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Matrix Attachment Region Binding Protein MFP1 Is Localized in Discrete Domains at the Nuclear Envelope

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Recently, it has been suggested that nuclear processes, such as replication, transcription, and splicing, are spatially organized and associated with a nuclear framework called the nuclear matrix, a structure of unknown molecular composition. It has been shown that chromatin is attached to the nuclear matrix via specific DNA fragments called matrix attachment regions (MARs). We have begun to dissect the plant nuclear matrix by isolating a DNA binding protein with specific affinity for MARs. Here, it is shown that MAR binding filament–like protein 1 (MFP1) is associated with speckle-like structures at the nuclear periphery that are part of isolated nuclei and the nuclear matrix. A predicted N-terminal transmembrane domain is necessary for the specific targeting of MFP1 to the speckles, indicating an association with the nuclear envelope–endoplasmic reticulum continuum. In addition, it is shown that a marker protein for plant microtubule organizing centers, which has been shown to be localized on the outside of the plant nuclear envelope, is also part of the nuclear matrix. These findings indicate a close and previously undescribed connection in plants between the nuclear envelope and the internal nuclear matrix, and they suggest a function for MFP1 in attaching chromatin to specific sites at the nuclear periphery.

INTRODUCTION

The nuclear matrix hypothesis proposes a structural framework for the eukaryotic nucleus that is similar to the cytoskeleton. Biochemically, the nuclear matrix is defined as the insoluble material that remains after extraction of nuclei with high-salt solutions (Berezney and Coffey, 1974) or with the chaotropic agent lithium diiodosalicylate (Mirkovitch et al., 1984) and treatment with DNases. Electron microscopy has shown a network of fibers of ~10 nm in diameter. These fibers resemble the intermediate filaments of the cytoskeleton. Because they can be detected only under certain preparation conditions (He et al., 1990), their in vivo existence has been somewhat controversial.

The isolated nuclear matrix binds specifically to certain DNA elements called matrix attachment regions (MARs). MARs are generally AT-rich DNA sequences that are several hundred base pairs long and are localized in the noncoding regions of the DNA, often flanking genes (Gasser and Laemmli, 1987). MARs have a positive effect on gene expression (Allen et al., 1993, 1996; Mlynárová et al., 1996) that is believed to be due to the establishment of independent and transcriptionally active chromatin domains between two MARs (Spiker and Thompson, 1996).

Although the nuclear matrix was identified more than 20 years ago (Berezney and Coffey, 1974), none of its structural components has been isolated and molecularly characterized from any organism. One strategy to identify such components has been to isolate proteins that specifically bind to MARs. A small number of MAR binding proteins have been purified and cloned from animals, and they subsequently have been shown to be localized in the nuclear matrix (von Kries et al., 1991; Dickinson et al., 1992; Renz and Fackelmayer, 1996; Göhring et al., 1997). Thus far, a single MAR binding protein has been identified from plants (Meier et al., 1996). In contrast to the animal proteins, the plant protein MFP1 (for MAR binding filament–like protein 1) has a filament protein–like structure that makes it a good candidate for a MAR binding constituent of the observed nuclear filaments.

In addition to the animal MAR binding proteins isolated by their DNA binding activity, some filament-like proteins, which had been identified previously in a different context, subsequently have been shown to have MAR binding activity. The nuclear lamins are a group of intermediate filament proteins that constitute the nuclear lamina, which is a filamentous protein network that lines the inner membrane of the animal nuclear envelope (McKeon et al., 1986). It has been shown that rat lamin A and lamin B have MAR binding activity (Luderus et al., 1992, 1994) and that the polymerized state of the lamins is necessary for their DNA binding specificity (Zhao et al., 1996). These findings suggest a role for the nuclear lamins in attaching chromatin to the nuclear envelope.

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A second type of MAR binding filament–like protein from animals is the nuclear mitotic apparatus protein (NuMA), which was first identified as a protein localized at the centrosomes during mitosis (Lydersen and Pettijohn, 1980). NuMA is also present in the interphase nucleus, but thus far the only function established for the protein is during the completion of mitosis (Yang and Snyder, 1992; Compton and Cleveland, 1993). Whereas the MAR binding activity of lamins fits very well with their localization and proposed function, the biological significance of the MAR binding activity of NuMA (Luderus et al., 1994) has not been established.

To date, no homologs of these proteins have been found in plants, despite some earlier efforts to identify laminlike proteins in plant nuclei (Beven et al., 1991; McNulty and Saunders, 1992; Minguez and Moreno Diaz de la Espina, 1993). Interestingly, the yeast genome contains no genes encoding laminlike proteins (Mewes et al., 1998), indicating that different types of chromatin binding structural proteins might have evolved that replace the nuclear lamins in nonanimal eukaryotes. An interesting feature of MFP1 in this context is the presence of a predicted N-terminal transmembrane domain (Meier et al., 1996). Whereas lamins are attached to the nuclear envelope via farnesylation and interactions with integral membrane proteins (Schafer and Rine, 1992; Furukawa et al., 1995), the presence of both a predicted transmembrane domain and a MAR binding domain on MFP1 might indicate an alternative possibility for the attachment of chromatin to the nuclear envelope in plants.

To investigate whether MFP1 might represent a functional homolog of one of the animal filament-like MAR binding proteins and to gain more information about its possible biological function, we have performed a detailed analysis of its subcellular localization. Here, we describe the localization of MFP1 in discrete specklelike structures associated with the nuclear envelope and the nuclear matrix and the involvement of the predicted transmembrane domain in this localization. A marker protein for plant microtubule organizing centers (MTOCs) has been shown previously to be localized on the plant nuclear envelope. We show that this protein is also part of the nuclear matrix, indicating a close connection between MTOCs, the nuclear envelope, and the nuclear matrix in plants.

RESULTS

A Variable Amount of MFP1 Is Tightly Associated with the Nuclear Matrix

Previous results have shown that MFP1 is present in a nuclear matrix preparation derived from tobacco NT-1 suspension-cultured cells (Meier et al., 1996). However, we noticed that the fraction of total MFP1 detectable in the nuclear matrix varied from experiment to experiment (data not shown). Potential artifacts of this type of fractionation experiment could be impurities of the isolated nuclei, resulting in cofractionation of nonnuclear material and inefficient extraction during nuclear matrix preparation, which could lead to the cofractionation of soluble nuclear proteins. To exclude these possibilities, we carefully monitored the fractionation steps both by confocal laser scanning microscopy of the isolated structures and by comparing the protein profiles of the different extracts.

