Theor Appl Genet (1988) 76:305–310

Cytoplasmic male sterility in *Petunia hybrida*: factors affecting mitochondrial ATP export in normal and cytoplasmically male sterile plants

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Received February 27, 1988; Accepted March 10, 1988
Communicated by H. F. Linskens

Summary. In view of accumulating evidence that cytoplasmic male sterility (CMS) in some species results from an inability to generate the high ATP/ADP ratios required for specific stages of differentiation in the reproductive cycle, a number of aspects of ATP metabolism are being examined in CMS and male fertile plants.

In experiments designed to test mitochondrial efficiency in ATP export, organelles from CMS plants performed very poorly when compared with normal lines. It is proposed that although most of the molecules involved in mitochondrial ATP production are nuclear encoded, the lesions in mitochondrial (mt)DNA known to accompany the CMS phenotype may be expressed as small modifications within the architecture of the mitochondrial membrane. To detect whether such changes could affect the ADP-ATP translocator in the membrane, two sets of experiments were carried out to determine a 'Km' for the translocator. The two methods employed were based on different precepts, but nevertheless indicated a 'Km' for the mitochondrial translocator in CMS lines which differed dramatically from that of male fertile plants. The view that CMS in *Petunia hybrida* thus might result from small differences in mtDNA encoded membrane proteins is considered in the light of the cytological changes seen to accompany CMS in these plants, as well as in the context of current theories advanced to explain CMS in other species.

Key words: ADP-ATP translocator – Cytoplasmic male sterility – Mitochondrial inner membrane – Pollen development

Introduction

Cytoplasmic male sterility (CMS) is defined as a maternally-inherited trait that results in the abortion of male cells during microsporogenesis. This phenomenon has been extensively reviewed (Edwardson 1956, 1970; Laser 1972; Overman 1972; Pearson 1981; Kumer 1984). While CMS varies both in character and in the stage at which it takes its effect, it is nevertheless surprising that the first deviation of normal development normally takes place in the tapetal cells – rather than in the pollen mother cells themselves (Grant 1986; Heslop-Harrison 1968; Nishiyama 1970). Thus in CMS lines of *Petunia hybrida*, the first deviation in tapetal development may be detected far earlier than the first necrotic changes in the pollen mother cells, although the latter cells are fully aborted by the end of meiotic prophase (Bino 1985a, b). These early changes in tapetal cells of CMS *Petunia* do not involve the normal indications of necrosis, but seemingly isolated effects on DNA synthesis, nuclear division and the organisation of the endoplasmic reticulum (ER) (Liu et al. 1987). Later stages in development display the more traditional signs of necrosis, including the generation of large vacuoles. It has been suggested that these early effects are commensurate with an inability of tapetal mitochondria to provide the high ATP/ADP ratios required for the rapid synthesis of DNA which characterises these stages in this tissue (Liu et al. 1987).

In actively metabolising plant cells, ATP is generated principally in the mitochondria, from whence it is transferred to the cytosol for utilisation. From a functional point of view, ATP production can thus be regarded as including transport across the mitochondrial membranes, known to be performed by a specific ADP-ATP translocator (Klingenberg 1964). This molecule is located in the inner mitochondrial membrane and comprises two protein subunits encoded by nuclear genes (Leaver 1980, 1982; Pfaff 1969; Heldt 1976; Weidemann 1970; Hawkesford et al. 1987; Pfanner 1987). It is also considered possible that this translocator also serves to regulate phos-

phorylation in the mitochondria via a feedback system which controls the ATP/ADP ratio within the matrix (Mattoon et al. 1979). Although its nuclear genetic control suggests that the translocator itself is unlikely to be affected directly in CMS lines, the data available do suggest that the capacity of the tapetal cells in these plants is in some way unable to sustain high ATP/ADP ratios when required. We therefore consider it a strong possibility that the function of this translocator is indeed impeded in these lines, perhaps through small modifications to one or more of the mtDNA encoded membrane proteins situated in close proximity to the translocator. We record here a range of experiments designed to test the efficiency of the mitochondrial ADP-ATP translocator in normal and CMS lines of *Petunia hybrida*.

Materials and methods

Plant materials

The sources of the CMS and normal lines of *Petunia hybrida* used in this work have been described in detail elsewhere (Liu et al. 1987). Only young expanding leaves were used for the extraction of mitochondria, and in all experiments normal and CMS tissues were handled simultaneously.

