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## Dual location of MAR-binding, filament-like protein 1 in Arabidopsis, tobacco, and tomato

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**Abstract** Matrix attachment region-binding filament-like protein 1 (MFP1) is a plant-specific long coiled-coil protein that binds double-stranded DNA. While originally identified as a component of the tobacco nuclear matrix, it was subsequently shown that the majority of MFP1 resides in mature chloroplast where it is located at the stroma side of the thylakoids and is able to bind to nucleoids. On the other hand, a 90 kDa MFP1-like protein from onion has been convincingly shown to be an intrinsic component of the onion meristematic nuclear matrix. Here, we have expanded the analysis of the subcellular location of MFP1 by using high-resolution confocal immunofluorescence microscopy and immunogold electron microscopy. Two different antisera raised against MFP1 from two species were used on isolated nuclei and chloroplasts from tomato, tobacco, and Arabidopsis. Our data show that both antibodies detect a signal in both compartments in all three species. An Arabidopsis *MFP1* T-DNA insertional mutation abolishes both nuclear and chloroplast signals, indicating that the nuclear and plastidic antigens are derived from the same gene. We therefore suggest that MFP1 is a protein with a dual location, in both nuclei and chlo-

roplasts, consistent with prior findings in onion and the dicot species investigated here.

**Keywords** Arabidopsis · Chloroplasts · Confocal and electron microscopy · Matrix attachment region-binding filament-like protein 1 (MFP1) · Nuclear matrix · Subcellular distribution (MFP1) · Tobacco · Tomato

**Abbreviations** BSA: bovine serum albumin · CK2: casein kinase 2 · DAPI: 4',6' diamidino-2-phenylindole · DTT: dithiothreitol · GFP: green fluorescent protein · MAR: matrix attachment region · MFP1: MAR-binding filament-like protein 1 · NLS: nuclear localization sequence · PBS: phosphate saline buffer

### Introduction

Matrix attachment region-binding filament-like protein 1 (MFP1) is a plant-specific, long coiled-coil protein (Meier et al. 1996) with non-specific DNA-binding activity. Orthologs of MFP1 are present in several plant species, however, its functionality is not well understood (Harder et al. 2000; Jeong et al. 2003, 2004). The protein consists of an N-terminal domain containing two conserved hydrophobic domains, a central coiled-coil rod domain and a C-terminal DNA-binding domain. Several conserved casein kinase 2 (CK2) phosphorylation motifs are present, and it has been shown that the DNA-binding domain of MFP1 is an in vitro target for CK2 and that CK2 phosphorylation inhibits DNA-binding (Meier et al. 1996, Jeong et al. 2004). Expression of MFP1 is high in light and in shoots and low in dark and in roots (Jeong et al. 2003). While first identified as located in the nuclear matrix of tobacco (Meier et al. 1996) we more recently found that the majority of the protein in tobacco and Arabidopsis is present in chloroplasts, where it is associated with the stroma side of the thylakoids. Its co-purification with

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nucleoids in *Arabidopsis* chloroplasts suggested a function for MFP1 at the interface between the nucleoids and the thylakoid membranes (Jeong et al. 2003).

An antibody raised against tomato MFP1 (serum 288) detected an 80-kDa band in nuclei and a nuclear matrix fraction from tobacco (Meier et al. 1996). The same antiserum when used in immunofluorescence microscopy detected a strong, speckle-like signal around the nuclear periphery, and a very faint staining in the nuclear interior. The speckles were recovered in a nuclear matrix preparation (Gindullis and Meier 1999). A MFP1-GFP fusion protein was located in speckles at the nuclear periphery, which were subsequently identified as the proplastids of the tobacco suspension cultured cells (Gindullis and Meier 1999; Jeong et al. 2003). While the latter data seemed to indicate that the original nuclear matrix labeling could have been caused by a contamination with plastid material, we also demonstrated that a 90-kDa antigen recognized by the 288 antibody in onion was a component of a more stringently isolated nuclear matrix fraction and that this antigen is associated with specific nuclear bodies, indicating that at least in proliferating onion cells an MFP1-like protein exists in the nucleus and nuclear matrix (Samaniego et al. 2001).