Figure 1 shows the results of a typical experiment. The confocal images of unfixed NT-1 cells, nuclei, and a nuclear matrix preparation are shown in Figure 1A. The identity of the nuclei in intact cells and of the isolated nuclei was shown by 4′,6-diamidino-2-phenylindole (DAPI) staining (data not shown). The larger, round structure inside the nucleus is the nucleolus, which sometimes contained smaller, round structures of unknown identity. This is a typical appearance of tobacco suspension-cultured cell nuclei. The images show that both nuclei and nuclear matrices were free of any visible cellular debris. Figure 1B shows the protein profiles of whole NT-1 cells, isolated nuclei, and nuclear matrices after SDS-PAGE. The absence of detectable histone bands (arrowheads in Figure 1B) in the nuclear matrix fraction indicates that it is essentially free of chromatin-associated and soluble nuclear proteins (Hall et al., 1991). Figure 1C shows the result of an immunoblot experiment with a replica gel and an antibody directed against the central portion of MFP1 (a288; Meier et al., 1996). A single band of ~80 kD, representing tobacco MFP1, was detected in nuclei as well as the nuclear matrix fraction (arrow in Figure 1C). An additional band of a lower molecular mass in the total cell extract represents either a second cross-reacting protein or a breakdown product of MFP1. No signal was detected with the preimmune serum at the same dilution (data not shown). In this experiment, ~10 to 20% of total MFP1 was detected in the nuclear matrix. This indicates that MFP1 is associated with the insoluble nuclear matrix but that a significant fraction of the protein is also present in extranuclear material.

The Majority of Total Cellular MFP1 Is Localized in Nuclear Matrix–Associated Speckles at the Nuclear Periphery

To further investigate the subcellular localization of MFP1, we conducted immunolocalization experiments with fixed tobacco NT-1 protoplasts. Figures 2A and 2B show that the strongest signal detected with the a288 antibody was localized at discrete specklelike structures at the nuclear periphery. The speckles have a diameter of ~1 μm, and ~15 speckles were localized in the cross-section shown (Figure 2B). Under the same conditions, no signal was detected with the preimmune serum (data not shown). A second antibody, which was directed against the C-terminal portion of MFP1 (amino acids 491 to 718) and did not cross-react with

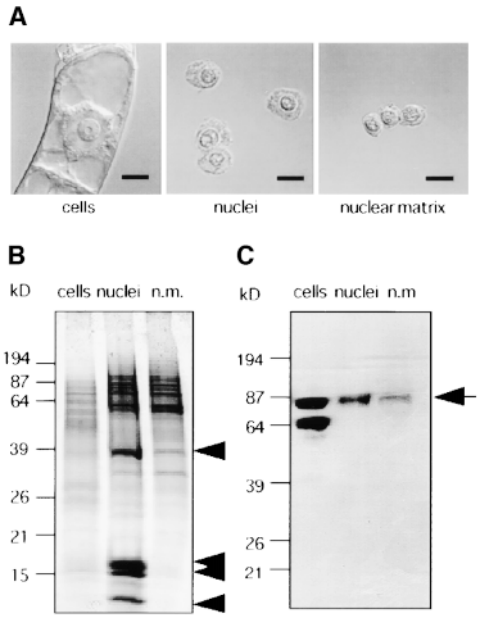

Figure 1. MFP1 Is Partially Localized in the Plant Nuclear Matrix.

(A) Differential interference contrast images of tobacco NT-1 suspension-cultured cells, purified NT-1 nuclei, and an NT-1 nuclear matrix fraction. Bars = 10 μm.
(B) Approximately equal amounts of protein, as determined by Bluprint Fast–PAGE stain (Gibco BRL), of NT-1 cells, NT-1 nuclei, and a NT-1 nuclear matrix (n.m.) fraction were separated on a 12% SDS–polyacrylamide gel. Arrowheads indicate the bands corresponding to histone H1, histones H2A and H2B, histone H3, and histone H4 (from top to bottom) (Hall et al., 1991).
(C) Samples identical to the ones shown in **(B)** were separated on a 10% SDS–polyacrylamide gel and subjected to an immunoblot with the a288 anti-MFP1 antibody. The arrow indicates the 80-kD band corresponding to tobacco MFP1.
In **(B)** and **(C)**, numbers at left indicate molecular mass markers in kilodaltons.

the a288 antigen, showed the same decoration pattern (data not shown). The extent of the nucleus was determined by DAPI staining (data not shown).

To investigate whether the speckles are a part of the nucleus and the nuclear matrix, we performed immunolocalization experiments on fixed isolated nuclei and nuclear matrix preparations. Figure 2 shows that a288 decorates speckles of the same shape and size on the rim of isolated nuclei, indicating that they are closely associated with the nuclear envelope (Figures 2C and 2D). Whereas, for some nuclei, the number of speckles was comparable to that observed in whole protoplasts, other nuclei contained significantly fewer speckles (compare the three nuclei in Figure 2D). The antigen also is detectable in a nuclear matrix preparation (Figures 2E and 2F). In contrast to the isolated nuclei, the specklelike structures now are detected inside the nuclear matrices. This redistribution of the antigenic material indicates that a structure associated with the nuclear envelope collapses during nuclear matrix isolation and that this structure contains MFP1. No signal was obtained under identical conditions with the preimmune serum (data not shown). The identity of the isolated nuclei was shown by DAPI staining (data not shown). The localization pattern observed with isolated nuclei and nuclear matrices explains the results of immunoblot experiments, such as the results shown in Figure 1. A portion of the MFP1-containing structures was copurified with isolated nuclei and nuclear matrix preparations, but a variable amount of the immunoreactive material was lost during the fractionation, explaining the reduced and variable intensity of the signal in nuclei and in the nuclear matrix compared with whole cells. Together, these data indicate that MFP1 is associated with specklelike structures at the nuclear periphery that are a part of isolated nuclei as well as of the insoluble nuclear matrix.

