotenone was added to specifically inhibit NADH: ubiquinone oxidoreductase.
(b) Cytochrome *c* oxidase activity was monitored by recording the oxidation of cytochrome *c* at 550 nm (100% value, 2.7 ± 0.19 μmol min^{-1} μ^{-1}g). In the two right-hand lanes, 1 mM KCN was added.
(c) Mitochondrial proteins (100 μg) were electrophoresed and analysed by Western blotting using anti-alternative oxidase antiserum.

nad9 was found on a fragment that was about 0.4 kb shorter than that bearing the *Nv nad9*.

Sequence analysis

In beet, the *cox2* gene consists of two exons coding for a 260 amino acid protein (Senda *et al.*, 1991). In the present study, the *G cox2* sequence obtained by PCR revealed four single point mutations in the intron and one in exon 2, in which the TTA codon encoding leucine[253] was changed to the STOP codon, TGA. As a result, the *G* COX2 subunit does not contain the 8 C-terminal amino acid residues, which are highly conserved in dicotyledonous species and include leucine[253], which is conserved across several distant species (Figure 3). The loss of these 8 amino acid residues explains the size difference between *Nv* and *G* COX2 on Western blots.

The two copies of *nad9* in the *G* cytoplasm (G*nad9*–1 and G*nad9*–2) were cloned by inverse PCR and sequenced (Figure 4a). Compared with *Nv nad9,* 10 single point mutations, 5 of which resulted in amino acid substitutions, were found in G*nad9*–1. One of these resulted in the substitution of leucine for phenyalanine[175], which is part of a highly conserved motif (QEFR) in NAD9 polypeptides. A T/G substitution of the *nad9* STOP codon resulted in extension of the coding sequence by 14 codons (Figure 4b). This gene was fused at its 3′ extremity with an 80-bp fragment sharing 95% homology with the 5′ extremity of the beet *atp9* gene (Xue *et al.*, 1989). An 11 nucleotide stretch, common to *nad9* and *atp9*, is probably involved in this recombination. Furthermore, a 50-bp fragment, 92% homologous with the *coxI* gene (Kubo *et al.*, 1999), was found 122 bp downstream of the G*nad9*–1 *orf*.

The G*nad9*–2 copy shared the same point mutations with G*nad9*–1 (Figure 4a), suggesting that the duplication of this gene occurred following mutations. G*nad9*–2 had the same genomic organization as *Nv nad9* (Kubo *et al.*, 1995) except for a 390-bp deletion in the 3′ extremity that includes the NAD9 C-terminal coding sequence. This 390 bp sequence is directly flanked by two short repeat

sequences (5′-TCGATT-3′), probably involved in the rearrangement leading to its deletion. The new *orf* putatively encodes a shorter 21.7 kDa polypeptide. Given the size of 25 kDa for *Nv* NAD9 as determined by Western blotting, the larger NAD9 (27 kDa) found in the *G* cytoplasm must correspond to the G*nad9*–1 product. No shorter product corresponding to G*nad9*–2 was seen.

Northern blot analysis of the cox2 *and* nad9 *transcripts*

Northern blotting using a *cox2*-exon1 probe revealed the presence of a single transcript in the *Nv* and *G* cytoplasms, the G*cox2* transcript being 0.6 kb larger than the *Nv cox2* transcript (Figure 5a). This was not surprising, since Southern blotting showed that modification occurred around this gene in *G* cytoplasm.

Although two copies of *nad9* were found in *G* cytoplasm, Northern blotting revealed only a single transcript which most probably corresponds to G*nad9*–1 (Figure 5b), since G*nad9*–2 would encode a smaller, rather than a larger polypeptide product. The G*nad9*–2 gene therefore seems not to be expressed at a detectable level, possibly as a result of the 390 bp deletion in its 3′ region which could destabilize the transcript (Stern *et al.*, 1991). Interestingly, *G nad9* mRNA is four times larger than *Nv nad9* mRNA, suggesting possible cotranscription of G*nad9* with another mitochondrial gene, as is occasionally the case, for instance, in wheat (Gualberto *et al.*, 1991).

Separation of mitochondrial complexes on blue native gels

The NAD9 and COX2 subunits belong, respectively, to complexes I and IV of the mitochondrial respiratory chain. These complexes were separated on blue native gels (Figure 6a), giving a pattern similar to that described for potato mitochondria (Jansch *et al.*, 1996). Complex I was found to be present in both the *G and Nv* cytoplasms. When this band was further analyzed by SDS-PAGE and Western blotting using anti-NAD9 antiserum, the variant NAD9 was clearly identified as part of the complex (Figure 6b,c). In contrast, complex IV was only seen in the *Nv* cytoplasm. Its identification was confirmed by N-terminal microsequencing of a 32-kDa polypeptide obtained after separation by second dimension SDS-PAGE which gave a sequence corresponding to the first 10 amino acids of the mature form of the beet COX2 subunit (data not shown). These results suggest that the *G* complex IV is unstable under the conditions used for solubilization and/or blue native gel electrophoresis.