The amino acid sequence of *Arabidopsis* MFP1 contains both a predicted *N*-terminal transit peptide for chloroplast import and a predicted *C*-terminal nuclear localization sequence (NLS) (Jeong et al. 2003). Deleting the 125 *N*-terminal amino acids of a MFP1-GFP fusion, or fusing GFP to the *N*-terminus of full-length MFP1 both leads to cytoplasmic localization, confirming that the *N*-terminus acts as chloroplast import signal (Gindullis and Meier 1999). In contrast, none of the GFP-fusion proteins tested showed nuclear localization, indicating that at least in the context of *N*-terminal and *C*-terminal GFP fusions, the NLS is not functional in classic nuclear import.

Here, we wished to investigate thoroughly by immunofluorescence studies where the antigens for the 288 antibody are located by comparing immunofluorescence signals obtained from nuclei and chloroplasts of tobacco, tomato, and *Arabidopsis*. In addition, we have used a new antibody that was raised against *Arabidopsis* MFP1 (OSU91 serum) in tomato and tobacco. To find out whether the antigen in both locations is a gene product of *MFP1*, we further investigated if a T-DNA insertional mutation in the *Arabidopsis MFP1* gene abolishes both nuclear and chloroplast immunofluorescence signals.

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## Materials and methods

### Isolation of nuclei and chloroplasts and nuclear matrix preparation

Leaf sections from plants of *Lycopersicon esculentum* L. cv Rutgers, *Nicotiana tabacum* L. cv Xanthi nc (pro-

vided by Dr. M.T. Serra, Laboratory of Plant Viral Pathology, Department of Plant Biology, CSIC, Madrid, Spain) and *Arabidopsis thaliana* L. cv Columbia (provided by Plant Biotechnology Center, Ohio State University, Columbus, OH, USA) were homogenized at 4°C using an Ultra-Turrax homogenizer (20,000 rpm) in extraction buffer (25 mM Tris-HCl, pH 7.8; 0.5 mM EDTA; 8 mM  $\beta$ -mercaptoethanol; 4 mM *n*-octanol; 2% (w/v) arabic gum; 1.25% (w/v) ficoll; 2.5% dextran; 30% (w/v) glycerol; 1.2 mM phenyl-methylsufonyl fluoride (PMSF); 2 mg/ml aprotinin), sequentially filtered through 100, 50, and 30  $\mu$ m filters and centrifuged at 1,400 g for 10 min. The resulting pellet, containing isolated nuclei, chloroplasts, and other co-purified organelles of similar density was washed twice in cold extraction buffer.

Nuclear matrices were extracted as described (Samaniego et al. 2001). Briefly, isolated nuclei were incubated in cytoskeleton buffer [CSK: 10 mM Pipes, pH 6.8; 100 mM KCl; 300 mM sucrose; 3 mM MgCl<sub>2</sub>; 1 mM EGTA; 1.2 mM PMSF; 2 mg/ml aprotinin; 20 mM dithiothreitol (DTT)] containing 0.5% Triton X-100. After 5 min, nuclear soluble proteins were removed by centrifugation at 1,400 g for 10 min and collected in the supernatant. The pellet containing the nuclear insoluble proteins was digested with 200  $\mu$ g/ml RNase-free DNase I in digestion buffer (DB: 10 mM Pipes, pH 6.8; 50 mM KCl; 50 mM NaCl; 300 mM sucrose; 3 mM MgCl<sub>2</sub>; 1 mM EGTA; 1.2 mM PMSF; 2 mg/ml aprotinin; 20 mM DTT; 0.5% Triton X-100) for 30 min at room temperature. About 1 M of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solved in DB was added to a final concentration of 0.25 M. DNA and its associated proteins were collected in the supernatant. Nuclear structures were further extracted with 4 M NaCl solved in DB to a final concentration of 2M. This step releases the outer nuclear matrix proteins and reveals the core filaments. Except when mentioned, all steps were performed at 4°C.