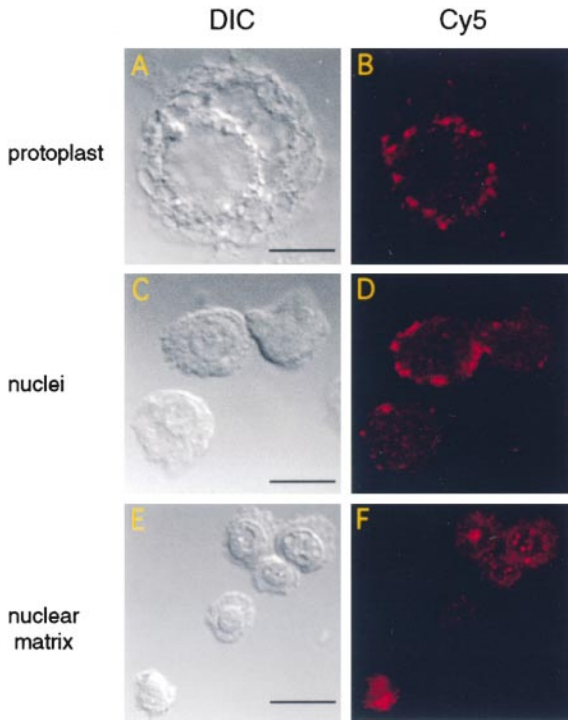

Figure 2. Immunolocalization of MFP1 in NT-1 Protoplasts, NT-1 Nuclei, and the NT-1 Nuclear Matrix.

(A), **(C)**, and **(E)** Differential interference contrast (DIC) images of fixed NT-1 protoplasts **(A)**, NT-1 nuclei **(C)**, and the NT-1 nuclear matrix **(E)**.
(B), **(D)**, and **(F)** Detection of MFP1 with a288 and a Cy5-conjugated goat anti–rabbit secondary antibody in NT-1 protoplasts **(B)**, NT-1 nuclei **(D)**, and NT-1 nuclear matrix **(F)**.
Bars in **(A)**, **(C)**, and **(E)** = 10 μm for **(A)** to **(F)**.

The MFP1-Containing Structures Are Globular and Are Arranged in a Sphere at the Nuclear Periphery

To further characterize the localization of MFP1 at the nuclear periphery, we used the expression of MFP1–green fluorescent protein (GFP) fusion proteins in transiently transformed NT-1 protoplasts. Figure 3A illustrates the fusion proteins used for these experiments. A mutant GFP with optimized codon usage for plants (mGFP; von Arnim et al., 1998) was used. In addition to an N-terminal and a C-terminal fusion of MFP1 to the mGFP (MFP1–mGFP and mGFP–MFP1 in Figure 3A), the unfused mGFP was used as a control. Figure 3B shows the results of the GFP localization experiments in live protoplasts. Consistent with the immunolocalization data, MFP1–mGFP is nearly exclusively localized in small speckles at the nuclear periphery. In contrast, mGFP–MFP1 shows a diffuse localization in the cytoplasm and no accumulation at the nuclear periphery. Figure 3B shows that the mGFP is localized in the cytoplasm as well as in the nucleus of a transiently transformed NT-1 protoplast. Due to its small size of 27 kD, the mGFP can passively diffuse into the nucleus, even in the absence of a nuclear localization signal (Görlich and Mattaj, 1996; Grebenok et al., 1997b). Figures 4A to 4R show the three-dimensional distribution of MFP1–mGFP in a representative tobacco protoplast. Optical sections were taken at 0.6-μm intervals. The digital images shown correspond to the consecutive sections in which mGFP fluorescence was detected. The majority of speckles observed are localized on a sphere at the nuclear periphery, with a few speckles located at greater distance from the nucleus (e.g., Figures 4D and 4E). A total of ~100 to 150 speckles per cell was estimated from the optical sections.

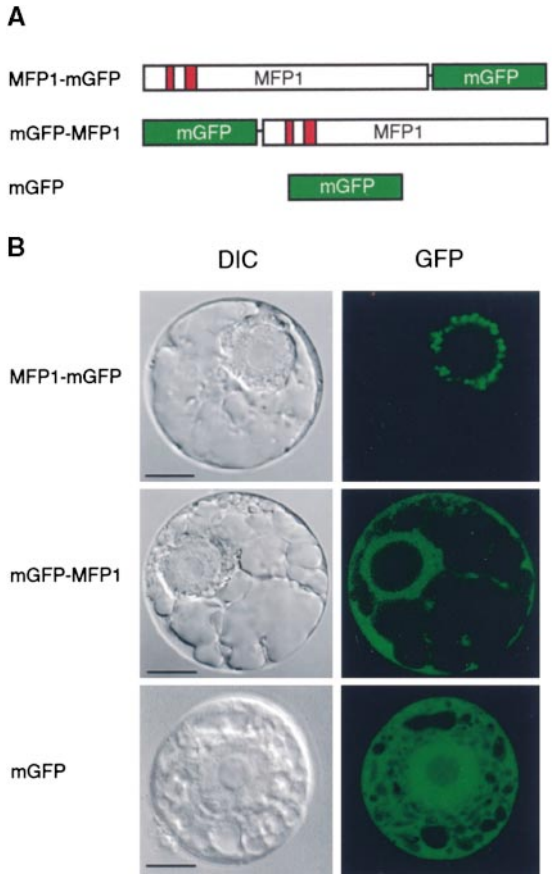

Figure 3. Localization of MFP1–GFP Fusion Proteins in NT-1 Protoplasts.

(A) Schematic representation of the proteins that were transiently expressed in NT-1 protoplasts after polyethylene glycol (PEG) transformation. The red bars indicate the hydrophobic domains of MFP1.
(B) Localization of GFP fluorescence in confocal images of protoplasts expressing the proteins indicated in **(A)**. DIC, differential interference contrast; GFP, GFP fluorescence. Bars = 10 μm.

These data indicate that the MFP1-containing speckles are roughly spherical structures that are closely, but not exclusively, associated with the nuclear periphery. The fact that only the N-terminal MFP1–mGFP fusion protein is localized to the speckles suggests that the free N terminus of MFP1 is necessary for its proper localization. In addition, the exclusion of mGFP–MFP1 from the interior of the nucleus indicates that no classic nuclear localization signal is present on MFP1.

