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Mitochondria-targeted GFP highlights the heterogeneity of mitochondrial shape, size and movement within living plant cells

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Abstract

Little is known concerning the heterogeneity of mitochondrial shape, size, number, cytoplasmic distribution, and motility *in planta*. Ultrastructural studies using the electron microscope have shown a variety of mitochondrial shapes and sizes within fixed cells, however, it is not possible to dismiss the possibility that any heterogeneity observed resulted from preparation or fixation artefacts. Unambiguous demonstration of the extent and nature of mitochondrial heterogeneity *in vivo* necessitates the use of a truly *in vivo* mitochondrial detection system. Green fluorescent protein is an excellent *in vivo* marker for gene expression and protein localization studies. It is particularly useful for real-time spatiotemporal analysis of intracellular protein targeting and dynamics and as such is an ideal marker for analysing mitochondria *in planta*. Stably transformed *Arabidopsis* lines have been generated with GFP targeted to the mitochondria using either of two plant mitochondrial signal sequences from the β-ATPase subunit or the mitochondrial chaperonin CPN-60. Mitochondrially targeted GFP, which is easily detectable using an epifluorescent or confocal microscope, highlights heterogeneity of mitochondrial shape, size, position, and dynamic within living plant cells.

Key words: Mitochondria-targeted GFP, subcellular dynamics, mitochondrial heterogeneity.

Introduction

Mitochondrial morphology and dynamics have been studied extensively in non-plant eukaryotic cells and are known to vary considerably depending on the cell type and organism studied. Shapes range from the classic textbook oval, to spherical, to elongated worm-like or even branched structures. Lewis and Lewis, using simple bright field microscopy, were among the first to describe mitochondrial shape changes and movement (Lewis and Lewis, 1914). Since that time there have been numerous studies, particularly on animal cells, investigating mitochondrial polymorphism and behaviour (for a review see Tyler, 1992). However, even in these systems many questions, for example, the mechanisms underlying mitochondrial movements and whether morphological heterogeneity is concomitant with functional heterogeneity, remain unresolved (Bereiter-Hahn and Voth, 1994). Few studies have been conducted on higher plants and therefore little is known about morphological polymorphism and the dynamics of plant mitochondria.

Green fluorescent protein from the jellyfish *Aquorea victoria* has become a very popular reporter protein for studies of plant gene expression. Its unique property in forming a chromophore of three amino acids within its primary structure requiring no co-factors or substrates other than molecular oxygen makes it a perfect marker for *in vivo* studies *senso stricto*. In particular, GFP is an ideal reporter for monitoring dynamic processes within a cell or organism. In the past four years GFP has been used very successfully as a reporter for gene expression and protein trafficking in living cells (Chalfie and Kain, 1998). During this time improvements over wild-type GFP have been engineered, differing in their excitation and emission wavelengths, stability and quantum yield, and specific mutants have been developed for expression *in planta* (for a review see Haseloff and Siemering, 1998).

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Using these plant-optimized proteins, GFP has been targeted to four intracellular compartments within the plant cell: endoplasmic reticulum, nucleus, chloroplast, and mitochondria. Previous reports resulting from the targeting of GFP to plant mitochondria (Kohler *et al*., 1997; Niwa *et al*., 1999) have concentrated on the successful targeting and observation of GFP within mitochondria. No data have been presented on the fascinating insight into mitochondrial morphological heterogeneity and dynamics that is possible using this technology.

Materials and methods

Construct assembly

cDNAs encoding the mitochondrial presequences from *Arabidopsis thaliana* CPN-60 (nucleotides 74–186) and *Nicotiana plumbaginifolia* β-ATPase (nucleotides 387–666) were PCR-amplified using primers containing 5′-*BamH*I and 3′-*Spe*I restriction enzyme sites. pBIN*mGFP5*-ER was a kind gift of Dr J Haseloff. The *mGFP5* sequence was PCR-amplified clear of the chitinase signal sequence and HDEL ER retention sequence using a 5′ primer containing a *SpeI* site and translation start codon and a 3′ primer containing a 3′-*Sac*I site and a stop codon. Presequence cDNA and the *mGFP5* gene were ligated into the *BamH*I and *Sac*I sites within pBIN121 downstream of the CaMV-35S promoter in place of the GUS gene to create pBINmgfp5-cpn60 or pBINmgfp5-atpase. *Agrobacterium tumefaciens* was transformed with these vectors using standard techniques.