Enzymatic activity assays

No consistent differences could be detected in the NADH dehydrogenase activity of submitochondrial particles from *Nv* and *G* cytoplasms and, in both cases, the activity was reduced by 25% in the presence of 60 μM rotenone (Rasmusson *et al.*, 1994) (Figure 7a). However, assay of cytochrome *c* oxidase (complex IV) revealed that the activity of this complex was reduced by 50% in the *G* cytoplasm compared with *Nv*, and that, in both cytoplasms, cytochrome *c* oxidase activity was totally inhibited by 1 mM KCN (Figure 7b); when the assay was carried out using fresh mitochondria with 1.5% *n*-dodecylmaltoside and 500 mM aminocaproic acid used in the blue native gel sample buffer, although the overall activity was lower, the same 50% difference in activity was still seen (data not shown), indicating that cytochrome *c* oxidase integrity was probably lost during the actual blue native gel electrophoresis, rather than during solubilization in the sample buffer.

Alternative oxidase Western blotting

Given the reduced activity of the cytochrome *c* oxidase in *G* cytoplasm, we wondered whether alternative oxidase (AOX), an alternative ubiquinone electron acceptor, might be up-regulated. As shown in Figure 7(c), Western blotting revealed the presence of two AOX subunits in beet leaves, with the 32 kDa subunit being present in similar amounts in both cytoplasms and the 34.5 kDa subunit being more highly expressed in *G* than in *Nv*. The existence of two AOX subunits of different sizes in leaves has already been documented in several species (Elthon *et al.*, 1989; Finnegan *et al.*, 1997).

Discussion

Altered respiratory chain in G cytoplasm does not affect beet vegetative growth

We have identified mutations of the *nad9* and *cox2* genes of the CMS *G* cytoplasm of wild beet that result in the production of modified proteins that belong to complex I and IV, respectively. Complex I activity was not affected in leaves whereas complex IV activity was reduced by 50% and seemed to be unstable under electrophoretic conditions. These two mitochondrial complexes have previously been shown to be altered in some cytoplasms. Alloplasmic wheat with a reduced amount of *coxI* transcript is characterized by reduced growth and partial male sterility (Ikeda and Tsunewaki, 1996). In maize, in which deletions in the *cox2* gene have been described in the NCS5 (Newton *et al.*, 1990) and NCS6 (Lauer *et al.*, 1990) mutants, the COX2 subunit is not expressed in the mutated mitochondria and complex IV is therefore non-functional and plants can survive only through heteroplasmy. Several mitochondrial mutations affecting complex I have also been characterized in plants. Maize NCS2 mutant has a *nad4*-

nad7 chimeric gene (Marienfeld and Newton, 1994), while *N. sylvestris* CMSI (Gutierres *et al.*, 1997) and CMSII (Lelandais *et al.*, 1998) exhibit deletions in *nad7* leading to impaired development and modified mitochondrial metabolism.

In all these cases, mutations result from mitochondrial DNA rearrangements and are associated with abnormal development. However, in *G* cytoplasm the modified size of COX2 and NAD9 was a direct result of DNA single point mutations, which is most unusual. Discrete mutations leading to complexes that are still active might explain why the male sterile *G* plants do not exhibit abnormal development and are maintained in natural populations.

The G *complex I containing the mutated NAD9 subunit remains functional at the vegetative stage*

Complex I is L-shaped and is integrated in the mitochondrial inner membrane with its peripheral arm in the matrix (Hofhaus *et al.*, 1991). In several plant species, it is made up of more than 30 subunits (Herz *et al.*, 1994; Leterme and Boutry, 1993; Rasmusson *et al.*, 1994), the function of most of which are unknown and nine subunits, including NAD9, are mitochondrially encoded (Lamatina *et al.*, 1993). The NAD9 subunit is part of the peripheral arm and, in *Neurospora,* inactivation of the nuclear *nuo30.4* gene (fungal *nad9* homologue), prevents peripheral arm assembly (Duarte *et al.*, 1998). Furthermore, labelling of the NAD9 homologue in beef by photoaffinity with ^{32}P-NAD(H) suggests that some amino-acids can be implied in NADH binding with complex I (Yamaguchi *et al.*, 2000). In the present study, the *G nad9* DNA sequence was found to contain 10 mutations that do not correspond to editing sites (Kubo *et al.*, 1993), but, instead, result in 5 amino acid substitutions and C-terminus extension. Although some residue changes occurred in highly conserved motifs in NAD9, these changes had no effect on either complex I assembly or NADH dehydrogenase activity. However, since the role of this well-conserved protein is unknown, we cannot exclude the possibility that these mutations may affect an undetermined complex I function linked to NAD9.

