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ORIGINAL PAPER

Comparison of expression of three different sub-cellular targeted GFPs in transgenic Valencia sweet orange by confocal laser scanning microscopy

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Abstract The green fluorescent protein (GFP) has become an ideal visual marker to monitor and quantify the expression of the transgene. It can be targeted to specific subcellular locations, including the endoplasmic reticulum, mitochondria, actin cytoskeleton and nuclei through the addition of signal peptides. Our previous work has resulted in transgenic citrus plants expressing cytoplasmic targeted GFP (Cy-GFP) or endoplasmic reticulum targeted GFP (Er-GFP) gene. To evaluate the localization of three different subcellular targeted GFP, i.e., Cy-GFP, Er-GFP and mitochondria targeted GFP (Mt-GFP) in citrus tissues and to utilize cell lines containing Mt-GFP for basic research in cell fusion, the plasmid pBI-mgfp4-coxIV encoding the Mt-GFP gene was successfully transferred into embryogenic callus of Valencia sweet orange (*Citrus sinensis* (L.) Osbeck) via *Agrobacterium tumefaciens*-mediated transformation. Furthermore, we compared the specific expression of these three different subcellular localized GFP constructs in cells of different mature leaf tissues (upper epidermis, palisade parenchyma, spongy parenchyma and lower epidermis) by a confocal laser scanning microscope (CLSM). Cytoplasmic-localized GFP expression was observed throughout the cytoplasm but appeared to accumulate within the nucleoplasm. The Er-GFP occurred within a layer very close to the cell wall. In addition, a stable fluorescence on the ER network throughout the guard cells was detected. Interestingly, the Mt-GFP specifically expressed in the guard cells to particles of about 1–2 μm within the cytoplasm in this case. To verify that the fluorescent particles observable in the guard cells are indeed mitochondria, we co-localize the Mt-GFP fusion protein with a mitochondrial-specific dye in citrus protoplasts. These results demonstrate that the subcellular distribution of the three subcellular targeted GFP is very distinct in citrus leaf cells and the cell lines containing Mt-GFP gene can be further used in citrus basic cell fusion research.

Keywords Agrobacterium tumefaciens · Citrus · Co-localization · Confocal laser scanning microscope · Green fluorescent protein

Abbreviations

AS	Acetosyringone
CLSM	Confocal laser scanning microscope
Cy-GFP	Cytoplasmic targeted GFP
Er-GFP	An endoplasmic reticulum targeted GFP
GFP	Green fluorescent protein
MT medium	Murashige and Tucker (1969)
Mt-GFP	Mitochondrial targeted GFP
NPT II	Neomycin phosphotransferase II

Introduction

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been proven to be an effective tool for the monitoring and quantification of transgene expression in real time under living conditions. It has been demonstrated as an ideal visual marker as compared to the most widely used visual reporter marker, β-glucuronidase, which needs substrate addition (De Ruijter et al. 2003;

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Haseloff and Siemering 2006). Direct visualization of gene expression in individual cells is therefore possible without cell lysis and subsequent biochemical analysis, and tissue distortion caused by fixation, staining and section. By fusing the protein to specific targeting peptides or to sequences of complete proteins (Haseloff et al. 1997; Kohler et al. 1997; Voigt et al. 2005), it is now possible to observe the subcellular localization of proteins, protease action, transcription factor, Ca^{2+} sensitivity, protein and organelle diffusion and movement within the cell, and protein-protein interactions by fluorescence resonance energy transfer (Zacharias et al. 2000; Zimmer 2002; Lippincott-Schwartz and Patterson 2003; Lo et al. 2004; Pozzan and Rudolf 2009). Moreover, visual selection using fluorescent protein is a powerful tool in cases where the callus proliferation or regeneration step is inefficient under antibiotic selection, because it enables selection and regeneration of transgenic plant cells without any additional selection pressure (Hraska et al. 2006).

Previously, organelle identification was commonly studied by electron microscopy, as it is a direct way to define the cellular structures of interest (Singh et al. 2010). However, identification of plant organelles via electron microscopy was limited to several well-characterized organelles including ER, Golgi apparatus, chloroplast, mitochondrion, vacuole, and protein body (Tse et al. 2009). Another disadvantage of using electron microscopy is that it depicts only momentary states of its structure. This situation changed rapidly after the discovery and application of GFP, as well as the development and improvement of the confocal laser scanning microscope (CLSM), which permits collection of focused images of single planes within a thick specimen, and can be used to visualize fluorescent signals within a narrow plane of focus, with exclusion of out-of-focus blur (Yoneda et al. 2007). By fusing GFP with the target protein to form a chimeric fluorescent reporter expressing in transgenic cells or plants, the trafficking of the GFP reporter and the dynamics of the GFP-marked organelles in living transgenic cells can be easily traced and studied in high resolution with CLSM (Dhanoa et al. 2006).