Plant transformation

Wild-type *Arabidopsis thaliana* (ecotype Columbia) plants were grown under constant environment conditions (20 °C, 16/8 h light/dark). Plants were transformed by *Agrobacterium*-mediated transformation using vacuum infiltration essentially as described previously (Bechtold *et al*., 1993). Seeds (T2) from these plants were collected, stratified, surface-sterilized, and germinated on selection media containing 50 μg ml^{-1} kanamycin and 200 μg ml^{-1} timentin. An identical procedure was used for the production of further generations. Highly-fluorescent T2 seedlings were identified by epifluorescent microscopy (see below) and grown to seed for the production of further generations. Results are presented from the analysis of T2, T3 or T4 generations.

Visualization of mitochondria in vivo

Epifluorescent microscopy was performed using a Leica DM RB microscope equipped with DAPI (UV) and FITC (blue) filter cubes. Tissue samples were simply mounted in distilled water on a glass slide and overlaid with a coverslip. Confocal microscopy was performed using a BioRad MRC-600 confocal laser-scanning microscope in fluorescent mode. This was attached to a Nikon microscope equipped with a 60× NA 1.4 oil immersion objective. The excitation wavelength was set at 488 nm. Images of GFP fluorescence only were captured using a LP515 nm filter. For dual channel imaging of GFP and chlorophyll autofluorescence a 522/35 nm band pass filter was used for the green channel with a 605/40 nm band pass filter for the red channel.

Intracellular localization

Leaves from T3 plants transformed with pBINmgfp5-atpase were homogenized in 0.3 M sucrose, 50 mM MOPS pH 7.8, 5 mM $MgCl_2$, 2 mM EDTA, 1% (w/v) BSA, 0.6% (w/v) PVP-40, and 20 mM cysteine. The post-5000 *g* supernatant was layered onto a 38 ml linear 30–50% (w/w) sucrose gradient, centrifuged at 100 000 *g* for 2 h and fractionated into 1.5 ml fractions. Aliquots of each fraction were mixed with Laemmli sample buffer and separated by SDS-PAGE. After Western blotting, immunodetection was carried out using polyclonal antisera raised against GFP (1:100, Clontech), the mitochondrial adenine nucleotide transporter (ANT) (1:5000) or mitochondrial HSP-70 (1:5000).

Results

CPN-60 or β-ATPase presequences target GFP to mitochondria

Positive transformants, irrespective of construct, were identified by highly visible punctate green fluorescence when viewed by epifluorescent microscopy using FITC (Fig. 1A) or DAPI filter cubes (not shown). Although mGFP5 is not the most highly-fluorescent GFP mutant available, use of the CaMV 35S RNA promoter was sufficient for expression levels high enough to enable easy visualization of the GFP signal in cells of the hypocotyl and root (Fig. 1) and even against the high chlorophyll autofluorescence of leaf cells (not shown).

Green fluorescence was restricted to numerous spherical, sausage-shaped or spaghetti-like bodies within all leaf and root cell-types examined. In order to confirm that these bodies were mitochondria, subcellular fractionation was performed to enable co-localization of GFP with native mitochondrial proteins. As the data in Fig. 2 show, GFP and the two mitochondrial proteins mit-HSP70 and ANT co-locate at a peak density of 42–45% (w/w) sucrose confirming that stable transformants expressing mitochondrial-targeted GFP (mit-GFP) have been produced.

Mitochondrial expressed GFP highlights mitochondrial number, position, shape, and dynamics

One of the most useful features of GFP is its ability to be used as an *in vivo* marker in real time without causing any perceptible tissue damage. Three facts are apparent when viewing mitochondria expressing GFP: (i) mitochondrial are not distributed evenly within the cytoplasm; (ii) mitochondria are not all the same size or shape; and (iii) mitochondria can be highly dynamic structures, changing shape and position within a second. The GFP signal within *Arabidopsis* tissue is most clearly viewed by epifluorescence microscopy of hypocotyl and root tissues where there are few or no chloroplasts. These tissues also produce clear confocal images due to the longitudinal orientation of the cells. While GFP-tagged mitochondria are visible in stem and leaf tissue using epifluorescent