The Hydrophobic N Terminus of MFP1 Is Required for Its Correct Localization

Interestingly, the N terminus of MFP1 has a predicted secondary structure that diverges greatly from the rest of the protein. Whereas the majority of MFP1 is highly α helical and predicted to form a coiled-coil structure (Meier et al., 1996), the first 150 amino acids lack a coiled-coil structure but contain two hydrophobic domains (indicated as red bars in Figures 3 and 5). The first hydrophobic domain is similar to transmembrane domains from a variety of membrane attached proteins (Meier et al., 1996), indicating that the N terminus of MFP1 might act as a membrane-attachment domain. To test whether the hydrophobic domains are involved in the targeting of MFP1 to the speckles, we constructed two N-terminal deletion mutants of MFP1–mGFP (Figure 5A). In MFP1Δ79–mGFP, the N-terminal 79 amino acids, including the first hydrophobic domain, were deleted. MFP1Δ125–mGFP lacks the N-terminal 125 amino acids, deleting both hydrophobic domains. Figure 5B shows the results of the transient transformation experiments compared with the full-length MFP1–mGFP fusion protein in live protoplast. Deletion of the N-terminal 79 amino acids leads to loss of the speckle structures and an enrichment of the fusion protein at the nuclear periphery, where it appears to be localized in vesicle-like structures (Figure 5B, MFP1Δ79–mGFP). In addition, a portion of the fusion protein accumulates in the cy-

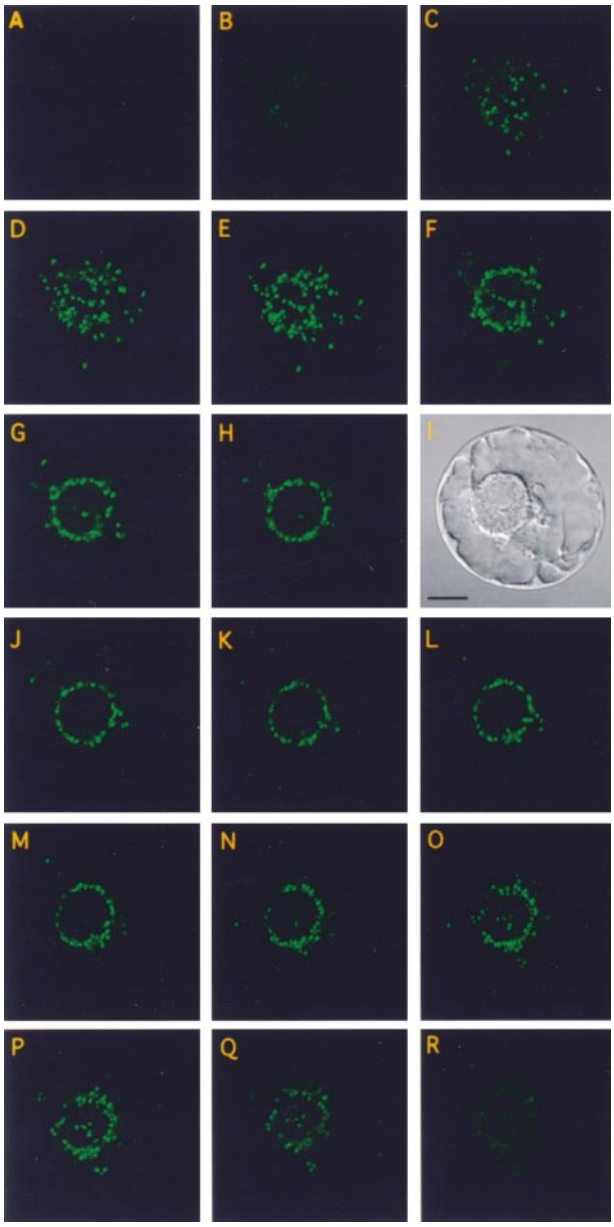

Figure 4. Three-Dimensional Distribution of the MFP1–mGFP Fusion Protein in NT-1 Protoplasts.

(A) to **(H)** and **(J)** to **(R)** Optical sections of a representative MFP1–mGFP—expressing NT-1 protoplast were taken at 0.6-μm intervals. Only the consecutive sections showing GFP fluorescence are presented.

(I) Differential interference contrast image corresponds to the fluorescence signal in **(J)**.

Bar in **(I)** = 10 μm for **(A)** to **(R)**.

toplasm. Deletion of the N-terminal 125 amino acids leads to a diffuse localization of the fusion protein in the cytoplasm, indistinguishable from the localization of mGFP–MFP1 (Figure 3B). These data indicate that the N-terminal 125 amino acids of MFP1 are necessary for the targeting of the protein to the speckles at the nuclear periphery and that this targeting most likely involves membrane attachment.

MFP1 and a Centrosome-like Component Define a Plant Nuclear Envelope–Nuclear Matrix Connection

Because the MFP1-containing speckle structures were somewhat reminiscent of nuclear-associated endoplasmic reticulum (ER; Restrepo-Hartwig and Ahlquist, 1996; Schaad et al., 1997), we compared the localization pattern of MFP1–mGFP with the staining pattern obtained with 3,3′-dihexyloxacarbocyanide iodide ($DiOC_6$), a vital fluorescent stain for ER membranes (Knebel et al., 1990; Staehelin, 1997). Because $DiOC_6$ renders protoplasts extremely unstable, intact NT-1 suspension-cultured cells were used for this experiment. After transient transformation with the MFP1–mGFP expression plasmid, the same arrangement of MFP1-containing speckles was observed at the nuclear periphery of the intact cell (Figures 6A and 6B), as has been observed in protoplasts (Figure 3B). This indicates that the localization of MFP1–mGFP at the nuclear periphery is not an artifact of the protoplast preparation, for example, due to disturbance of cytoskeletal structures after cell wall removal. A comparison of MFP1–mGFP fluorescence (Figures 6A and 6B) with the total fluorescence of ER membranes, as observed by using $DiOC_6$ staining (Figures 6C and 6D), shows that the MFP1-associated speckles are comparable in size and shape to some ER material at the nuclear periphery but that MFP1–mGFP clearly is excluded from the majority of ER in the cytoplasm of the cell.

The fact that the N-terminal domain of MFP1 is required for its localization, together with the $DiOC_6$ staining results, indicates that the specklelike structures might be a specific substructure of the nuclear envelope–ER continuum (Staehelin, 1997). Nevertheless, the fact that MFP1 can be detected at the periphery of nuclei that were isolated in the presence of Triton X-100 and therefore at least should be free of the outer nuclear envelope and attached ER material (Watson and Thompson, 1986) indicates that additional non-membrane-attachment interactions connect MFP1 with the nuclear periphery and the nuclear matrix. Due to the filament-like structure of MFP1, it is tempting to speculate that a connection with cytoskeletal and/or nuclear matrix filaments causes this association.