Cytochrome c *oxidase properties are modified in* G *mitochondria*

Plant complex IV is composed of 10 subunits (Jansch *et al.*, 1996). The COX1 and COX2 subunits constitute the core of the enzyme in which oxido-reduction reactions and proton transfer occur (Covello and Gray, 1990). In *G* COX2, the normally highly-conserved 8 C-terminal residues are lost. According to the crystallographic structure of beef cytochrome *c* oxidase (Tsukihara *et al.*, 1996), the COX2 C-terminus is localized in the intermembrane space of the mitochondrion and it is here that cytochrome *c* binds to the complex and electrons are transferred to the active site via Cu_A and cytochrome *a* (Saraste, 1999). Although the *G* complex IV was not observed on blue native gels, the complete inhibition of cytochrome *c* oxidation by cyanide showed that the 50% reduced activity could be attributed to complex IV. The observation that the amount of immunodetected COX2 protein was not reduced in *G* cytoplasm suggests that the lower cytochrome *c* oxidase activity is due to a qualitative defect, rather than to reduced synthesis of COX2. Thus, the presence of the modified COX2 does not lead to total complex inactivation or breakdown. The loss of 8 amino acids at the C-terminus of COX2 might locally modify complex IV conformation and/or its affinity for cytochrome *c* and lead to reduced activity and complex instability during electrophoresis.

The reduced cytochrome c *oxidase activity might explain the increased expression of alternative oxidase*

In plants, alternative oxidase is encoded by a small gene family. Three to four different subunits have been described in different species (Finnegan *et al.*, 1997; Saisho *et al.*, 1997) and form AOX dimers (Umbach and Siedow, 1993). Differential tissue-specific and/or stress-induced expression of AOX genes has been reported (McIntosh, 1994). In the present study, we identified two subunits (32 and 34.5 kDa) in beet leaves and found that the 34.5 kDa subunit was more highly expressed in *G* cytoplasm than in *Nv* cytoplasm (Figure 7c). In the plant respiratory chain, ubiquinone, reduced by complexes I and II and internal and external NAD(P)H dehydrogenases, can be oxidized via cytochrome *c* or alternative oxidase. In *G* mitochondria, diverting the electron flux from the cytochrome *c* pathway to the over-expressed alternative oxidase may compensate mitochondrial respiration for the impaired cytochrome *c* oxidase activity. Alternative oxidase is not coupled to phosphorylation but its over-expression may preserve TCA cycle turnover by maintaining electron flux through the respiratory chain. Moreover, it prevents blockage of the metabolism by continuous regeneration of NAD(P) and accelerates glycolysis for cytosolic ATP production (Sabar *et al.*, 2000). However, the role of this alternative pathway is not fully understood and many factors are involved in its regulation (McIntosh, 1994). Recently, cytochrome *c* oxidase-defective mutants of the fungus, *Podospora anserina*, revealed a possible link between alternative oxidase and senescence in terms of the prevention of the production of reactive oxygen species (ROS) by complex III (Dufour *et al.*, 2000). An increase in alternative oxidase and decrease in cytochrome *c* oxidase activity might result in less ROS production (Maxwell *et al.*, 1999), and protect mitochondrial metabolites and genetic information against oxidative stress

during plant life (Wagner and Moore, 1997). According to our observations, this particular electron partitioning in *G* beets does not seem to affect plant development, except possibly for the male reproductive trait, and may contribute to a selective advantage of this cytoplasm under given environmental conditions.

Are the modified cox2 *and* nad9 *involved in G cytoplasmic male sterility?*

Several lines of evidence suggest that plant male gametogenesis requires high mitochondrial activity (Huang *et al.*, 1994; Lee and Warmke, 1979; Smart *et al.*, 1994). Moreover, mitochondrial mutations that affect respiratory chain performance disturb plant development and are strongly associated with impaired gametogenesis. Several hypotheses can be put forward for a putative link between modified respiratory complexes and CMS:

(1) We are not able to attribute any enzyme dysfunction at the vegetative stage to mutated NAD9. Since respiratory chain subunits (De Paepe *et al.*, 1993; Huang *et al.*, 1994) and regulation of mitochondrial gene expression (Conley and Hanson, 1994; Li *et al.*, 1996; Smart *et al.*, 1994) can be anther-specific, we cannot exclude the possibility that *G* complex I impairment is limited to reproductive tissues. Indeed, the role of subunit 9 remains unknown and various complex I alterations are strongly associated with pollen production deficiency (Rasmusson *et al.*, 1998). Interestingly, the *Neurospora* mutants deleted for the *nad9* homologue had a partially assembled complex I (Duarte *et al.*, 1998), exhibited normal growth, but failed to produce ascospores, suggesting an essential role of complex I in the reproductive phase.