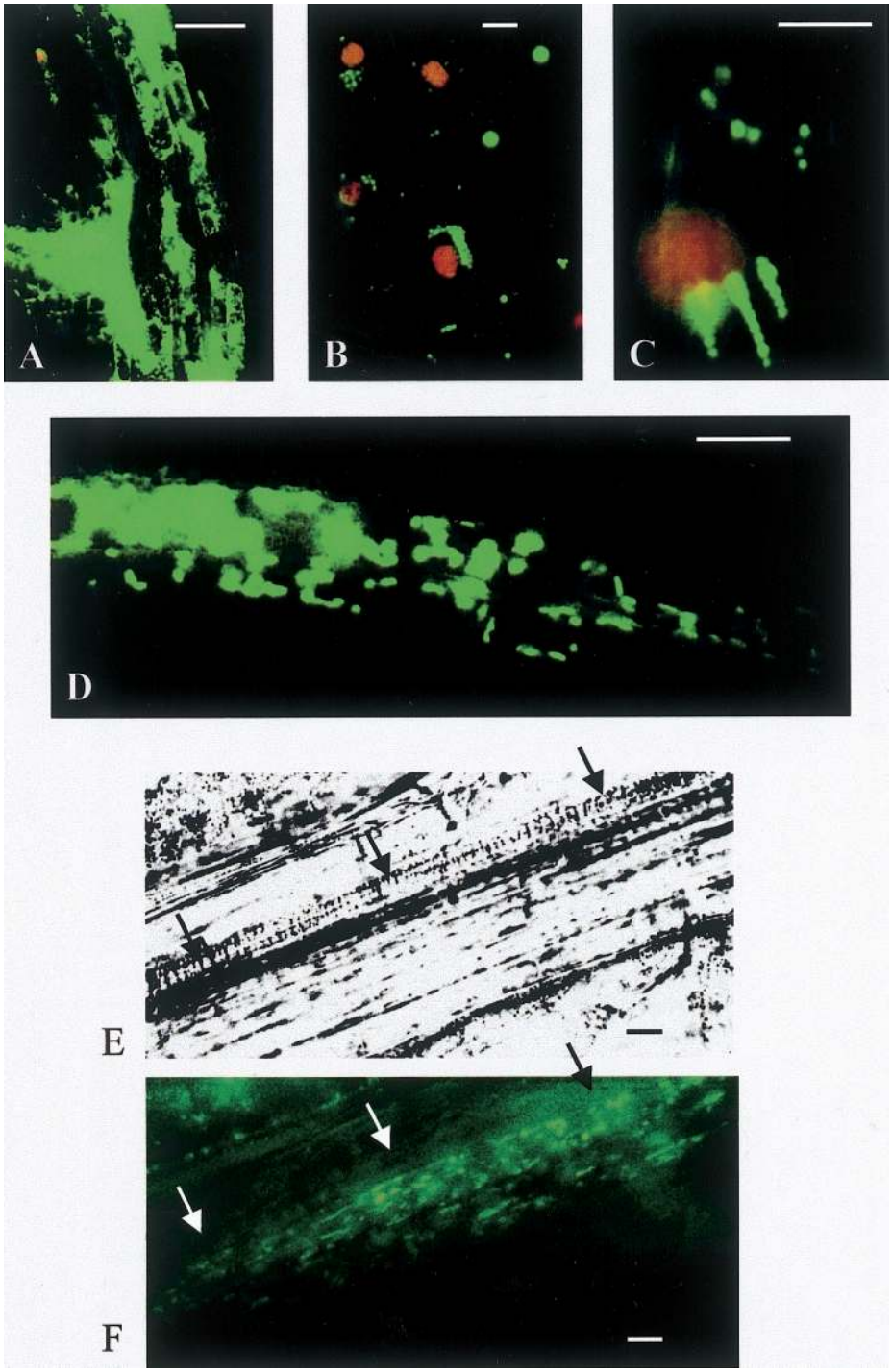

Fig. 1. Epifluorescent micrographs of mit-GFP *Arabidopsis* viewed using the FITC filter set. (A) Low magnification image of root of T2 plant transformed with pBIN*mgfp5-cpn60*. Scale bar = 50 μm. (B) Mitochondria (green) and chloroplast (red) within a cell from the hypocotyl of a plant transformed with pBIN*mgfp5-atpase* (line 4/3/A/9). Scale bar = 5 μm. (C) Another hypocotyl cell from a plant line 4/3/A/9. Scale bar = 5 μm. (D) Sausage-shaped mitochondria within the vasculature of a plant of line 4/3/A/9. Scale bar = 5 μm. (E) Bright-field image of a section of root from line 4/3/A/9 showing position of vascular bundle: arrows show the position of the xylem tissue identifiable by lignification. (F) Epifluorescent image of the section shown in (E), the long worm-like mitochondria are located within cells of the vasculature. Scale bar in (E) and (F) = 5 μm.