A first indication for a connection between the nuclear envelope–ER continuum and cytoskeletal components in plants was the identification of MTOCs at the plant nuclear periphery (Staehelin, 1997). Plant cells do not contain centrosomes or spindle pole bodies like animal or fungal cells (Stoppin et al., 1994). Instead, the nuclear surface appears to be a

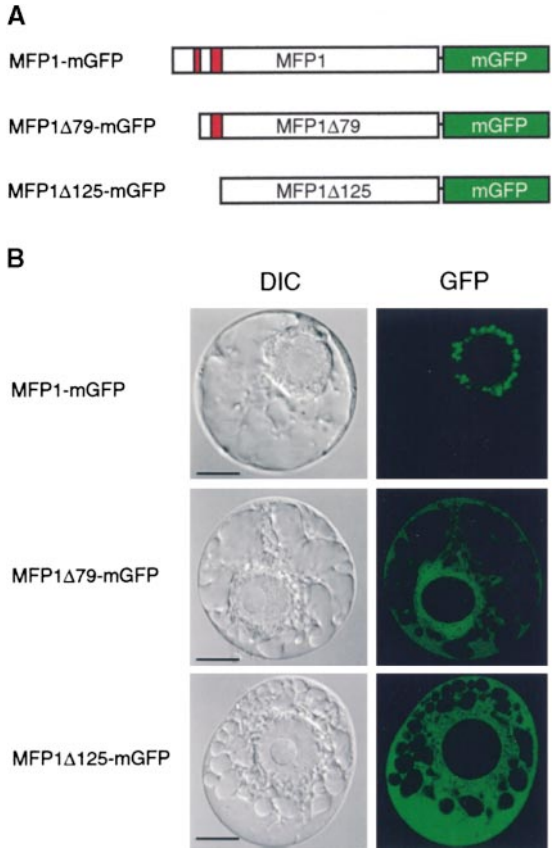

Figure 5. Effect of N-Terminal Deletions on the Localization Pattern of MFP1–mGFP.

(A) Schematic representation of the fusion proteins that were transiently expressed in NT-1 protoplasts after PEG transformation. The red bars indicate the hydrophobic domains of MFP1.
(B) Localization of GFP fluorescence in confocal images of protoplasts expressing the proteins indicated in **(A)**. DIC, differential interference contrast; GFP, GFP fluorescence. Bars = 10 μm.

major site of MTOCs in plant cells (Stoppin et al., 1994, 1996). A monoclonal antibody raised against isolated native calf thymus centrosomes (mAb6C6; Chevrier et al., 1992) has been shown to decorate the periphery of isolated maize nuclei (Stoppin et al., 1994, 1996) and to colocalize with the origins of microtubule clusters (Chevrier et al., 1992). Here, we used mAb6C6 to compare the localization pattern of its plant antigen on isolated nuclei and nuclear matrix preparations with the localization of MFP1.

Figures 7A and 7B show that mAb6C6 detects an antigen on isolated tobacco nuclei and that the mAb6C6 antigen is, like MFP1, localized in small specklelike structures at the nuclear periphery. This is consistent with the colocalization of the mAb6C6 antigen with the origins of microtubule clusters that were observed on the nuclear surface of Haementhus cells (Chevrier et al., 1992). Surprisingly, mAb6C6 also decorates specklelike structures on isolated nuclear matrices (Figures 7C and 7D). In contrast to MFP1, the speckles associated with the mAb6C6 antigen are localized on the surface of the nuclear matrices and are not detected in its interior, indicating that the two proteins are components of separate nuclear matrix–associated structures. These data provide definitive evidence that in plants, potentially cytoskeleton-associated proteins, which are localized on the nuclear periphery, are tightly connected to the nuclear matrix and indicate that the operationally defined nuclear matrix contains material located on the outer surface of the nuclear envelope.

DISCUSSION

High-Resolution Subcellular Localization of MFP1 by Using the GFP

The GFP from jellyfish has been developed into a powerful marker for the in vivo localization of proteins at high resolution and in real time in living cells (Ellenberg et al., 1997). Although original problems with expressing the GFP at suffi-

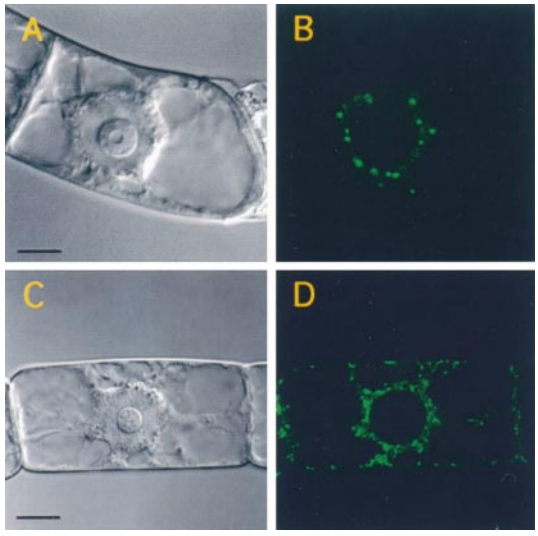

Figure 6. Comparison of MFP1 Localization and the Localization of the ER in NT-1 Suspension-Cultured Cells.

(A) and **(C)** Differential interference contrast images of the NT-1 cells shown in **(B)** and **(D)**.
(B) GFP fluorescence of a cell transiently expressing MFP1–mGFP after particle bombardment–mediated transformation.
(D) Fluorescence of a cell stained with $DiOC_6$, a vital fluorescence stain for ER membranes.
Bars in **(A)** and **(C)** = 10 μm for **(A)** to **(D)**.

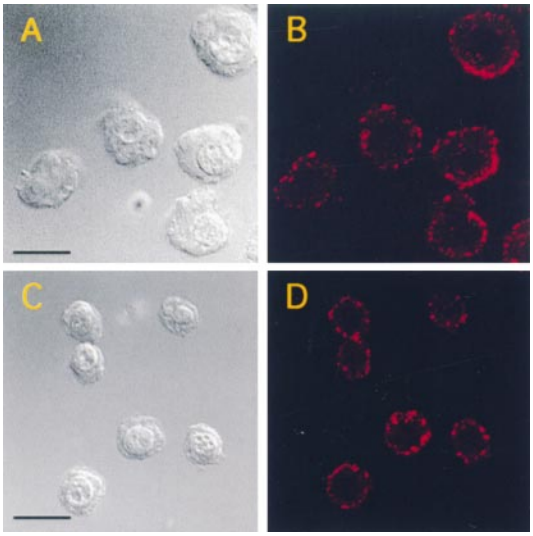

Figure 7. Immunolocalization of the mAb6C6 Antigen in Isolated NT-1 Nuclei and an NT-1 Nuclear Matrix Fraction.