Isolation of mitochondria

Mitochondria were isolated from leaves according to the method of Moore and Proudlove (1983). 10 gms of leaves were placed in a pre-cooled mortar together with 25 ml of homogenising medium (0.4 M mannitol, 1 mM EDTA, 0.4% BSA, 0.6% insoluble PVP, 17 µl/100 ml β-mercaptoethanol and 20 mM HEPES adjusted to pH 7.8 with KOH). The leaves were cut into 1 cm squares under the surface of the medium, and then homogenised with a pre-cooled pestle and mortar. This homogenate was then passed through four layers of muslin and two layers of Miracloth. The filtrate was centrifuged at 3,000 g at 0°C for 10 min, and the supernatant recentrifuged at 11,000 g at 0°C for a further 10 min. The pellet was carefully suspended in 5 ml of modified homogenisation medium (omitting β-mercaptoethanol and PVP, and adjusted to pH 7.2). Finally, these centrifugation steps were repeated once more and the pellet gently suspended in 1 ml of the modified homogenisation medium. In the radio-labelling experiment the pellet was resuspended in a different medium, comprising 0.4 M mannitol and 20 mM triethanolamine, pH 7.2 (MT buffer).

The intactness of the isolated mitochondria was estimated by assaying the permeability of the outer membrane to cytochrome C (Douce et al. 1972). Fully intact mitochondria will not reduce exogenously supplied cytochrome C in the presence of succinate and KCN, whilst ruptured organelles will do so (Douce et al. 1973). 0.05 ml of the resuspended pellet was added to 0.85 ml of reaction medium (0.4 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 50 µM cytochrome C, 1 mM KCN, 0.2 mM ATP and 10 mM phosphate buffer at pH 7.2) in a 1 ml spectrophotometer cuvette. Two groups of samples were taken, with one set containing 5 µl of 10% Triton X100 per cuvette. The reaction was initiated by the addition of 0.1 ml of 10 mM succinate, and aliquots of 0.1 ml distilled water were added to the controls. Reduced cytochrome C was measured at 550 nm using a double-beam spectrophotometer, and the percentage of intact mitochondria was calculated according to the following formula:

$$\text{Intact mitochondria (\%)} = \left[1 - \frac{\text{(reduced cyto. C (before treatment)}}{\text{(reduced cyto. C. (after treatment)}}\right] \times 100$$

The total cytochrome C content of isolated mitochondria was determined according to the method of Tolbert et al. (1968) using mitochondrial cytochrome C oxidase. 0.05 ml of the mitochondrial pellet was added, together with 5 µl of 10 Triton X100, to 0.945 ml of 50 µM reduced cytochrome C in 0.01 M phosphate buffer at pH 7.0. Absorbance was measured in a double beam spectrophotometer at 550 nm, and a reaction mixture containing a sample treated at 100°C for two minutes was used as a blank. The cytochrome C used in the experiment was reduced using sodium dithionite until a A550/565 ratio of between 9 and 10 was achieved.

Determination of mitochondrial ATP export

The method employed in this work is based on the precept that when isolated intact mitochondria containing, and producing ATP are provided with a given concentration of ADP, ATP will be exported from the organelles by means of the ADP-ATP translocator, and the performance of this carrier will depend upon its affinity for ADP and ATP. ATP production was measured using the following reaction system (Brolin 1983; Deutsch 1983).

(1) $\text{ATP} + \text{Glucose} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-phosphate} + \text{ADP}$

(2) $\text{Glucose-6-phosphate} + \text{NADP} \xrightarrow{\text{G6P dehydrogenase}} \text{NADPH} + \text{6-phosphogluconate}$

The final levels of NADPH resulting from these reactions was measured in the spectrophotometer at 340 nm (NADPH: $E340 = 6.22 \times 10^{-3}\ \text{mol}^{-1}\ \text{cm}^{-1}$).

Thus, 0.05 ml aliquots of mitochondrial pellet were added to 0.85 ml of reaction medium (0.4 M mannitol, 1.0 mM EDTA, 3 mM α-D-(+)-glucose, 3 mM K_3PO_4, 10 mM succinate, 5 mM $MgCl_3$, 0.02 µM $CaCl_3$, 20 mM HEPES [pH 7.2], 2 units/ml of glucose-6-phosphate dehydrogenase and 6 units/ml hexokinase) in a 1 ml quartz cuvette. Reactions were initiated with 0.1 ml aliquots of 1.0 mM ADP and 3 mM NADP. Three sets of samples were examined in these experiments: (a) mitochondria ruptured using 1% Triton X100; (b) intact mitochondria; (c) a control using intact mitochondria, but omitting the ADP substrate.