(2) In the vegetative stage, reduced cytochrome *c* oxidase activity can be compensated for by increased glycolysis and AOX activity. However, this second pathway does not contribute to the transmembrane potential and thus ATP production. In contrast to the situation in vegetative tissues, the high ATP/ADP ratio observed during pollen development (Bergman *et al.*, 2000) may not be achieved in flower buds with the *G* cytoplasm, and this might result in male sterility. For example, in transgenic potatoes containing complex I 55 kDa subunit antisense DNA in which the amount of this protein was reduced by 50%, vegetative growth was unchanged, but pollen maturation was markedly affected, and the authors suggested that this might be due to insufficient respiratory chain activity (Heiser *et al.*, 1997).

(3) We cannot exclude the hypothesis that smaller COX2 and larger NAD9 are both required for the expression and/or maintenance of this CMS in natural populations. Both modified subunits are indeed associated in all accessions of *G* cytoplasm encountered in natural populations (Ducos, E., unpublished data). Moreover, none of the 20 different mitotypes characterized in the beets of Western Europe (Desplanque *et al.*, 2000) contain the smaller COX2 or larger NAD9 specific to *G*.

Conclusions

Until now, alterations in plant mitochondria respiratory chain have been studied by analysis of mutants that would be unable to survive in natural conditions as a result of the developmental changes. To the best of our knowledge, the present study is the first to describe a plant mitochondrial mutant affecting the respiratory chain in which vegetative growth is not affected and which is maintained in natural populations. Moreover, since this CMS has been identified in various beet populations in Western Europe, we assume that the mutations described in this paper are not deleterious to the plants, except perhaps for the male reproductive trait. Interestingly enough, restored CMS *G* plants coexist with male sterile plants in beet populations. This material will be useful in studying the male sterility mechanism and putative selective advantages of the metabolic changes under natural selection. Finally, this study opens up fields of investigation for metabolism diversity encountered in natural populations.

Experimental procedures

Plant material

The two sugar beet lines used were provided by INRA (Dijon, France). They constitute a near nuclear isogenic pair and differ in their cytoplasm, which is *G* for the male-sterile line and *Nv* (*Normal vulgaris*) for the maintainer line. The donor parent of the *G* cytoplasm is a male-sterile *Beta vulgaris* ssp. *maritima* derived from seeds harvested in 1989 from an open pollinated male-sterile plant in a wild population in Saint-Nazaire (Brittany, France). The line bearing the *G* cytoplasm results from 6 successive backcrosses with the recurrent parental line, INRA 660. The reference fertile cytoplasm, *Nv* (also called *TK81-O* (Senda *et al.*, 1991)), is the most frequently used in sugar beet breeding programs. Plants were grown to an age of 2–4 months in a greenhouse under standard conditions (20°C; 16 h light).

Mitochondrial protein synthesis

Mitochondria were isolated under sterile conditions from young leaves by centrifugation on a Percoll gradient and labeled *in organello* with [^{35}S]methionine according to the method described by Boutry *et al.* (1984). For pulse-chase experiments, after 7 min of *in organello* labeling in 100 μl of synthesis medium, 10 μl of 25 mM unlabeled methionine was added and incubation continued for 40 min.

Protein gel electrophoresis and Western blotting

In organello labeled mitochondria (30,000–40 000 cpm) were subjected to electrophoresis on 12–17% SDS gels, electroblotted

onto nitrocellulose membrane, and autoradiographed. For Western blotting, the amount of mitochondrial protein electrophoresed was 5 μg when using antisera against maize COX2 (Kumar *et al.*, 1995) or wheat NAD9 (Lamatina *et al.*, 1993) and 100 μg when using a monoclonal antibody against *Sauromatum guttatum* alternative oxidase (Elthon *et al.*, 1989). For blue native gels, 200–300 μg of proteins of freshly purified mitochondria were used, following the protocol described by Jänsch *et al.* (1996); for the second dimension, the blue native gel was incubated for 3 × 30 min in denaturation buffer (80 mM Tris, 2% SDS, 10% glycerol, 0.005% bromphenol blue, 1.5% dithiothreitol, pH 6.8), then regions containing mitochondrial complexes stained with Coomassie blue were cut out of the gel, placed above the stacking gel of a standard 15% SDS-PAGE gel and sealed in position with melted 1% agarose in 50 mM Tris, 400 mM glycine, 2.5 mM EDTA, pH 7.0.

Protein N-terminal microsequencing

Proteins separated on two-dimensional blue native/SDS PAGE and electroblotted onto a PVDF membrane were sequenced by automated Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer (model 477 A, Foster City, CA, USA).