In higher-plant cells, mitochondria and endoplasmic reticulum play important roles in a variety of cellular events, including cell division, morphogenesis, and cell differentiation (Scott and Logan 2008; Sparkes et al. 2009). Surprisingly, despite a large number of articles employing GFP and specific targeting peptides to reveal the distribution and heterogeneity of organelles in protoplasts in vivo (Gupton et al. 2006; Sheahan et al. 2007), we found very few well-documented studies on nondividing cells of different mature leaf tissues. For example, the localization and distribution of organelles in cells from different mature leaf tissue remain unresolved. Herein, the expression of three different subcellularly targeted GFP markers, i.e., Cy-GFP, Er-GFP or Mt-GFP was studied in citrus leaves by CLSM. In our previous study, transgenic citrus plants expressing Cy-GFP or Er-GFP have been obtained (Guo et al. 2005; Liu et al. 2006). In this study, the Mt-GFP version was for the first time transformed into citrus via the embryogenesis pathway, which is believed to be a more efficient *Agrobacterium-mediated* transformation procedure for regeneration of transgenic plants from callus. The performance of the three different subcellularly targeted GFPs was compared in living citrus leaf cells, and we further confirmed that the fluorescent particles observed in the guard cells are indeed mitochondria by co-localizing the Mt-GFP fusion protein with a mitochondrial-specific dye in citrus protoplasts.

Materials and methods

Plant materials

Transgenic Valencia sweet orange (*C. sinensis* (L.) Osb.) plants expressing Cy-GFP were previously obtained through PEG-mediated protoplast transformation (Guo et al. 2005). Transgenic Valencia plants containing Er-GFP gene, were obtained through *Agrobacterium*-mediated callus transformation (Liu et al. 2006). For transformation, embryogenic callus lines of Valencia sweet orange (induced from undeveloped ovules in 1982, and kindly provided by Dr Jude Grosser, University of Florida, USA) were subcultured on solid MT (Murashige and Tucker 1969) basal medium containing 40 g/l sucrose. The transgenic plants with normal and vigorous growth were transferred to the greenhouse.

Bacterial strain and vector

Agrobacterium tumefaciens strain EHA105 harboring the binary plasmid pBI-mgfp4-coxIV was used as the transformation vector system. The plasmid containing Mt-GFP gene was kindly provided by Dr. Maureen R. Hanson (Cornell University, USA). Construction of the plasmid has been described by Kohler et al. (1997).

Transformation and regeneration of plants expressing Mt-GFP

Before being inoculated with *Agrobacterium*, the callus was cultured in liquid MT medium containing 0.5 g/l malt extract, 1.5 g/l L-glutamine, 40 g/l sucrose for 4 days. A fresh single colony of *A. tumefaciens* strain was selected and multiplied on solid LB (Luria-Bertani) medium with 50 mg/l kanamycin (Km) for 48 h at 28°C. The bacterial

cells were collected and transferred to liquid MT medium in an orbital shaker at 28°C and 180 rpm for 2 h, and then adjusted to an absorbance A600 = 0.5–0.8. Callus was immersed in the adjusted *Agrobacterium* suspension for 30 min. It was blotted dry on sterile filter paper and transferred to solidified MT medium with 100 μM AS. After a 3 days co-cultivation period in the dark at 23°C, the callus was transferred to selection medium (MTS basal medium supplemented with 400 mg/l cefotaxime (Cef) and 50 mg/l kanamycin (Km), and subcultured every 3 weeks in darkness at 28°C until resistant callus formed.

The resistant callus was transferred to MT medium supplemented with 2% glycerol (Vardi et al. 1989) for the induction of somatic embryos. Then, the mature embryos were transferred to shoot regeneration medium (basal MT medium plus 0.5 mg/l benzyladenine (BA), 0.5 mg/l kinetin (KT), 0.2 mg/l α-naphthaleneacetic acid (NAA), and 30 g/l sucrose) (Guo et al. 2002) without antibiotics. Explants were subcultured every 3 weeks on the same medium until shoots were regenerated. Regenerated shoots were further transferred to shoot elongation medium consisting of solid MT with 0.1 mg/l BA, 0.25 mg/l gibberellic acid (GA_3), 0.1 mg/l indoleacetic acid (IAA) and 0.2 mg/l activated charcoal. The elongated shoots of 0.5–0.8 cm length were then excised and transferred to root induction medium containing half-strength MT medium supplemented with 0.5 mg/l NAA, 0.1 mg/l indolebutyric acid (IBA), 25 g/l sucrose and 0.5 g/l activated charcoal or in vivo grafted onto trifoliate orange rootstock (*Poncirus trifoliata*) to produce transgenic plants.