microscopy, interference from chloroplast autofluorescence produces a washed-out image of the GFP signal. Within epidermal and cortex cells the mitochondria are restricted to a narrow band of cytoplasm appressed to the plasma membrane along with other cytosolic components due to the presence of the large central vacuole (Fig. 3). Within this limited area mitochondria frequently show a clumped distribution. In cells of the hypocotyl, for

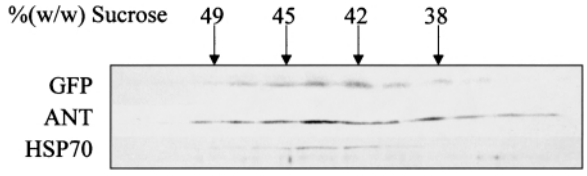

Fig. 2. Co-localization of GFP and two matrix-localized mitochondrial proteins. Subcellular fractionation was performed on sucrose gradients followed by SDS-PAGE electrophoresis of gradient fractions, protein blotting and probing with polyclonal antisera raised against GFP, the adenine nucleotide transporter (ANT) and the mitochondrial chaperonin HSP-70.

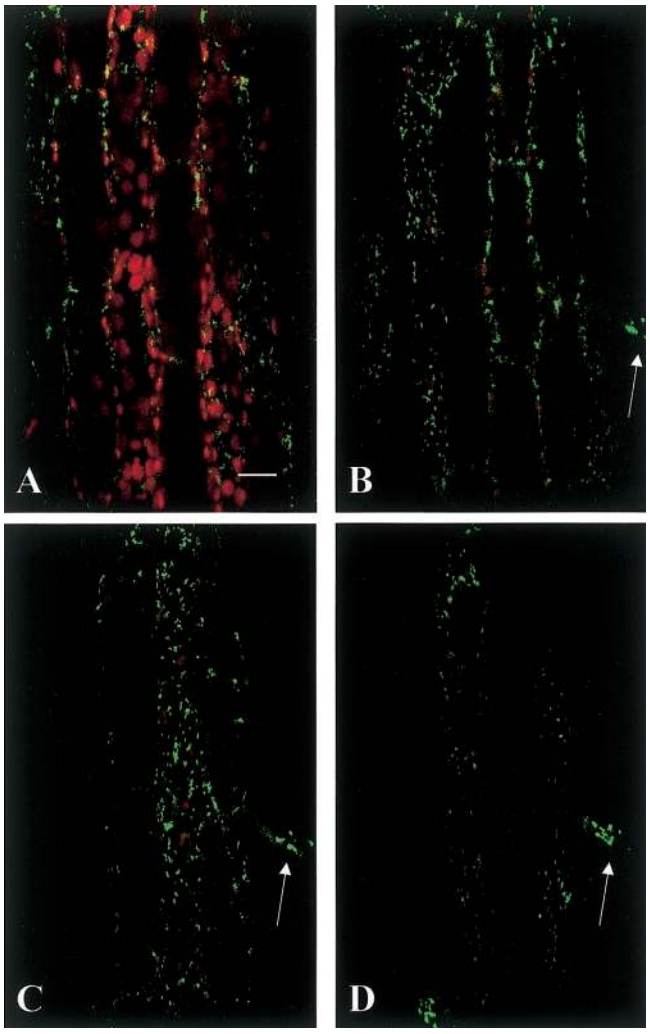

Fig. 3. Confocal microscope images of the hypocotyl/root region of a mit-GFP (pBIN*mgfp5-atpase*) transformed *Arabidopsis* seedling. Optical sections (B), (C) and (D) are of the same region as (A), but 5, 10 and 15 μm deeper into the tissue, respectively. The arrow highlights a root hair. The images have been false coloured: GFP signal, green; chlorophyll autofluorescence, red. Scale bar = 10 μm.

example, where only a few chloroplasts are visible, mitochondria often appear to aggregate around the chloroplast either in clumps or lines (Fig. 1 B, C). Mitochondria in *Arabidopsis* also show heterogeneity in size and shape. From these observations mitochondria are typically spherical structures of uniform diameter (Fig. 1B, C). However, in certain cell types within the vascular tissue mitochondria range from sausage-shaped (Fig. 1D) to longer worm-like forms (Fig. 1F).