Determination of a 'Km' value for the ADP-ATP translocator

Using the method described above the 'Km' of the ADP-ATP translocator was estimated by measuring ATP export rates in the presence of a range (0.30–1.20 mM) of concentrations of ADP, and 3 mM NADP. The rate of the first order reaction was recorded for each concentration and the reaction rate (Y axis) was plotted against the ADP concentration (X axis) for normal and CMS derived materials. Finally, linear regressions of double reciprocal plots of these rates (Y axis) against the corresponding ADP concentrations (X axis) were drawn with computer assistance. The 'Km' value was interpreted as the intercept on the X axis of the reciprocal of the straight line produced. It should be emphasised that normally the Km of an enzyme is calculated on the basis of its affinity for the substrate, and this is clearly not exactly the case here. However, since the ADP-ATP shuttle is known to bind both ADP and ATP, its response to changes in ADP concentration is likely to reflect its affinity for ATP.

Determination of a 'Km' value for the ADP-ATP translocator using a radio-labelling method

This work employed a modified version of the method of Weidemann (1970) and Pfaff (1969) to determine the 'Km' value of the ADP-ATP translocator. Since extracted mitochondria contain endogenous ADP and ATP, which block the ingress of externally supplied labelled ATP into the organelles, these endogenous nucleotides have to be removed before the organelles were supplied with labelled ATP in the absence or presence of sodium atractyloside, an inhibitor of the translocator. Mitochondria thus labelled were then extracted and the radio tracer present assayed in a scintillation counter.

Depleting endogenous nucleotides in mitochondria. Extracted mitochondria were suspended in 5 ml depleting medium, comprising 5 mM sodium arsenate and 1 mM EDTA in MT buffer. Following incubation at 20 °C for 45 min, the mixture was centrifuged at 11,000 g at 0–2 °C for 10 min. The precipitate was gently suspended in fivefold excess of MT buffer, and the centrifugation procedure repeated. Finally, the precipitate was carefully resuspended in 8 ml MT buffer (the final mixture contained approximately 1.7–2.0 mg protein/ml) and maintained in ice.

Mitochondrial labelling with U-14C ATP. 0.5 ml of the mitochondrial extract was mixed with 0.49 ml incubation medium (10 mM ado-3'-P, 120 µg/ml oligomycin, 0.5 mM EDTA, 0.4 M mannitol and 20 mM triethanolamine, adjusted to pH 7.2 with KOH) containing atractyloside to a final concentration of 10 µM. Following incubation for 60 s in 1 ml Eppendorf tube frozen in ice, the temperature was raised to 5 °C and 10 µl U-^{14}C ATP was added. After a further 120 s, the mixture was centrifuged for one minute at 13,000 rpm on MSE Micro Centaur (rotor pre-cooled using Arcton 12). Following centrifugation, the supernatant was carefully removed using a 1 ml syringe and the precipitate resuspended in 1 ml ice cold MT buffer. After a second centrifugation, the pellet was extracted by shaking 0.1 ml of double distilled water, and then mixing rapidly with 0.4 ml of 1 M $HClO_4$. To ensure complete extraction, the mixture was held in a 5 °C refrigerator overnight. In a second set of samples, 0.5 ml of the mitochondrial extract was mixed with 0.49 ml of incubation medium, and 10 µl of U-^{14}C ATP, and allowed to incubate for 120 s at 5 °C. At this point 10 µM (final volume) sodium atractyloside was mixed into the solution, which was allowed to stand for one further minute at 0 °C. The extraction was then performed as for the previous samples.

Following extraction, all samples were centrifuged at 13,000 rpm in a MSE Micro Centaur for 5 min, the supernatants removed into 20 ml plastic vials, and the pellets washed in 1 ml distilled water accompanied by vigorous shaking. Following a further centrifugation, the supernatant was again removed into plastic vials. The radiolabel at present in each of these samples was then measured by mixture with 10 ml of 'Ecoscint' fluid in a LKB 1215 Back Beta liquid scintillation counter.