DNA extraction and molecular analysis for transformed plants expressing Mt-GFP

Genomic DNA was extracted from fresh young leaves (200 mg) of transformed plantlets from independent lines and untransformed control plantlets using the CTAB method according to Cheng et al. (2003). To detect the presence of the Mt-GFP gene in the transgenic plants, PCR experiments were performed using specific primers for Mt-GFP, i.e., 5′-CTAGATATCTGCTTCAGCAAAAACCC-3′ and 5′-TGCCATGTGTAATCCCAGCA-3′. The PCR amplification program was as follows: pre-heating at 95°C for 4 min, and then 30 cycles of 30 s denaturation at 95°C, annealing of primers pairs at 55°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. The PCR products were analyzed on 2% agarose gel.

Visualizing GFP expression

GFP expression in the co-cultivated callus, embryogenic tissues, and regenerated plantlets was visualized under a fluorescence stereo-microscope (MZ FLIII; Leica Microsystems, Heerbrugg, Switzerland) equipped with a 480/40 nm exciter filter, a 505 nm LP dichromatic beam splitter and a 510 nm LP barrier filter. Fluorescence photographs were taken with a Nikon E4500 digital camera.

Fully expanded fresh leaves from different subcellularly targeted GFP transgenic plants and untransformed control plants were imaged using a LSM 510 CLSM (Carl Zeiss, Jena, Germany) with a 40X water-immersion objective. GFP was excited at a laser wavelength of 488 nm and detected through a filter for a fluorescence wavelength of 505–530 nm. Thin sections of leaf samples were mounted in distilled water on a glass slide and overlaid with a coverslip for microscopy. Confocal images from the outer epidermal wall through to the spongy mesophyll cells were merged using Zeiss LSM Image Examiner software (Carl Zeiss Image Microscopy Release 4.0) from the outer epidermal wall through to the spongy mesophyll cells. Untransformed plant tissues were used as negative controls to adjust the parameters to ensure the exclusion of autofluorescence in nontransformed leaf. All images were taken under the same parameters and were processed with Adobe Photoshop CS software to facilitate visualization.

Mito tracker staining

Protoplasts were isolated and purified from the transformed and untransformed callus of Valencia orange, basically following the method of Grosser and Gmitter (1990). Co-localization experiments were carried out according to the manufacturer's instructions, using the mitochondrial-specific fluorescent dye, MitoTracker Orange (CMTMRos, Invitrogen). The protoplasts were examined with the same CLSM equipped with 63 × 1.4-numerical aperature oil immersion objective. For dual imaging of GFP and the mitochondrial-specific dye, GFP was visualized with a 488 nm Ar laser and a BP505-530 filter, and MitoTracker was viewed with a 543 nm He:Ne laser and a BP565-615 filter.

Results

Transformation of the Mt-GFP gene and plant regeneration

After growth on selective medium for 5 weeks, calluses of Valencia orange, co-cultivated with *A. tumefaciens* EHA105 carrying the binary vector pBI-mgfp4-coxIV, produced kanamycin-resistant callus lines that kept growing while the others became necrotic and died (Fig. 1a). The highest level of GFP expression was detected in the kanamycin-resistant callus under the fluorescence stereomicroscope with 480 nm excited light (Fig. 1b). Calluses

emitting green fluorescence were isolated and subcultured at 10–20 days intervals in fresh MT medium for multiplication and then transferred to embryoid-induction medium. On this medium, resistant callus differentiated into embryos (Fig. 1c), although some malformed embryoids were observed. 4 weeks later, well-developed somatic embryoids with green fluorescence were observed (Fig. 1d). These embryoids were then selected and transferred to the shoot induction medium for 1–2 months culture. Whole plantlets were recovered after shoots were cultured on root induction medium (Fig. 1e). Different expression levels of the GFP gene were observed. In young tissues (shoot apices, young leaves), full green fluorescence was shown (Fig. 1f), while in old tissues, red fluorescence was observed, possibly because chlorophyll accumulation partially masked the green fluorescence (data not shown). Finally, when plants with well-developed root systems were regenerated, they were enclosed in a plastic bag, watered, and monitored (Fig. 1g) or transgenic shoots were grafted onto rootstocks in vivo to expedite their growth (Fig. 1h). Five in vivo grafted plantlets were recovered and they are almost of normal morphology while six self-rooted plantlets were recovered and they are not normal and grow slowly. These in vivo grafted and self-rooted transgenic plantlets were finally acclimatized, hardened and repotted in greenhouse for subsequent analyses.