Mitochondria are highly dynamic organelles undergoing rapid movement within the cell. Movements range from small continuous oscillations of no more than a few hundred nanometres to large-scale displacements of tens of micrometres in seconds (Figs 4, 5 and data not shown). Within a cell, neighbouring mitochondria are capable of independent movement, one may oscillate or undergo rapid larger scale movements whilst others are static (Fig. 4). The worm-like mitochondria were observed to be particularly dynamic being in constant motion, undergoing snake-like turning and folding movements (data not shown).

Discussion

Previous studies investigating mitochondrial morphology and behaviour have been hampered by limitations of the available technology. All methods required preparation of the tissue or resulted in damage to it. The earliest studies made use of bright field microscopy, however, contrast was very low and could only be increased by diffraction which limited resolution. Newer observation methods such as phase contrast required relatively high light intensities that were damaging to cells. Prior to GFP the most advanced techniques for the study of mitochondria behaviour in living cells made use of fluorescence chromophores such as rhodamine-123. However, use of rhodamine-123 also involves invasive procedures and thus observations are not free from possible artefacts. In contrast, GFP is a true *in vivo* marker, its expression allows real time analysis of subcellular events, it is highly stable and relatively non-toxic compared to alternative fluorescent dyes.

Expression studies with wild-type GFP, while successful in some plants, failed in *Arabidopsis* (Haseloff and Amos, 1995; Hu and Cheng, 1995; Sheen *et al*., 1995). Haseloff and Amos were the first to demonstrate efficient expression in *Arabidopsis* when they discovered that it was necessary to destroy a cryptic intron within the wild-type *gfp* gene by changing codon usage to create *mgfp*4 (Haseloff and Amos, 1995). The highly active version of GFP used in this study, GFP5 differs from GFP4 and the commonly-used GFP(S65T) by having two excitation peaks (maxima at 395 and 473 nm) and a thermotolerant phenotype. These properties confer several advantages for the screening of bacterial and plant transformants using a long-wavelength UV lamp (Haseloff and Siemering, 1998). In this study, expression of GFP5 driven by a single CaMV-35S promoter was sufficient for easy detection of fluorescence in transformed *Arabidopsis* tissues using an epifluorescent microscope equipped with