(A) and **(C)** Differential interference contrast images of fixed nuclei **(A)** and a fixed nuclear matrix fraction **(C)** corresponding to the immunofluorescence images in **(B)** and **(D)**.

(B) and **(D)** Immunofluorescence images of isolated NT-1 nuclei **(B)** and an NT-1 nuclear matrix fraction **(D)** probed with the mouse monoclonal antibody mAb6C6 and a Cy5-conjugated anti–mouse secondary antibody.

Bars in **(A)** and **(C)** = 10 μm for **(A)** to **(D)**.

ciently high levels in plants have been overcome (Haseloff et al., 1997; Davis and Vierstra, 1998), its application for high-resolution subcellular localization is still less widespread than it is in the animal field. This is due in part to the fact that its excitation wavelength also causes strong red fluorescence of chlorophyll, which can partially obscure the green fluorescence signal (Grebenok et al., 1997a). In addition, the fact that the protein diffuses passively into the nucleus due to its small size has discouraged researchers from using the GFP routinely for nuclear localization experiments (Grebenok et al., 1997a, 1997b). Here, we demonstrate that the GFP can be used in combination with confocal laser scanning microscopy to obtain high-resolution information about subcellular protein localization in plants. The use of transiently transformed cells or protoplast of a heterotrophic suspension culture provides a versatile system with very little background fluorescence, due to the absence of chlorophyll. In addition, our data extend previous findings (Grebenok et al., 1997b) demonstrating that GFP fusion proteins with a size larger than ~50 kD are excluded from the nucleus and are targeted to their correct location.

We have used two different methods for the localization of MFP1: immunolocalization in fixed protoplasts and the localization of GFP fusion proteins in living protoplasts and cells. The combination of both methods guards against any misinterpretation that might arise from using one approach. For example, immunocytochemistry is sensitive to changes in subcellular structures due to the fixation process. At the same time, GFP localization experiments alone can lead to uncertainties resulting from the overexpression of the fusion protein (Ellenberg et al., 1997). Here, both techniques have revealed the identical pattern of localization for MFP1, indicating that the accumulation of the protein in speckles at the nuclear envelope reflects the in vivo situation.

The Hydrophobic N Terminus of MFP1 Is a Targeting Domain

The N-terminal 125 amino acids of MFP1 are necessary for the correct targeting of the protein (Figure 5). This domain contains two stretches of hydrophobic amino acids, between positions 61 and 73 and between positions 103 and 124. Both hydrophobic sequences are predicted to be transmembrane domains (von Heijne, 1992; Meier et al., 1996), and their sequence and spacing is highly conserved between MFP1 sequences from tomato, tobacco, and Arabidopsis (P. Harder and I. Meier, unpublished results).

One possible function for the N-terminal domain is that of a signal peptide for cotranslational ER import. Results of the SignalP signal peptide prediction program (Nielsen et al., 1997) are ambiguous for MFP1 and are more supportive of the N terminus being a signal anchor sequence (uncleaved signal peptide) than a real signal peptide. However, if a cleavage site is present, the program predicts it to be located between positions 77 and 78, immediately after the first hydrophobic domain. If the N-terminal 77 amino acids act as a signal peptide, then the second hydrophobic domain most likely would represent a “stop transfer” sequence, which would anchor MFP1 into the membrane with an orientation placing the N terminus into the ER lumen and the C terminus into the cytoplasm. If instead the N terminus constitutes a signal anchor sequence, the protein could be inserted in either orientation (Galili et al., 1998). Alternatively, MFP1 might be inserted into membranes post-translationally. An example for this alternative pathway in plants is the targeting to ER membranes of the 6-kD protein of the tobacco etch potyvirus (Schaad et al., 1997).

Recently, it has been shown in animal systems that transmembrane domains of post-translationally inserted proteins can determine retention of a membrane-attached protein in the ER in the absence of an ER retention signal. In two cases, a short transmembrane domain of 17 amino acids determined ER retention, whereas lengthening of the domain to between 21 and 26 amino acids caused the protein to be localized in the Golgi apparatus or on the cell surface (Pedrazzini et al., 1996; Yang et al., 1997. These data support the notion that lipid composition–dependent differences in the thickness of lipid bilayers might play a

determining role in protein sorting (Bretscher and Munro, 1993). Thus far, no information exists about transmembrane domains involved in the specific sorting of proteins to the nuclear envelope. Although the first hydrophobic domain of MFP1 has convincing sequence similarity to a number of transmembrane domains (Meier et al., 1996), its length of 13 amino acids appears to be too short for a canonical α-helical transmembrane domain. Hence, it is tempting to speculate that a specific composition of the lipid bilayers close to the nucleus might be involved in determining the localization of proteins with short hydrophobic domains, such as MFP1. This can now be tested by investigating if the N terminus of MFP1 is sufficient to target a heterologous protein to the nuclear periphery and what influence a successive extension of the first hydrophobic domain has on such a localization.

MFP1 and the Plant Nuclear Envelope–ER Continuum

Both the specklelike localization of MFP1 at the nuclear periphery and the fact that a potential membrane-attachment domain is necessary for this localization support the idea that the site of function of MFP1 is a specific subdomain of the nuclear envelope–ER continuum. Four specific types of ER subdomains at the plant nuclear envelope have been distinguished in the literature: the lamin receptor domain, the nuclear envelope–ER gates, the microtubule–nucleation domains, and the nuclear pore complexes (Staehelin, 1997). The lamin receptor domain is thus far purely hypothetical in plants, because no lamins and lamin receptor–like proteins have been identified. Although it would be easy to imagine MFP1 as a protein of the inner nuclear membrane that attaches chromatin to the nuclear envelope in a laminlike fashion, one would expect MFP1 to be distributed evenly at the nuclear periphery in such a simple model. Nuclear envelope–ER gates are membrane structures forming the connection of the ER to the outer membrane of the nuclear envelope. Localization of MFP1 in such structures is not very likely, because they should be removed from the isolated nuclei and, in particular, from the nuclear matrix fraction that contains the MFP1-associated speckles (Watson and Thompson, 1986; Verheijnen et al., 1988).