DNA cloning and sequencing

The sequence of G*cox2* was determined by direct sequencing of the PCR product using specific infra-red detection (IRD)-labeled primers on a Li-Cor automated DNA sequencer 4000 l. For *nad9*, inverse-PCR using internal primers was performed on *Hind*III-digested and re-ligated DNA (Ochman *et al.*, 1988; Triglia *et al.*, 1988). The amplified fragments were cloned using the pGEM-T Vector Cloning System (Promega, Madison, WI, USA), and sequenced. Specific primers were then defined for each copy of *nad9*. The products of three independent PCR were sequenced per gene.

Southern blotting

DNA isolation and hybridization were performed as described by Saumitou-Laprade *et al.* (1991). Homologous *nad9* and *cox2* probes were produced by PCR and labeled using the Boehringer (Mannheim, Germany) PCR DIG labeling mix.

Northern blotting

Total RNA was isolated from green leaves in the presence of aurintricarboxylic acid (Stern and Newton, 1986). Transcripts were hybridized with the *cox2*-exon 1 or *nad9* probes obtained by PCR and labeled with [^{32}P]dNTP.

Enzymatic activity assay

Cytochrome *c* oxidase activity was monitored at 550 nm the oxidation of 0.1% (w/v) reduced cytochrome *c* in 40 mM NaH_2PO_4, 0.1% *n*-dodecylmaltoside, 1 mM EDTA, pH 7.2. NADH dehydrogenase activity was measured by monitoring at 550 nm the reduction of 0.1% cytochrome *c* in 40 mM NaH_2PO_4, 0.1% *n*-dodecylmaltoside, 1 mM EDTA, 1 mM KCN, 2.5 mM NADH, pH 7.2. NADH:ubiquinone oxidoreductase was specifically inhibited by 60 μm rotenone dissolved in ethanol (Leterme and Boutry, 1993). Measurements began after addition of 10 or 50 μg of submitochondrial particles (Kotlyar and Vinogradov, 1992) for the cytochrome *c* oxidase assay or NADH dehydrogenase assay, respectively.

Acknowledgements

We thank J. Cuguen for his helpful comments on the manuscript, I. Small, J.-M. Grienenberger and T. Elthon for gifts of antibodies and H. Degand for protein sequencing. This work was supported by grants from Région Nord-Pas de Calais, FEDER, the Tournesol Programme (Belgium/France) and a CNRS-FNRS International Cooperation Program, and from the Belgian National Fund for Scientific Research and the Interuniversity 'poles of attraction' program of the Belgian Government Office for Scientific, Technical and Cultural Affairs. E. D. received fellowships from the European Community (PTP142 and the ESF Plant Adaptation Program) and the University of Lille I (BQR).

The G*cox2*, G*nad9*-1, G*nad9*-2 sequences have been deposited in the Genbank database. Accession numbers: G*cox2*: AF276430; G*nad9*-1: AF276428; G*nad9*-2: AF276429.

References

Akagi, H., Sakamoto, M., Shinjyo, C., Shimada, H. and Fujimura, T. (1994) A unique sequence located downstream from the rice mitochondrial atp6 may cause male sterility. *Curr. Genet.* **25**, 52–58.

Bergman, P., Edqvist, J., Farbos, I. and Glimelius, K. (2000) Male-sterile tobacco displays abnormal mitochondrial *atp1* transcript accumulation and reduced floral ATP/ADP ratio. *Plant Mol. Biol.* **42**, 531–544.

Boutry, M., Faber, A.-M., Charbonnier, M. and Briquet, M. (1984) Microanalysis of plant mitochondrial protein synthesis products: detection of variant polypeptides associated with cytoplasmic male sterility. *Plant Mol. Biol.* **3**, 445–452.

Conley, C.A. and Hanson, M.R. (1994) Tissues-specific protein expression in plant mitochondria. *Plant Cell*, **6**, 85–91.

Covello, P.S. and Gray, M.W. (1990) RNA sequence and nature of the CuA-binding site in cytochrome *c* oxidase. *FEBS Lett*, **268**, 5–7.

Cuguen, J., Wattier, R., Saumitou-Laprade, P., Forcioli, D., Mörchen, M., Van Dijk, H. and Vernet, P. (1994) Gynodioecy and mitochondrial DNA polymorphism in natural populations of *Beta vulgaris* ssp. *Maritima. Genet. Sel. Evol.* **26**, 87–101.

De Paepe, R., Forchioni, A., Chetrit, P. and Vedel, F. (1993) Specific mitochondrial proteins in pollen: presence of an additional ATP synthase beta subunit. *Proc. Natl Acad. Sci. USA*, **90**, 5934–5938.