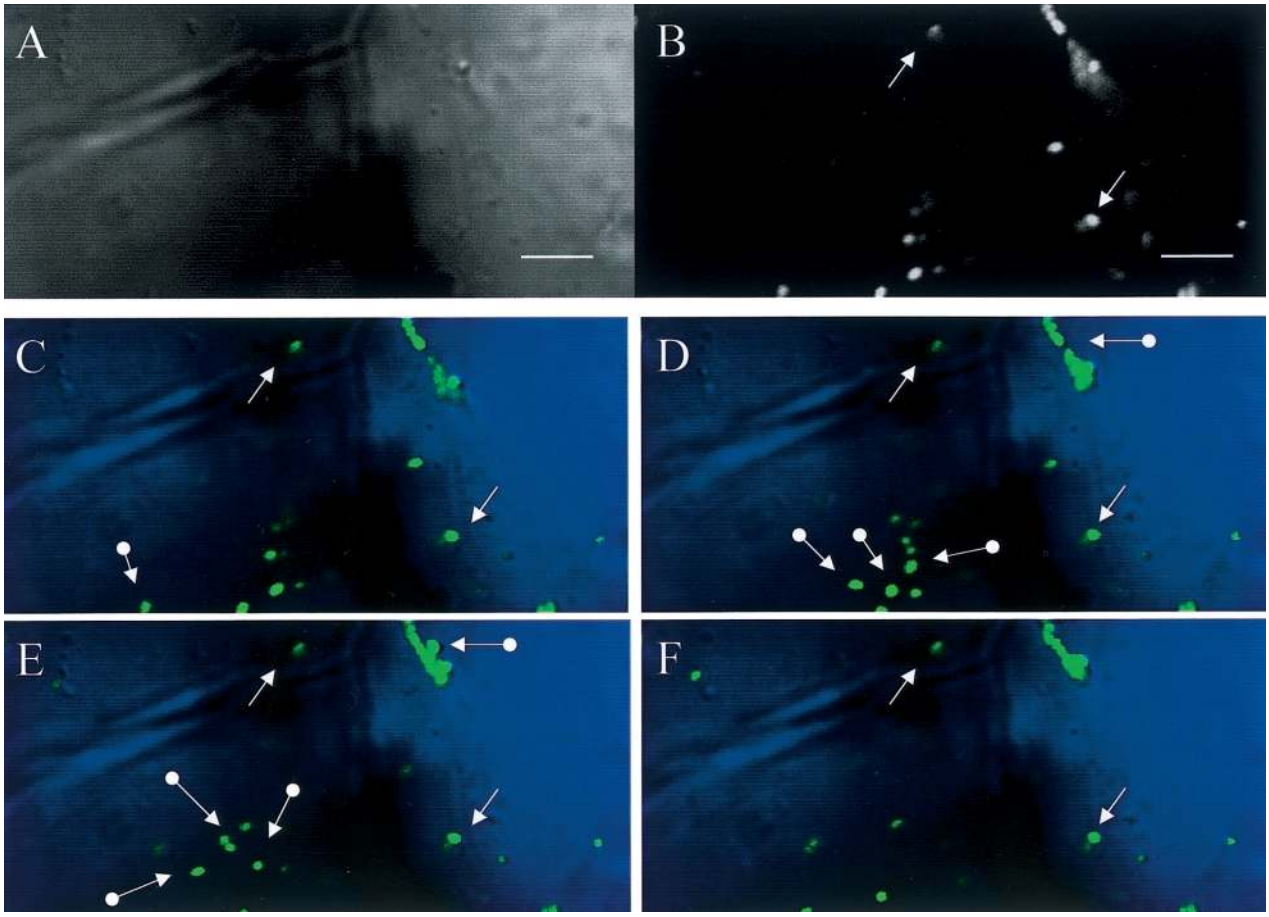

Fig. 4. Movement of leaf mitochondria in real time. Confocal images of epidermal cells of a leaf of the *mgfp5-atpase* line 4/3/A/9. (A) Bright-field image of the leaf surface; (B) the corresponding confocal image. (C) Image as (B), false coloured for the GFP signal (green) and leaf surface (blue). (D, E, F) Images of the same region captured 2, 4 and 6 s later, respectively. The plain arrows indicate examples of non-motile mitochondria, while those starting with a solid circle indicate examples of mitochondria that have moved between the capture of that image and the proceeding one. Scale bar = 2.5 μm.

DAPI or FITC filter cubes. The ability to use both wavelengths is particularly useful for minimizing interference from autofluorescence.

The observations of intracellular location and polymorphism of mitochondria described in this paper reflect those first made in studies using animal cells. Early reports of mitochondrial dislocation, shape changes, fission, and fusion described previously (Lewis and Lewis, 1914) have been followed by more in-depth studies relating mitochondrial dynamics to cellular processes. Mitochondria are known to associate with energy-consuming organelles and structures; in particular, around the base of sperm flagella (Bawa and Werner, 1988) and other structures of similar function such as in algal zoospores (Hatano and Ueda, 1988). Similarly, associations with the nuclear envelope, rough endoplasmic reticulum and plasma membrane have all been described (for a review see Bereiter-Hahn, 1990). It is assumed that such associations overcome limitations on the diffusion of adenine nucleotides possibly due to microcompartmentation (Friedrich, 1985). It is suggested that the observations from this study of the association of mitochondria with chloroplasts provide another example of this phenomenon. Apart from the exchange of adenine nucleotides, which may be particularly important for chloroplasts in the hypocotyl where photophosphorylation may be limited, it is likely that mitochondria group around chloroplasts due to oxygen and carbon dioxide gradients. The activity of such gradients has been inferred by other authors (Bereiter-Hahn and Voth, 1994) with the suggestion that mitochondria cluster around the nucleus to create hypoxia to reduce the risk of free radical attack. It is also possible that the clustering of mitochondria around chloroplasts establishes gradients of metabolites interchanged between the two.