The microtubule–nucleation domain on the nuclear envelope is specific to plants. In contrast to animal or fungal cells, plants do not contain centrosomes or spindle pole bodies. Recent evidence has shown that the outer surface of the nuclear envelope serves as an MTOC (Chevrier et al., 1992; Stoppin et al., 1994, 1996). An antibody directed against calf thymus centrosomes reacts with a 100-kD protein that colocalizes with microtubule clusters on the plant nuclear surface (Chevrier et al., 1992; Stoppin et al., 1994, 1996) and that redistributes to the centrosome-kinetochores during anaphase (Schmit et al., 1994). Here, we have shown that the specklelike structures on the nuclear surface that are associated with this antigen are a part of the plant nuclear matrix. Their localization differs from the localization of MFP1 in that they remain on the periphery of the nuclear matrix, whereas the speckles associated with MFP1 collapse onto its interior after removal of chromatin and soluble nuclear proteins. This difference in behavior during nuclear matrix preparation of MFP1 and the mAb6C6 antigen indicates that despite their similar appearance on the nuclear periphery, they are not part of the same protein complex. Hence, it is unlikely that MFP1 is a component of a plant MTOC.

Plant nuclei contain several hundred nuclear pores that have a diameter of ~100 nm. Animal and yeast nuclear pore complexes (NPCs) consist of ~100 different proteins, of which only 25 to 30% are known (Heese-Peck and Raikhel, 1998). No plant genes encoding NPC proteins have been cloned, and no proteins with sequence similarity to the vertebrate and yeast NPC proteins have been found in plant databases, although candidate glycoproteins localized at the nuclear periphery have been identified recently (Heese-Peck and Raikhel, 1998).

Electron microscopy of NPCs has shown cytoplasmic as well as nuclear fibrils connected to the central pore complex, and it has been suggested that these peripheral structures connect nucleoplasmic and cytoplasmic cytoskeletal elements (Davis, 1995; Heese-Peck and Raikhel, 1998). Several NPC proteins from vertebrates and yeast contain α-helical coiled-coil domains, which might allow them either to form the filamentous structures of the NPC or to associate with the structural proteins forming them (Heese-Peck and Raikhel, 1998). Some of the yeast and vertebrate NPC proteins have structures that should allow them to bind to DNA or RNA. Vertebrate Nup153p contains several zinc fingers and has been shown to bind to *Escherichia coli* DNA in an in vitro gel blot assay (Sukegawa and Blobel, 1993). In addition, three known NPC proteins are transmembrane proteins (Heese-Peck and Raikhel, 1998) and are believed to be involved in anchoring the NPC to the membranes of the nuclear envelope.

A protein like MFP1, possessing a membrane-attachment domain, filament protein structure, as well as the ability to specifically bind to DNA, is an interesting candidate for a plant NPC-associated protein. Whereas NPCs appear somewhat too small and too abundant, as shown by using electron microscopy, to account for the MFP1-associated speckles, the localization of a protein containing a plant nuclear localization signal to isolated tobacco nuclei shows a pattern of speckles of ~0.5 μm in diameter, which is strikingly similar to the localization pattern of MFP1 (Hicks et al., 1996). Hence, the actual distribution of NPC-associated proteins at nuclear pores might be larger than the structures visible by using electron microscopy. It is intriguing to speculate that the function of a MAR binding protein localized at the vicinity of NPCs might be to organize chromatin such that genes that are transcribed are tethered to an area close to the NPCs for easy export of RNA. Once nuclear pore complex proteins from plants are characterized, coimmunolocalization experiments with MFP1 will allow us to determine more precisely its relationship to plant NPCs.

MFP1 and the mAb6C6 Antigen Define a Connection between the Plant Nuclear Rim and Nuclear Matrix

Whereas MFP1 and the mAb6C6 antigen seem to belong to different nuclear structures, they have in common their close association with the nuclear matrix, although they are localized at the nuclear rim. This supports the hypothesis that cytoskeletal structures penetrate the nuclear pores and connect structural protein systems of the nucleus and the cytoplasm (Verheijnen et al., 1988) and demonstrates a close connection in plants between the nuclear matrix, the nuclear envelope, and MTOCs. These data should caution us in our interpretation of the biochemical properties of nuclear matrix preparations. These properties often are attributed to proteins localized in the interior of the nucleus. For example, the DNA binding activity of nuclear matrix preparations is widely used to identify MARs, which are thought to form the bases of chromatin loops inside the interphase nucleus and are expected to influence the expression of flanking genes (Spiker and Thompson, 1996). The DNA binding activity of the nuclear matrix includes the activities of proteins, such as MFP1, which are localized at the nuclear rim but connected to the nuclear matrix in a fashion that is not understood. The biological function of their DNA binding activity and their potential influence on the expression of associated genes remain to be elucidated.

All the nuclear envelope–associated structures discussed here are poorly understood on a molecular level in plants, and some of them are poorly understood in any organism. The localization pattern of MFP1 described here defines a specific substructure of the nuclear periphery that is most likely associated with membranes, the structural proteins that constitute the basis of the insoluble nuclear matrix, and with DNA. Indications are emerging that the cellular membrane systems and the cytoskeleton are in close association. Whereas in animals, both the ER and the Golgi apparatus are connected to microtubules, such an association seems to be provided by an interaction of the membrane systems with actin filaments in plants (Boevink et al., 1998). No proteins providing these associations have been identified, and no information exists about interactions of the nuclear envelope membranes and the cytoskeleton in plants. Compartmentalization of molecular events is the emerging theme in many research areas both for nuclear events, such as replication or transcription (Wei et al., 1998), and, more recently, for cytoplasmic events, such as mitogen-activated kinase pathways (Whitmarsh et al., 1998). In contrast, our knowledge about the cellular structures possibly providing such a compartmentalization and about their dynamic interactions is still in its infancy. The analysis of novel proteins, such as MFP1, which is localized at specific, nuclear matrix–connected domains of the nuclear periphery, might provide insight into the fine structure of cellular compartments involved in the orchestration of complex molecular processes.