Desplanque, B., Viard, F., Bernard, J., Forcioli, D., Saumitou-Laprade, P., Cuguen, J. and Van Dijk, H. (2000) The linkage disequilibrium between chloroplast DNA and mitochondrial DNA haplotypes in Beta vulgaris ssp. maritima (L.). *Mol. Ecol.* **9**, 141–154.

Dewey, R.E., Timothy, D.H. and Levings III, C.S. (1987) A mitochondrial protein associated with cytplosmic male sterility in the T cytoplasm of maize. *Proc. Natl Acad. Sci. USA*, **84**, 5374–5378.

Duarte, M., Mota, N., Pinto, L. and Videira, A. (1998) Inactivation of the gene coding for he 30.4-kDa subunit of respiratory chain NADH dehydrogenase: is the enzyme essential for Neurospora? *Mol. Gen. Genet.* **257**, 368–375.

Dufour, E., Boulay, J., Rincheval, V. and Sainsard-Chanet, A.

(2000) A causal link between respiration and senescence in Podospora anserina. *Proc. Natl Acad. Sci. USA*, **97**, 4138–4143.

Elthon, T.E., Nickels, R.L. and McIntosh, L. (1989) Monoclonal antibodies to the alternative oxidase of higher plant mitochondria. *Plant Physiol.* **89**, 1311–1317.

Finnegan, P.M., Whelan, J., Millar, A.H., Zhang, Q., Smith, M.K., Wiskich, J.T. and Day, D.A. (1997) Differential expression of the multigene family encoding the soybean mitochondrial alternative oxidase. *Plant Physiol.* **144**, 455–466.

Grelon, M., Budar, F., Bonhomme, S. and Pelletier, G. (1994) Ogura cytoplasmic male-sterility (CMS) -associated *orf138* is translated into a mitochondrial membrane polypeptide in male-sterile *Brassica* cybrids. *Mol. Gen. Genet.* **243**, 540–547.

Gualberto, J.M., Bonnard, G., Lamattina, L. and Grienenberger, J.M. (1991) Expression of the wheat mitochondrial nad3-rps12 transcription unit: correlation between editing and mRNA maturation. *Plant Cell*, **3**, 1109–1120.

Gutierres, S., Sabar, M., Lelandais, C., Chetrit, P., Diolez, P., Degand, H., Boutry, M., Vedel, F., de Kouchovsky, Y. and de Paepe, R. (1997) Lack of mitochondrial and nuclear-encoded subunits of complex I and alteration of the respiraroty chain in *Nicotiana sylvestris* mitochondrial deletion mutants. *Proc. Natl Acad. Sci. USA*, **94**, 3436–3441.

Heiser, V., Rasmusson, A.G., Thieck, O., Brennicke, A. and Grohmann, L. (1997) Antisense repression of the mitochondrial NADH-binding subunit of complex I in transgenic potato plants affects male fertility. *Plant Sci.* **127**, 61–69.

Herrmann, J.M., Koll, H., Cook, R.A., Neupert, W. and Stuart, R.A. (1995) Topogenesis of cytochrome oxidase subunit II. Mechanisms of protein export from the mitochondrial matrix. *J. Bio. Chem.* **270**, 27079–27086.

Herz, U., Schroder, W., Liddell, A., Leaver, C.J., Brennicke, A. and Grohmann, L. (1994) Purification of the NADH: ubiquinone oxidoreductase (complex I) of the respiratory chain from the inner mitochondrial membrane of Solanum tuberosum. *J. Bio. Chem.* **269**, 2263–2269.

Hofhaus, G., Weiss, H. and Leonard, K. (1991) Electron microscopic analysis of the peripheral and membrane parts of mitochondrial NADH dehydrogenase (complex I). *J. Mol. Bio.* **221**, 1027–1043.

Horn, R., Köhler, R.H. and Zetsche, K. (1991) A mitochondrial 16 kDa protein is associated with cytoplasmic male sterility in sunflower. *Plant Mol. Biol.* **7**, 29–26.

Howad, W. and Kempken, F. (1997) Cell type-specific loss of atp6 RNA editing in cytoplasmic male sterile *Sorghum bicolor*. *Proc. Natl Acad. Sci. USA*, **94**, 11090–11095.

Huang, J., Struck, F., Matzinger, D.F. and Levings,C.S., III (1994) Flower-enhanced expression of a nuclear-encoded mitochondrial respiratory protein is associated with changes in mitochondrion number. *Plant Cell*, **6**, 439–448.

Ikeda, T.M. and Tsunewaki, K. (1996) Deficiency of cox1 gene expression in wheat plants with Aegilops columnaris cytoplasm. *Curr. Genet.* **30**, 509–514.

Iwabuchi, M., Kyozuka, J. and Shimamoto, K. (1993) Processing followed by complete editing of an altered mitochondrial *atp6* RNA restores fertility of cytoplasmic male sterile rice. *EMBO J.* **12**, 1437–1446.