### Immunofluorescence microscopy

The nuclei/chloroplast and the nuclear matrix fractions were fixed in 2% paraformaldehyde in PBS buffer (pH 7.4) containing 0.5% Triton X-100 for 30 min, centrifuged at 690 g for 10 min and washed in the same buffer 1×30 min. Pellets were suspended in 20 mM glycine for quenching aldehyde groups and blocked with 2% BSA (in PBS with 0.05% Tween-20) for 30 min. The anti-MFP1 sera 288 or OSU91 were added to the blocking buffer to a final concentration of 1:300–500, the anti-U2B antibody 4G3 (Organon Teknica, Durham, USA) to a final concentration of 1:50, incubated for 1 h at room temperature and washed 2×30 min in PBS with 0.05% Tween-20. Pellets were incubated with Alexa A488-coupled anti-rabbit secondary antibody (Molecular Probes, Leiden, The Netherlands) for 45 min under the same conditions and washed 2×30 min. Except when mentioned, all steps were performed at 4°C and with

constant shaking. The labeled fractions were finally layered on multi-wells slides, air dried and incubated with 1 µg/ml 4',6' diamidino-2-phenylindole (DAPI). Samples were examined in a Confocal Microscope Leica TCS-SP2-AOBS using the Leica-confocal software to process the images.

### Electron microscopy

Leaf sections and tobacco protoplasts were processed and embedded in LR White resin as described (Mínguez and Moreno Diaz de la Espina 1993). Post-embedding immunogold labeling was performed using 1:30 diluted anti-MFP1 serum OSU91 and 1:50 diluted 10 nm-gold particle-coated anti-rabbit secondary antibody (Sigma). Samples were contrasted with uranyl acetate and/or lead citrate and examined in a Jeol 1230 electron microscope at 80 kV.