Results and discussion

In a previous report, DNA synthesis and cytoplasmic differentiation have been described in the tapetal cells of male fertile and CMS plants of *Petunia hybrida*. This work indicated that CMS may result from mitochondria being unable to meet either the very specific synthesis requirements, or the very high energy needs of these rapidly differentiating cells (Liu et al. 1987). There is thus a strong possibility that the characteristic biochemical phenotype of CMS (Bino et al. 1986; van Went 1986) is related to the ATP/ADP ratios of the tapetal cells which is reflected either by the availability of ATP for metabolism, or by a controlling role of ATP/ADP ratio itself.

While it would thus seem essential to obtain an accurate picture of ATP/ADP levels within these cells, many current methods used to measure ADP and ATP production must be viewed with extreme caution. Most of these techniques measure only synthesised ATP, rather than the level of available to the cells. For this reason it seemed important to determine the rate of ATP export from the mitochondria, rather than the ability of those organelles to generate ADP to ATP through oxidative phosphorylation. Measurement of mitochondrial ATP export also compensates for the energy losses involved in the transport of ATP across the mitochondrial membrane. It is obviously of equivalent importance that experiments designed to measure the export of ATP from mitochondria should involve only intact organelles. In the work reported here, 98% of the normal, and 96.5% of the CMS mitochondria were found to be intact. Since this study essentially involves the measurement of mitochondrial performance, ATP production must clearly be related to the mitochondria present. For this reason, it would be foolish to consider ATP production in terms of total protein. Instead, we considered that the cytochrome C content of the extract was probably as good an indication as any of the numbers of mitochondria present.

The level of ATP exported from mitochondria of normal and CMS plants for given ADP concentrations is shown in Fig. 1. The two curves labelled *1* reveal the levels of ruptured organelles from both types of plants to be very similar. However, the two curves labelled *2* suggest that the export of ATP from intact mitochondria is far more efficient in normal than in CMS plants. The curves labelled *3* show that if the ADP substrate is not added to the reaction mixture, the ATP exported from the normal and CMS organelles is very low indeed. It must be strongly emphasised that these results may be affected by a number of factors. However, the two controls – curves *1* and *3* – together with the striking difference in export rate from intact organelles of normal and CMS plants would suggest that far less ATP is exported from mitochondria extracted from CMS lines of *Petunia hybrida*. Results from this experiment do not point unambiguously to any reason for this low level of export, but some lack of efficiency in the ADP-ATP translocator might produce this effect.

To investigate these possibilities further, the 'Km' value of the ADP-ATP translocator in CMS and normal mitochondria was determined. As is the case when the Km is determined for an enzyme, it is a parameter independent of environmental factors (Dixon 1979). Likewise, in this experiment, the 'Km' value is completely indepen-

dent of ADP levels within the mitochondria, and is dependent solely on the structure and properties of the ADP-ATP translocator and its environment within the membrane. There is, of course, the possibility that this 'Km' could be modified by the effect of temperature changes on the inner membrane, but these factors are unlikely to be significant during the experimental period. The method employed here effectively measures the affinity of the translocator for mitochondrial ADP and ATP as it transports ATP from the matrix into the cytosol. An alternative approach to the same problem involved using the methods of Pfaff (1969) and Weidemann (1970). Here the 'Km' value is determined by a radiotracer method, and uses the ADP-ATP translocator to transport ^{14}C-ATP from the cytosol into the matrix. It is, of course, quite possible that receptor affinity for both ATP and ADP on the component of the translocator located on the outer face of the inner membrane (C site), differs from that of the inner face (M site) (Klingenberg 1976). We readily agree that neither of these methods of measuring the translocators affinity for ATP is ideal, but the results obtained should reveal any gross differences in affinity between translocator molecules from normal and CMS lines of *Petunia*.

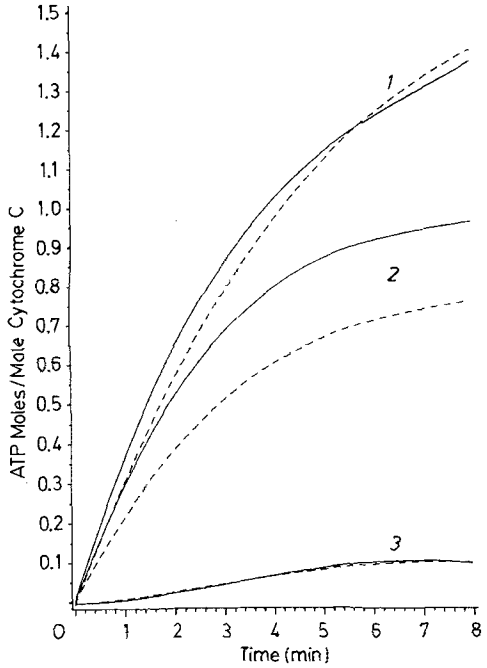

Fig. 1. ATP exported by mitochondria extracted from fertile and CMS lines of *Petunia hybrida*; *1* ruptured mitochondria, *2* intact mitochondria, *3* intact mitochondria without ADP substrate; —— male fertile plants, – – – CMS plants