Mitochondrial polymorphism and motility have also been related to the energy status of the organelle. Mitochondria are considered to exist in one of two conformational states, orthodox or condensed, depending on their internal morphology. In the condensed state the intracristal spaces are dilated and the matrix is electron

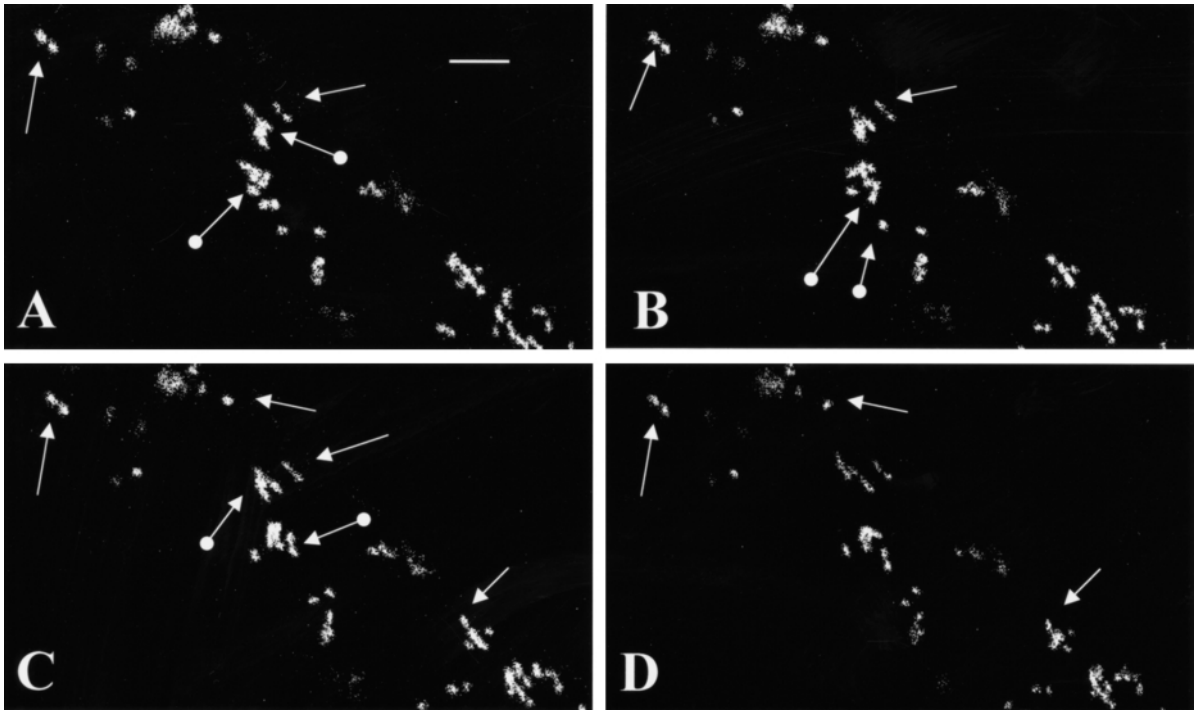

Fig. 5. Movement of root mitochondria in real time. Confocal images of a root cortex cell of the *mgfp5-atpase* line 4/3/A/9. (A, B, C, D) Images captured 2 s apart. The plain arrows indicate examples of non-motile mitochondria, while those starting with a solid circle indicate examples of mitochondria that have moved between the capture of that image and the proceeding one. Scale bar = 2.5 μm.

dense when viewed with the electron microscope. Conversely, in the orthodox state the matrix is expanded and less dense. Switching between these two states is modulated by the metabolic state of the mitochondria. Injection of ADP into a living cell causes mitochondria to adopt a condensed state while ATP causes an orthodox state (Bereiter-Hahn and Voth, 1993). Furthermore, in the condensed state the mitochondria become immobilized while in the orthodox state they are highly motile. It has been reported that variability in mitochondrial shape and size was particularly apparent in yeast cells where changes in mitochondrial proliferation occurs in response to changing environmental conditions such as oxygen availability, and carbon source (Van Der Klei *et al*., 1994). Changes in the external morphology of mitochondria are caused by rearrangement of cristae and likely result from changes in conformational state (Bereiter-Hahn, 1990; Tyler, 1992). It is therefore tempting to speculate that the largely immobile spherical mitochondria shown in Fig. 1A and B were in the condensed state while the sausage-shaped and especially the highly animated worm-like mitochondria (Fig. 1F and data not shown) were in the orthodox state. As shown in Fig. 1E and F the worm-like mitochondria are found in cells within the vascular parenchyma. Such elongated mitochondria have also been shown within the phloem parenchyma of pea leaf (Tobin *et al*.(1989). Further work is required to determine whether the worm-like morphology of these mitochondria, putatively assigned to the orthodox state, results from the presence of a high ATP concentration in the surrounding cytoplasm within these specialized and highly energetic cells.