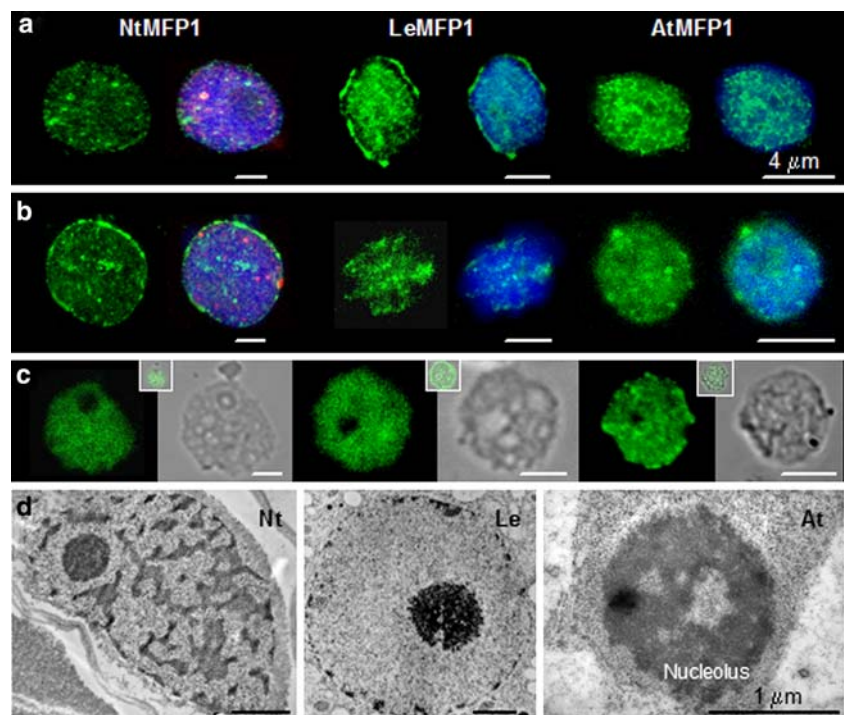
### Immunoblots

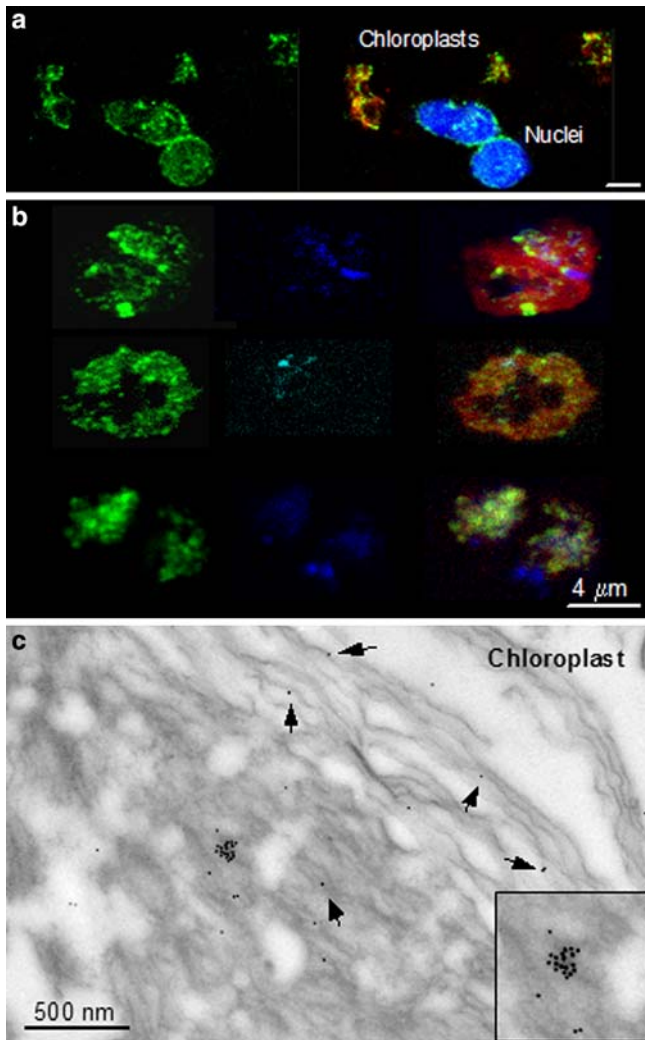
A 1:2,000 dilution of anti-MFP1 antiserum OSU91 and a 1:15,000 dilution of peroxidase-coupled anti-rabbit secondary antibody (Amersham) were used to perform immunoblot analyses, as described (Sambrook et al. 1989). OSU91 was raised against a  $\Delta$ 144AtMFP1, a deletion construct lacking the hydrophobic *N*-terminus of AtMFP1 (amino acids 1-144). It detects Arabidopsis, tomato, and tobacco MFP1, and does not cross-react with other proteins as shown in Jeong et al. (2003).

## Results

Immunofluorescence microscopy was performed with isolated nuclei from tobacco, tomato, and Arabidopsis and two antisera raised against tomato MFP1 (288; green fluorescence; Fig. 1a) and Arabidopsis MFP1 (OSU91; green fluorescence; Fig. 1b). In addition, tobacco nuclei were labeled with an antibody against the splicing protein U2B'' (4G3; red fluorescence; Fig. 1a, b). The projection of six optical sections is presented for the tomato and Arabidopsis nuclei, and a single virtual section at the center of the tobacco nuclei is shown. The data in Fig. 1a, b show that the general distribution pattern of MFP1 labeling is independent of the serum used in all three species. The only exception to this is a nuclear rim labeling detected with 288, but not OSU91 in tomato nuclei and with OSU91 but not 288 in tobacco, suggesting a re-location of the protein to different nuclear domains in these species, probably related with post-transcriptional modifications, as reported for other nuclear proteins in plants (Lorkovic et al. 2004). The labeling depicts a reticulate pattern with occasional large foci, which are similar to the onion nuclear bodies rich in the 90 kDa MFP1-like protein (Samaniego et al. 2001), but different from the Cajal bodies, as revealed by double labeling with 4G3 that detects the splicing protein U2B'' in the nucleoplasmic splicing network and the Cajal bodies (Cui and Moreno Díaz de la Espina 2003; Lorkovic et al. 2004). Counterstaining of nuclei with DAPI clearly shows that most of the 288 and OSU91 antigen is located in the nuclear interior. Figure 1c shows an immunolabeling experiment on isolated nuclear