Jansch, L., Kruft, V., Schmitz, U.K. and Braun, H.P. (1996) New insights into the composition, molecular mass and stoichiometry of the protein complexes of plant mitochondria. *Plant J.* **9**, 357–368.

Johns, C., Lu, M.Q., Lyznik, A. and Mackenzie, S. (1992) A mitochondrial DNA sequence is associated with abnormal pollen development in cytoplasmic male sterile bean plants. *Plant Cell*, **4**, 435–449.

Kaul, M.L.H. (1988). *Male Sterility in Higher Plants*. Berlin, Germany: Springer Verlag.

Kotlyar, A.B. and Vinogradov, A.D. (1992) Slow active/inactive transition of the mitochondrial NADH-ubiquinone reductase. *Biochim. Biophys. Acta.* **1019**, 151–158.

Kubo, T., Mikami, T. and Kinoshita, T. (1993) The sugar beet mitochondrial genome containe an ORF sharing sequence homology with the gene for 30-kDa subunit of bovine mitochondrial complex I. *Mol. Gen. Genet.* **241**, 479–482.

Kubo, T., Nishizawa, S. and Mikami, T. (1999) Alterations in organization and transcription of the mitochondrial genome of cytoplasmic male sterile sugar beet (Beta vulgaris L.). *Mol. Gen. Genet.* **262**, 283–290.

Kubo, T., Yanai, Y., Kinoshita, T. and Mikami, T. (1995) The chloroplast trnP-trnW-petG gene cluster in the mitochondrial genomes of Beta vulgaris, B. trigyna and B. webbiana: evolutionary aspects. *Curr. Genet.* **27**, 285–289.

Kumar, R., Lelu, M.-A. and Small, I. (1995) Purification of mitochondria and mitochondrial nucleic acids from embryogenic suspension cultures of a gymnosperm, Larix Ë leptoeuropaea. *Plant Cell Reports*, **14**, 534–538.

Lamatina, L., Gonzalez, D., Gualberto, J. and Grienenberger, J.M. (1993) Higher plant mitochondria encode an homologue of the nuclear-encoded 30-kDa subunit of bovine mitochondrial complex I. *Eur. J. Bioch.* **217**, 831–838.

Lauer, M., Knudsen, C., Newton, K.J., Gabay-Laughnan, S. and Laughnan, J. (1990) A partially deleted mitochondrial cytochrome oxidase 2 gene in NCS6 abnormal growth mutant of maize. *New. Biol.* **2**, 179–186.

Lee, S.-L.J. and Warmke, H.E. (1979) Organelle size and number in fertile and T-cytoplasmic make sterile corn. *Am. J. Bot.* **66**, 141–148.

Lelandais, C., Albert, B., Gutierres, S., De Paepe, R., Godelle, B., Vedel, F. and Chetrit, P. (1998) Organization and expression of the mitochondrial genome in the Nicotiana sylvestris CMSII mutant. *Genetics*, **50**, 873–882.

Leterme, S. and Boutry, M. (1993) Purification and preliminary characterization of mitochondrial complex I (NADH: ubiquinone reductase) from broad bean (Vicia faba L.). *Plant Physiol.* **102**, 435–443.

Levings, C.S. (1993) Thoughts on cytoplasmic male sterility in cms-T maize. *Plant Cell*, **5**, 1285–1290.

Li, X.-Q., Zhang, M. and Brown, G.G. (1996) Cell-specific expression of mitochondrial transcripts in maize seedlings. *Plant Cell*, **8**, 1961–1975.

Marienfeld, J.R. and Newton, K.J. (1994) The maize NCS2 abnormal growth mutant has a chimeric nad4-nad7 mitochondrial gene and is associated with reduced complex I function. *Genetics*, **138**, 855–863.

Maxwell, D.P., Wang, Y. and McIntosh, L. (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proc. Natl Acad. Sci. USA*, **96**, 8271–8276.

McIntosh, L. (1994) Molecular biology of the alternative Oxidase. *Plant Physiol.* **105**, 781–786.

Newton, K.J., Knudsen, C., Gabay-Laughnan, S. and Laughnan, J.R. (1990) An abnormal growth mutant in maize has a defective mitochondrial cytochrome oxidase gene. *Plant Cell*, **2**, 107–113.

Nivison, H.T. and Hanson, M.R. (1989) Identification of a mitochondrial protein associated with cytoplasmic male sterility in petunia. *Plant Cell*, **1**, 1121–1130.

Ochman, H., Gerber, A.S. and Hartl, D.L. (1988) Genetic

applications of an inverse polymerase chain reaction. *Genetics*, **120**, 621–623.

Owen, F.V. (1945) Cytoplasmically inherited male-sterility in sugar beets. *J. Agr. Res.* **71**, 423–440.