Very little is known about the genetic control of plant mitochondrial development, morphology and number; studies in these areas are hampered by hurdles to viewing mitochondria *in vivo*. In contrast, studies on chloroplast development benefit from the greater size of these organelles and simple staining procedures (Pyke and Leech, 1991). Stably transformed *Arabidopsis* lines expressing GFP targeted to the mitochondria provide a potentially very useful resource for identifying the physiological and genetic factors controlling mitochondrial shape, size and number. For example, mutagenesis of these lines is likely to result in the production of mitochondrial shape, size and number mutants that would be easily identifiable by fluorescence microscopy linked to image analysis. With the *Arabidopsis* genome-sequencing project nearing completion such a reverse genetics approach would provide a relatively simple route to the cloning of the relevant gene or genes responsible for the mutant mitochondrial phenotypes.

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References

Bawa SR, Werner G. 1988. Mitochondrial changes in spermatogenesis of the pseudoscorpion *Diplotemnus* sp. *Journal of Ultrastructure and Molecular Structure Research* **98,** 281–293.

Bechtold N, Ellis J, Pelletier G. 1993. *In planta Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *Critical Reviews of the Academy of Sciences Paris, Life Sciences* **316,** 1194–1199.

Bereiter-Hahn J. 1990. Behaviour of mitochondria in the living cell. *International Review of Cytology* **122,** 1–63.

Bereiter-Hahn J, Voth M. 1983. Metabolic control of shape and structure of mitochondria *in situ*. *Biology of the Cell* **47,** 309–322.

Bereiter-Hahn J, Voth M. 1994. Dynamics of mitochondria in living cells: shape changes, dislocations, fusion and fission of mitochondria. *Microscopy Research and Technique* **27,** 198–219.

Chalfie M, Kain S. 1998. *Green fluorescent protein: properties, applications and protocols*. New York: Wiley-Liss, Inc.

Friedrich P. 1985. Dynamic compartmentation in soluble multienzyme systems. In: Welch GR, Orlando E, eds. *Organized multienzyme systems*. New York: Academic Press, 141–176.

Haseloff J, Amos B. 1995. GFP in plants. *Trends in Genetics* **11,** 328–329.

Haseloff J, Siemering KR. 1998. The uses of green fluorescent protein in plants. In: Chalfie M, Kain S, eds. *Green fluorescent protein: properties, applications and protocols*. New York: Wiley-Liss, Inc., 191–220.

Hatano K, Ueda K. 1988. Changes in the shape of mitochondria during the asexual reproductive cycle in *Hydrodictyon reticulatum*. *European Journal of Cell Biology* **47,** 193–197.

Hu W, Cheng CL. 1995. Expression of *Aequorea* green fluorescent protein in plant cells *FEBS Letters* **369,** 331–334.

Kohler RH, Zipfel WR, Webb WW, Hanson MR. 1997. The green fluorescent protein as a marker to visualise plant mitochondria *in vivo*. *The Plant Journal* **11,** 613–621.

Lewis MR, Lewis WH. 1914. Mitochondria (and other cytoplasmic structures) in tissue cultures. *American Journal of Anatomy* **17,** 339–401.

Niwa Y, Hirano T, Yoshimoto K, Shimizu M, Kobayashi H. 1999. Non-invasive quantitative detection and applications of non-toxic, S65T-type green fluorescent protein in living plants. *The Plant Journal* **18,** 455–463.

Pyke KA, Leech RM. 1991. Rapid image analysis screening procedure for identifying chloroplast number mutants in mesophyll cells of *Arabidopsis thaliana* (L.) Heynh. *Plant Physiology* **96,** 1193–1195.

Sheen J, Hwang SB, Niwa Y, Kobayashi H, Galbraith DW. 1995. Green fluorescent protein as a new vital marker in plant cells. *The Plant Journal* **8,** 777–784.

Tobin AK, Thorpe JR, Hylton CM, Rawsthorne S. 1989. Spatial and temporal influences on the cell-specific distribution of glycine decarboxylase in leaves of wheat (*Triticum aestivum* L.) and pea (*Pisum sativum* L.). *Plant Physiology* **91,** 1219–1225.

Tyler DD. 1992. *The mitochondrion in health and disease*. Weinheim: VCH Publishing.

Van Der Klei IJ, Veenhuis M, Neupert W. 1994. A morphological view on mitochondrial protein targeting. *Microscopy Research and Technique* **27,** 284–293.