**Fig. 1** Immunofluorescence images corresponding to isolated nuclei from tobacco, tomato, and Arabidopsis leaves, labeled with 288 (a) or OSU91 (b) and 4G3 antisera. MFP1 signals are shown in green, DAPI fluorescence in blue, and U2B'' signals in red. c Isolated nuclear matrices from tobacco and tomato leaves labeled with 288 and from Arabidopsis leaves labeled with OSU 91 (from left to right: tobacco, tomato, Arabidopsis). MFP1 signals are shown in green next to the interference contrast images and the merged images (*inserts*). d Electron microscopy images of nuclei from tobacco (*Nt*), tomato (*Le*), and Arabidopsis (*At*) contrasted with uranyl acetate and lead citrate. Bars 4 µm (a-c), 1 µm (d)





**Fig. 2** Co-purified nuclei and mature chloroplast from tobacco leaves labeled with OSU91 (a). Isolated chloroplasts from tobacco (top) and tomato (middle) labeled with 288, and Arabidopsis (bottom) labeled with OSU91 (b). MFP1 in green, DAPI in blue, autofluorescence in red. c Mature chloroplast from tobacco leaves after immunogold-labeling with serum 288. Transversal sections of thylakoid membranes are occasionally decorated with 10 nm gold particles (arrows). Insert shows a magnification of the cluster of gold particles. Bars 4  $\mu\text{m}$  (a-b), 500 nm (c)

matrices, demonstrating that the 288 and OSU91 antigen is associated with the nuclear matrix in all three species.

Despite the similarity of distribution patterns, qualitative differences exist, which might reflect the different organization of the dynamic nuclear domains and their multiprotein complexes in these species (Bettinger et al. 2004; Lorkovic et al. 2004). To further examine this point, we compared electron micrographs of leaf nuclei of all three species (Fig. 1d). There are clear differences in the size and distribution of heterochromatin patches between the three species. Tobacco presents large heterochromatin patches forming an intranuclear reticulum, tomato has mostly nuclear envelope-associated chromocentres and Arabidopsis displays almost fully decondensed chromatin with a few chromocentres,

which are usually not observed in individual sections. The observed differences in heterochromatin distribution would correlate with a different location of the proteins involved in chromatin organization and regulation like MFP1 that locates at the interphase between heterochromatin and de-condensed chromatin (Samaniego et al. 2001).