Using the first methods the 'Kms' obtained for the translocator in normal and CMS plants are shown in Fig. 2. A 'Km' of 161.3 μM ADP for the translocator in CMS, and 87.3 μm ADP in normal plants is indicted by these graphs. Using the second method, the translocator in CMS plants emerges with a 'Km' of 200 μM ATP, while that of normal plants is far less at 100 μM ATP (Fig. 3). Although the two methods provide different data, in both cases the 'Km' of the CMS translocator is approximately twice as high as that of normal plants. Since a high Km value indicates a low substrate affinity, it is reasonable to conclude that the affinity of the ADP-ATP translocator for both ATP and ADP in normal plants is approximately twice as high as that found in CMS lines, an inference which correlates well with our observations on ATP export. Normally, the affinity of an enzyme for a substrate is dependent solely upon the structure of the enzyme itself, particularly the binding and reacting sites. A different situation may well pertain in the

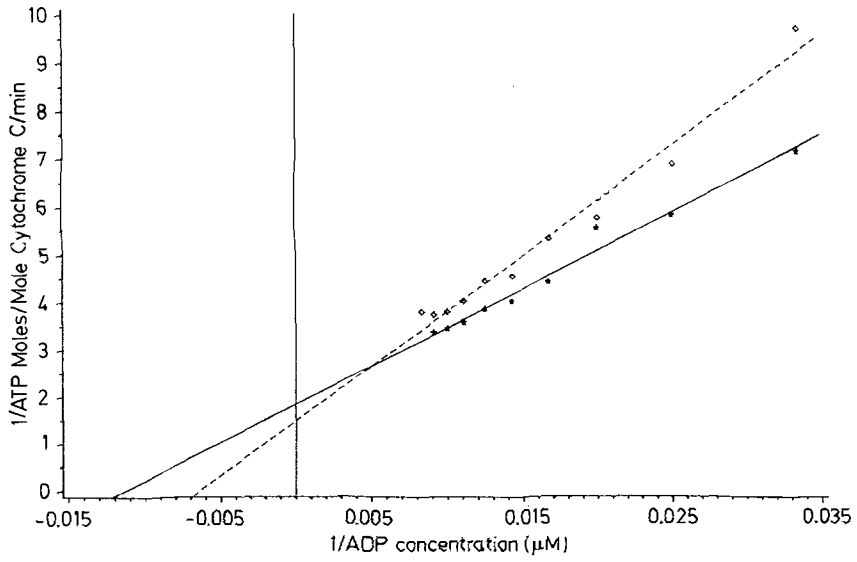

Fig. 2. Using the 'ATP/NADPH' method (see text □), double reciprocal plots indicating 'Km' values for the normal and CMS mitochondrial ADP-ATP translocators; —— male fertile plants ('Km' = 87.3 μM ADP), – – – CMS plants ('Km' = 161.3 μM ADP)