Pring, D.R., Tang, H.V., Howad, W. and Kempken, F. (1999) A unique two-gene gametophytic male sterility system in sorghum involving a possible role of RNA editing in fertility restoration. *J. Hered*, **90**, 386–393.

Rasmusson, A.G., Heiser, V., Zabaleta, E., Brennicke, A. and Grohmann, L. (1998) Physiological, biochemical and molecular aspects of mitochondrial complex I in plants. *BBA*, **1364**, 101–111.

Rasmusson, A.G., Mendel-Hartvig, J., Moller, I.M. and Wiskich, J.T. (1994) Isolation of rotenone-sensitive NADH-ubiquinone reductase (complex I) from red beet mitochondria. *Physiol. P.* **90**, 607–615.

Sabar, M., De Paepe, R. and de Kouchkovsky, Y. (2000) Complex I impairment, respiratory compensations, and photosynthetic decrease in nuclear and mitochondrial male sterile mutants of *Nicotiana sylvestris*. *Plant Physiol.* **124**, 1239–1249.

Saisho, D., Nambara, E., Naito, S., Tsutsumi, N., Hirai, A. and Nakazono, M. (1997) Characterization of the gene family for alternative oxidase from Arabidopsis thaliana. *Plant Mol. Biol.* **35**, 585–596.

Saraste, M. (1999) Oxidative phosphorylation at the fin de siècle. *Science*, **283**, 1488–1493.

Saumitou-Laprade, P., Pannenbecker, G., Boutin-Stadler, V., Michealis, G. and Vernet, P. (1991) Plastid diversity in natural population of *Beta maritima* showing additional variation in sexual phenotype and mitochondrial DNA. *Theor. Appl. Genet.* **81**, 533–536.

Saumitou-Laprade, P., Rouwendal, G.J.A., Cuguen, J., Krens, F.A. and Michaelis, G. (1993) Different CMS sources found in *Beta vulgaris* ssp *maritima*: mitochondria variability in wild population revealed by a rapid screening procedure. *Theor. Appl. Genet.* **85**, 529–535.

Schnable, P.S. and Wise, R.P. (1998) The molecular basis of cytoplasmic male sterility and fertility restoration. *Trends Plant Sci.* **3**, 175–180.

Senda, M., Harada, T., Mikami, T., Sugiura, M. and Kinoshita, T. (1991) Genomic organization and sequence-analysis of the cytochrome-oxidase subunit-ii gene from normal and male-sterile mitochondria in sugar-beet. *Curr. Genet.* **19**, 175–181.

Senda, M., Onodera, Y., Kinoshita, T. and Mikami, T. (1995) Mitochondrial gene variation and phylogenetic relationships in the genus *Beta*. *Theor. Appl. Genet.* **90**, 914–919.

Singh, M. and Brown, G.G. (1993) Characterization of expression of a mitochondrial gene region associated with the Brassica 'Polima' CMS: developmental influences. *Curr. Genet.* **24**, 316–322.

Smart, C.J., Moneger, F. and Leaver, C.J. (1994) Cell-specific regulation of gene expression in mitochondria during anther development in sunflower. *Plant Cell*, **6**, 811–825.

Stern, D.B. and Newton, K.J. (1986) Isolation of plant mitochondrial RNA. *Meth Enzymol.* **118**, 488–496.

Stern, D.B., Radwanski, R. and Kindle, K.L. (1991) A 3′ stem/loop structure of the Chlamydomonas chloroplast atpB gene regulates mRNA accumulation in vivo. *Plant Cell*, **3**, 285–297.

Triglia, T., Peterson, M.G. and Kemp, D.J. (1988) A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. *Nucl Acids Res.* **16**, 81–86.

Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Tamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. and Yoshikawa, S. (1996) The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 A. *Science*, **272**, 1136–1144.

Umbach, A.L. and Siedow, J.N. (1993) Covalent and non-covalent dimers of the cyanide-resistant alernative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. *Plant Physiol.* **103**, 845–854.

Wagner, A.M. and Moore, A.L. (1997) Structure and function of the plant alternative oxidase: its putative role in the oxygen defense mechanism. *Biosci Report*, **17**, 319–333.

Xue, Y.B., Thomas, C.M. and Davies, D.R. (1989) Nucleotide sequence and transcription of the sugar beet mitochondrial F0F1-ATPase subunit 9 gene. *Nucl Acids Res.* **17**, 8857.

Yamaguchi, M., Belogrudov, G.I., Matsuno-Yagi, A. and Hatefi, Y. (2000) The multiple nicotinamide nucleotide-binding subunit of bovine heart mitochondrial NADH: ubiquinone oxidoreductase (complex I). *Eur. J. Biochem.* **267**, 329–336.

GenBank accession numbers AF276428, AF276429 and AF276430.