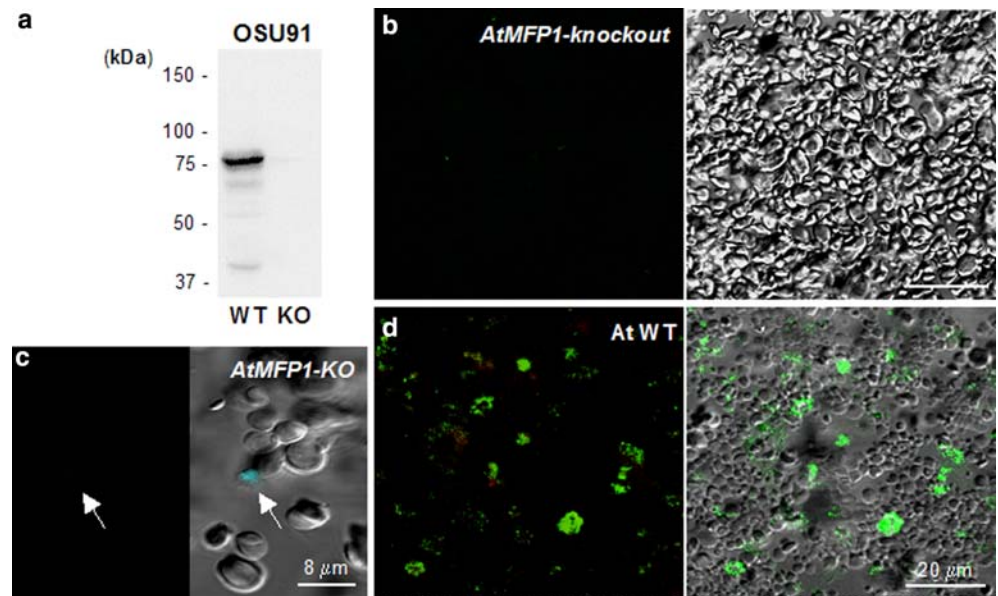
To directly compare the signals in nuclei and chloroplasts, a preparation was used that contains both nuclei and plastids (see Material and methods). Figure 2a shows co-purified nuclei and mature chloroplasts from tobacco leaves labeled with OSU91. Chloroplasts were identified by the far-red autofluorescence of chlorophyll, while nuclei were identified by strong DAPI staining. The OSU91 antigen is clearly detected in both compartments. Figure 2b shows a double labeling of MFP1 and chloroplast DNA, as visualized with DAPI. Larger accumulates of DAPI staining are distinct from MFP1 foci, however, a significant overlap was seen in the diffuse areas of labeling of MFP1 and DNA. The MFP1 signals clearly co-localize with the red autofluorescence of the thylakoid membranes. Figure 2c shows immunogold labeling with the 288 antibody on transversally sectioned thylakoid membranes (arrows). The densities of labeling in chloroplasts and nuclei were  $4.5 \pm 1.1$  and  $2.4 \pm 0.6$  particles/ $\mu\text{m}^2$ , respectively. Both clusters of gold particles (insert) and single gold particles were detected. It is likely that the clusters correspond to the foci of MFP1 also seen in the immunofluorescence images (Fig. 2b).

After confirming that the antigens detected by both sera are present in both nuclei and chloroplasts of tomato, tobacco, and Arabidopsis, we wished to confirm that they are derived from the same gene. Therefore, we used a previously described line of Arabidopsis, which contains a T-DNA insertion in the single *MFP1* gene. As discussed in Jeong et al. (2003), this line has no visible phenotype. Figure 3a shows an immunoblot with the OSU91 antiserum and total protein extracts from Arabidopsis wildtype Columbia (WT) and the T-DNA insertion line (KO), demonstrating that the 80 kDa At-MFP1 band is absent in the mutant. Figure 3b shows the results of an immunolabeling experiment on the mixed nuclei/chloroplast fraction derived from the knockout line in comparison with a preparation from wild type plants that were processed in parallel (Fig. 3d). No signals were detected in the mutant line. Figure 3c shows a magnification of a sector clearly containing a nucleus, as indicated by DAPI staining. No immunofluorescence signal was associated with the nucleus. This demonstrates that both the nuclear and the chloroplast antigen of the OSU91 antibody are absent from the T-DNA knockout line, indicating that they are both derived from the *AtMFP1* gene.

## Discussion

Both nuclear and plastidic localization of MFP1 have been suggested prior to this study. After first identifying

**Fig. 3** **a** Immunoblot of co-purified nuclei-chloroplast fractions from wild type (WT) and *AtMFP1*-knockout (*KO*) Arabidopsis leaves labeled with OSU91. **b–d** Low magnification immunofluorescence images showing a nuclei-chloroplast fraction labeled with OSU91. *AtMFP1* is shown in green. In **d** chloroplast autofluorescence is shown in red. *MFP1* and DIC images are merged on the right. **c** Detail of the knockout fraction, nucleus stained with DAPI (arrow). Bars = 20  $\mu$ m (**a, b, d**), 8  $\mu$ m (**c**)