METHODS

Plasmid Vectors

The 2.4-kb NcoI-BamHI fragment of pRSETA–MFP1 (Meier et al., 1996) containing the complete open reading frame of the matrix attachment region (MAR) binding filament–like protein 1 (MFP1) was cloned into the XbaI site of pBluescript II KS+ (Stratagene, La Jolla, CA) after filling in the overhangs with the Klenow fragment of DNA polymerase I to create pKS-MFP1. The 2.4-kb BamHI fragment of pKS-MFP1 was cloned into the single BglII site of pRTL2–mGFP S65T (von Arnim et al., 1998) to create a translational fusion of the mutant green fluorescent protein (mGFP) and MFP1 in the vector pmGFP–MFP1. To create pMFP1–mGFP, we amplified the open reading frame of MFP1 by using polymerase chain reaction (PCR) with the primers 5′-AGGAACCATGGCAACTTC-3′ and 5′-ATCCTCCATGGGTTCCTC-3′ and the vector pKS-MFP1 as the template. Using the internal NcoI sites in the primers, we cloned the PCR product into the single NcoI site of pRTL2–mGFP S65T. The N-terminal deletion clones pMFP1Δ79–mGFP and pMFP1Δ125–mGFP were generated in the same way by using PCR with the primers 5′-CCATGGGCTTGTCAACAGATTC-3′ and 5′-ATCCTCCATGGGTTCCTC-3′; or 5′-CCATGGCCTTGGCTCGAAATGAG-3′ and 5′-ATCCTCCATGGGTTCCTC-3′, respectively. The sequences of all PCR products and the correct translational fusions were verified by sequencing.

Plant Material

Suspension-cultured *Nicotiana tabacum* NT-1 cells were grown as described (Allen et al., 1996). For all experiments, aliquots from a midlog phase culture (days 5 to 6) were taken.

Isolation of NT-1 Total Protein, NT-1 Protoplasts, Nuclei, and Nuclear Matrices

A midlog NT-1 suspension culture was sedimented in a swing-out rotor for 5 min at 300*g*. NT-1 cells (~250 mg) were ground in liquid nitrogen to a fine powder. After adding 0.5 mL of extraction buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, and 1.4 M β-mercaptoethanol), the sample was vortexed for 30 to 60 sec and incubated for 10 min at 70°C. Debris was removed by centrifugation for 10 min at 4°C and 15,000 rpm in a tabletop centrifuge. The cleared supernatant was transferred to a fresh tube and stored at −80°C.

Protoplasts, nuclei, and nuclear matrices from NT-1 suspension-cultured cells were isolated as described by Hall et al. (1991). The quality of the samples was analyzed by microscopy and SDS-PAGE. For SDS-PAGE, nuclei and nuclear matrix samples were boiled for 10 min in 15 μL of SDS loading buffer (Sambrook et al., 1989) immediately before loading.

Antibodies

The production of a288 was described earlier (Meier et al., 1996). The antibody mAb6C6 has been described previously (Chevrier et al., 1992).

Immunoblot Analysis

A 1:3000 dilution of a288 antiserum and a 1:10,000 dilution of horseradish peroxidase–coupled anti–rabbit secondary antibody (Amersham Buchler, Amersham, UK) were used to perform immunoblot analyses, as described by Sambrook et al. (1989). Enhanced chemiluminescence detection was performed as described by the manufacturer (Amersham Buchler).

Fixation and Immunolabeling of NT-1 Protoplasts, Nuclei, and Nuclear Matrices

Samples were attached to gelatine-coated slides, fixed in 2% paraformaldehyde in PHEM (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 2 mM $MgCl_2$, pH 6.9), permeabilized in 0.5% (v/v) Nonidet P-40 (Sigma) in PHEM, and rinsed in a 1:1 mixture of acetone and methanol at −20°C. After rehydration in PBS (7.4 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, and 150 mM NaCl), samples were blocked overnight in 5% normal goat serum (Molecular Probes, Eugene, OR) and 2% BSA (Sigma) in PHEM. Samples were then incubated for 1 hr at room temperature in the primary antibody and diluted 1:50 for a288 or 1:300 for mAb6C6 in blocking solution. Samples were rinsed in PBS, PBS with 0.1% (v/v) Nonidet P-40, and PBS for 5 min each and then incubated for 1 hr in a 1:100 dilution of the appropriate Cy5-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Samples were rinsed as before and mounted with 3 μL of Slow Fade (Molecular Probes) with 50% glycerol. Endoplasmic reticulum (ER) membranes (Figure 6) of living NT-1 cells were stained with 0.05 μg/mL 3,3′-dihexyloxacarbocyanide iodide ($DiOC_6$) in NT-1 culture medium.

Transformation of NT-1 Cells and Protoplasts

Ballistic transformation of NT-1 cells was performed essentially as described by Allen et al. (1996). Approximately 1 μg of supercoiled plasmid DNA was used per bombardment. After bombardment, the Petri dishes were sealed and incubated overnight at 27°C. Aliquots of the bombarded cells were removed from the plates by resuspending them in 1 mL of NT-1 culture medium and analyzed for GFP expression. For polyethylene glycol (PEG)–mediated transformation, protoplasts were resuspended in 0.5 M mannitol, 15 mM $MgCl_2$, and 0.1% Mes at a density of 2×10^6/mL. Aliquots (250 μL) were distributed on 15-mL Falcon tubes and mixed with 15 μg of supercoiled plasmid DNA (1 μg/μL) and 250 μL of PEG solution (40% [w/v] PEG 4000 in 0.4 M mannitol and 0.1 M $Ca[NO_3]\cdot 4H_2O$, pH 8.0). Samples were incubated at room temperature for 5 min. Ten milliliters of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 5 mM glucose, pH 5.6 to 6.0) was added slowly over a 5-min period to the samples. Protoplasts were pelleted for 5 min at 100*g* in a swing-out rotor, resuspended in 2.5 mL of NT-1 culture medium supplemented with 0.5 M mannitol, and incubated overnight at 27°C.

Fluorescence Microscopy

Digitized confocal images of samples were acquired at either 512 × 512 or 1024 × 1024 pixel resolution with a ×100 oil objective (NA 1.4) on a Zeiss 410 confocal laser scanning microscope (Carl Zeiss Inc., Thornwood, NY) by using the 488-nm excitation line of an Omnichrome Ar-Kr laser (Omnichrome, Chino, CA) for GFP and $DiOC_6$, the 633-nm excitation line of the internal He-Ne laser (Uniphase, Manteca, CA) for Cy5, or the 364-nm excitation lines of a UV Ar laser (Coherent Enterprise, Santa Clara, CA) for 4′,6-diamidino-2-phenylindole (DAPI) and appropriate emission filters (495- to 515-nm bandpass for GFP and $DiOC_6$, 670- to 810-nm bandpass for Cy5, and 400- to 435-nm bandpass for DAPI). For differential interference contrast images, the excitation lines 488 or 586 nm were used. All plates were assembled electronically with Adobe Photoshop software (Adobe Systems Inc., Mountain View, CA).

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