Molecular identification of recovered plants

Putative transgenic plantlets expressing GFP were analyzed by PCR to verify the presence of the Mt-GFP gene in their genome. A predicted fragment of about 743 bp was amplified from 11 selected plants (Fig. 2). No amplification was detected in the DNA samples from regenerated nontransgenic control plant (Fig. 2 lane N), which emitted red autofluorescence under blue light (data not shown).

The expression of three different subcellularly targeted GFP constructs in citrus tissue

These three plasmid constructs containing Cy-GFP, Er-GFP and Mt-GFP were compared in an effort to evaluate their applicability for further research. No GFP fluorescence was visualized in nontransformed leaf as a control (data not shown). The significant differences in GFP expression and localization can be directly visualized in living plants by CLSM. In general, mitochondrial targeted GFP localizes specifically to particles of about 1–2 μm within the cytoplasm of stomatal guard cells of the stomata to particles (Fig. 3a, j), but no GFP expression was visualized in spongy parenchyma cells or palisade parenchyma cells (Fig. 3d, g). A narrow band of green fluorescence could be observed within epidermal cells at longitudinal optical sections (data not shown). A stable fluorescence of the ER network was detected throughout the guard cells (Fig. 3b), while the Er-GFP in the cells occurred within a layer close to the cell wall (Fig. 3b, k). Many fluorescent rings were observed specifically in the spongy tissue (Fig. 3e). Cytoplasmic-localized GFP was observed throughout the cytoplasm of the cell (data not shown), but it appeared to accumulate within the nucleoplasm (Fig. 3f) and it was excluded from vacuoles, chloroplast, and other bodies in the cytoplasm in epidermis and spongy mesophyll cells (Fig. 3c, l).

Co-localization of the Mt-GFP fusion protein with a mitochondrial-specific dye in citrus protoplasts

To verify that the fluorescent particles observable in the guard cells (Fig. 3a, j) are indeed mitochondria, it is necessary to investigate whether the Mt-GFP with 25-amino acid yeast coxIV transit peptide and with only four additional amino acids of the mature protein is able to localize GFP specifically to mitochondria in citrus. We stained citrus protoplasts with a mitochondrial-specific dye for living cells, MitoTracker CMTMRos, and compared the localization of the Mt-GFP fusion protein and the dye within the same cell. Due to differences in their spectral properties, the fluorescence of GFP and the dye can be distinguished (Fig. 4g, h). GFP fluorescence is only visible in the green channel (Fig. 4a) and shows no fluorescence in the red channel (Fig. 4b). Control cells stained with Mito Tracker appear only in the red channel (Fig. 4e) and show no fluorescence when observed through the green channel (Fig. 4d). Images taken of one cell observed through both channels can be superimposed to analyze whether the two fluorescences co-localize. Co-localization of the fluorescence of the Mt-GFP fusion protein and the mitochondrial-specific dye produce a yellow signal (Fig. 4i).

Discussion

In citrus, the effectiveness of transformation with GFP as a marker gene has been clearly demonstrated working with different transformation procedures and diverse genotypes (De Oliveira et al. 2009; Duan et al. 2010; Dutt et al. 2010). One of the advantages of using reporter marker genes, like *Escherichia coli uidA* or *Aequorea victoria gfp* gene, is to discriminate transgenic events by positive reporter expression in recovery lines (Saika and Toki 2009; Tan et al. 2009). Herein, we report a visual selection system for transformed cells using a GFP marker after *Agrobacterium*-mediated transformation in citrus. GFP-expressing callues could be detected at an early stage in vivo and were