*MFP1* by immunoblot analysis in nuclear and nuclear matrix fractions (Meier et al. 1996), a confusion about the localization pattern of the protein was created when strong green fluorescence from a C-terminal GFP fusion protein was detected in speckles lined up at the nuclear rim. Assuming that this localization pattern reflected the signal in the nuclear fraction, the speckles were interpreted as a component of the nuclear rim/nuclear envelope (Gindullis and Meier 1999). However, they were later identified as the small, white proplastids of the NT-1 tobacco suspension culture cells that had been used for the GFP study. For an unknown reason, these proplastids line up at the nuclear rim, leading to the interpretation that they were components of the nucleus. Only after a chloroplast marker was used in NT-1 cells to detect stromules was the peculiar location of the plastids in NT-1 cells revealed (Kohler and Hanson 2000). Following up on this finding, we discovered that *MFP1* was indeed located in plastids and associated with the thylakoid membranes (Jeong et al. 2003). This finding raised the question whether the original nuclear signal had been a contamination of the nuclear and nuclear matrix fractions with NT-1 plastids, or represented indeed a second location of the protein. The latter assumption was supported by independent findings of an *MFP-1* like protein in the onion nuclear matrix, which was localized both by light and electron microscopy to the internal nuclear matrix, unlikely to be contaminated with the plastid material (Samaniego et al. 2001).

In order to clarify if *MFP1* is a protein with dual location, we have investigated here the location of the native antigens of two different antibodies raised against tomato and Arabidopsis *MFP1* in nuclei and chloroplasts of tobacco, tomato, and Arabidopsis. Our data show that both antibodies detect similar signals in both cellular compartments of all three-plant spe-

cies. Importantly, both signals are absent in an Arabidopsis line with an insertional mutation in the *MFP1* locus. Together with the absence of any signals in controls with the respective preimmune sera (data not shown), this strongly suggests that both nuclear and plastidic signals are derived from a protein encoded by the *MFP1* gene. These data are consistent with our prior observation that in immunolabeling experiments with the 288 antibody and tobacco NT-1 protoplasts and nuclei, a weak signal was detected in the nuclear interior, in addition to the strong signal of the speckles at the nuclear rim (Gindullis and Meier 1999).

*MFP1* is not the first protein with a dual cellular location, but to our knowledge it is the first that is located both in the nucleus and in chloroplasts. At least two proteins are known that have a dual location in mitochondria and nuclei. *Trm1p* is a yeast protein that is located both in the inner nuclear envelope and the mitochondria (Rose et al. 1995). *MDDX28* is a DEAD-box helicase with a dual location in mitochondria and nucleus, and it has been suggested that the protein can be transported between the two compartments (Valgardsdottir et al. 2001). It contains a putative mitochondrial targeting signal (amino acids 3–18), nuclear export signal (*NES*, amino acids 180–191), and nuclear localization signal (*NLS*, amino acids 520–523). While *AtMFP1* has a clearly predicted chloroplast targeting signal (amino acids 1–41) and predicted *NLS* (amino acids 715–722), no *NES* is predicted on any of the available *MFP1* sequences, indicating that nuclear *MFP1* is not readily exported.

A remaining question is why the *MFP1*–GFP-fusion proteins are not at least partially located in the nucleus?. It is possible that GFP both at the N- and the C-terminals disrupts a signal required for nuclear import, or that GFP–*MFP1* lacks a post-translational modification

necessary for nuclear import. Alternatively, MFPI might be a protein that does not enter the nucleus through canonical importin-based nuclear import during interphase. If, for example, a round of cell cycle was required for the protein to assemble inside the nucleus, such assembly would not have been detected in the transient GFP-localization experiments. While little is known about such a mechanism, a number of structural nuclear proteins are recruited to the nucleus at an early stage of telophase before the nuclear envelope has closed. Specifically, Lamin B receptor, Nup153, and p62 have been shown to reconstitute around chromosomes very early in telophase prior to the recovery of nuclear import activity (Haraguchi et al. 2000). It is, therefore, conceivable that the nuclear fraction of MFPI is recruited at a stage of nuclear re-assembly not resolved in the GFP-fusion experiments.

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