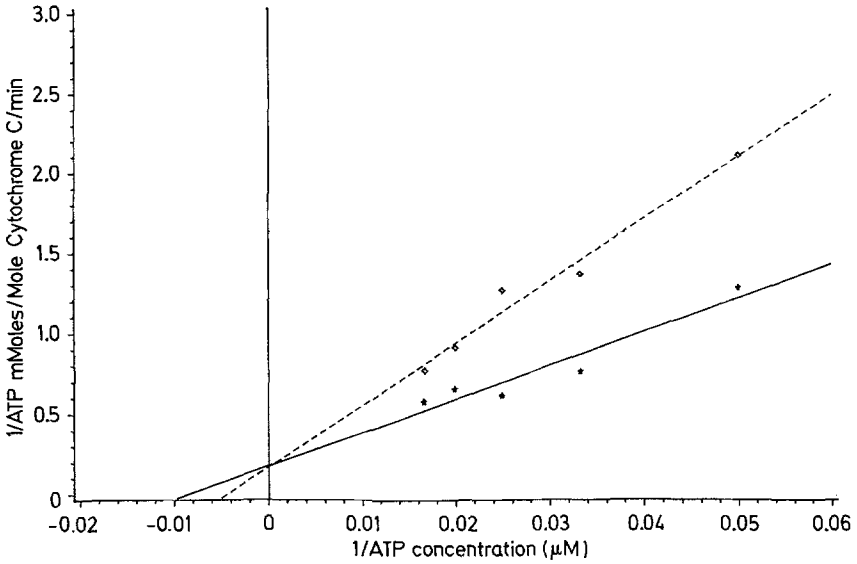

Fig. 3. Using the ^{14}C-ATP method (see text □), double reciprocal plots indicating 'Km' values for the normal and CMS mitochondrial ADP-ATP translocators; —— male fertile plants ('Km' = 100 μM ATP), – – – CMS plants ('Km' = 200.0 μM ATP)

case of the ADP-ATP translocator, which is associated with the other constituents of the inner mitochondrial membrane (Klingenberg 1979; Vignais 1976). Here the 'reacting site' of the translocator appears to be intimately associated with the inner membrane, with only the binding site operating independently. Thus, the low affinity of the ADP-ATP translocator for ATP and ADP in CMS mitochondria may be caused either by an alteration in translocator structure, or the interaction between this molecule and components of the inner membrane.

Further study of the results shown in Figs. 2 and 3 suggests that the decrease in affinity for both ATP and ADP by the CMS translocator resembles very closely that of normal enzymes in the presence of competitive inhibitors (Michal 1983). If this is the case the difference cannot have resulted from either low numbers of translocator molecules, or the decrease in a membrane area. Perhaps the four most likely reasons for an apparent competitive inhibition of the translocator in CMS lines are as follows:

(a) some structural modifications to the nuclear encoded translocator within the mitochondria.

(b) a change in organisation of the inner membrane proteins.

(c) the presence of nucleotide analogues in the mitochondria and

(d) differences in the electrical potential of the mitochondrial inner membrane.

Of the four possibilities, (a) and (b) are the most attractive. The ADP-ATP translocator is an unusual protein for, after the transcription and translation of nuclear genes, the precursor molecule – a two subunit protein – is transferred to the inner membrane of the mitochondria (Pfanner 1985, 1987). In the course of this procedure this precursor undergoes a number of conformational changes as it enters the bimolecular leaflet (Pfanner 1987). It is considered that the structure and properties of the functional ADP-ATP translocator is dependent not only upon the protein structure of the molecule per se, but also on its association with the complex architecture of the mitochondrial inner membrane. Thus, the conformational changes that occur to the translocator as it inserts into the inner membrane are essential for its activity, substrate affinity, and specificity. Significantly, many properties of the inner mitochondrial membrane are under the direct control of mitochondrial genes. A number of properties of the translocator – although encoded by the nuclear genes – may therefore be affected by changes in the mitochondrial genome.

If is, of course, valid to question whether the measurement of a 'Km' for the translocator by the method described here is an effective measurement of the molecule's efficiency in situ. Interestingly, work on the genetic modification of the mitochondrial translocator in *Saccharomyces cerevisii* by Mattoon et al. (1979) has been based on the same approach, and different 'Kms' have been obtained for the translocator in normal cells and in mutants with lesions in the genes coding for aspects of mitochondrial membrane structure.

Since it thus seems that the translocator in the mitochondrial membranes of CMS plants is less "efficient" than its counterpart in fertile lines, there is a strong likelihood that the tapetal cells in the CMS anther would be incapable of maintaining the high ATP/ADP ratio required during the early stages of meiosis. If is, of course, possible, that some unusual requirement is also made of the mitochondria of the pollen mother cells, but there is little structural evidence of this (Liu et al. 1987). One of the principle activities of the tapetal cells at this time is noted to be the synthesis of DNA, and this process would

probably be the first casualty of an inadequate ATP/ADP ratio and the consequent lowering of NADPH levels. Work is currently being carried out firstly to establish the molecular basis of the translocator inefficiency in CMS lines, and, secondly, to demonstrate that the tapetal cells of these plants are incapable of meeting the demands for energy put upon them early in meiosis.

Acknowledgements. This work was supported in part by the UK Agricultural and Food Research Council. The authors also wish to thank Prof. C. J. Leaver for a number of helpful discussions throughout the research.

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