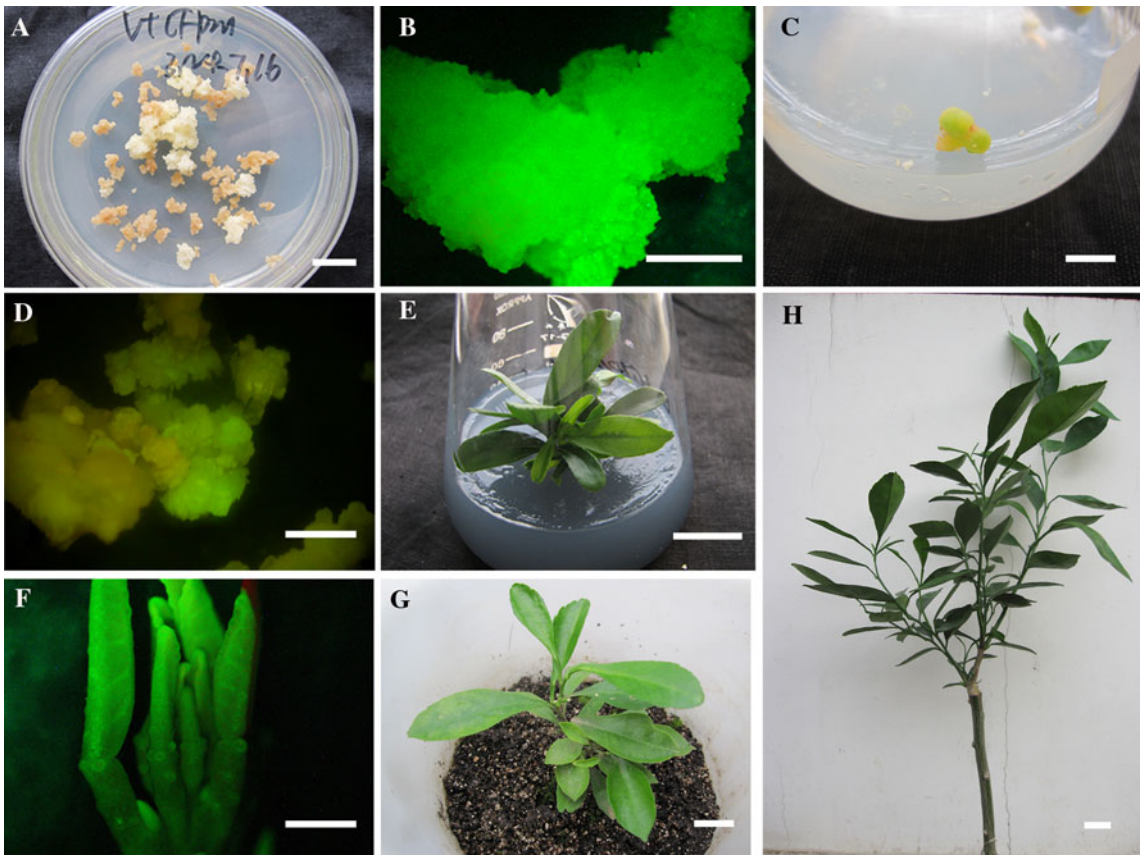

Fig. 1 Regeneration of Valencia orange plants expressing Mt-GFP. **a** Recovered kanamycin-resistant callus after 6 week culture on the selection medium; **b** Transformed callus expressing green fluorescence; **c** Kanamycin-resistant callus dedifferentiated into embryos on the embryoid-induction medium; **d** Regenerated embryoids expressing GFP; **e** Recovered plantlets; **f** Recovered shoots expressing GFP; **g** In vitro rooted transgenic plantlets growing in soil pot. **h** Shoot-tip in vivo grafted transgenic plantlet on trifoliate orange rootstock (*Poncirus trifoliata*). *Scale bar* 10 mm

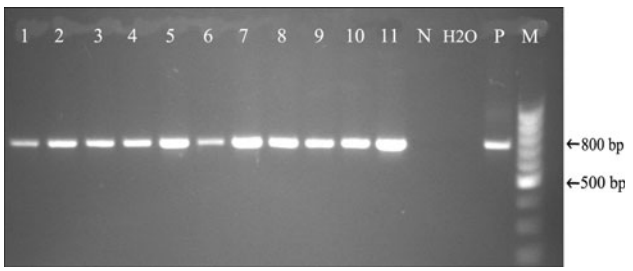

Fig. 2 PCR analysis of transgenic Valencia orange with specific primers for Mt-GFP gene. Lanes: M 100 bp DNA marker; P positive control; H2O negative control without DNA template (water only); N negative control (DNA from untransformed plants); 1–11 independent transgenic plants

transferred immediately for somatic embryo induction without antibiotics which reduced the toxicity of antibiotics. PCR analysis revealed that 100% of the callus with green fluorescence resulted from positive transgenic events. This demonstrates that though some silenced transgenic callus may have been lost, it is still a good marker gene to identify the transgenic events at an early stage in *Agrobacterium*-mediated transformation and is a great assistance for citrus breeding and gene function analysis.

Although GFP usage poses many advantages compared with other common marker genes frequently used in plant biology, monitoring its expression is coupled with some limitations and obstacles (Zhou et al. 2005). Firstly, the presence of some agent that can mask the GFP fluorescence, and/or is opaque to the excitation signal, represents an obstacle that could complicate the monitoring of the fluorescence emitted from particular tissue (Robic et al. 2009). Consequently, untransformed plant tissues were used as negative controls for adjusting the parameters to ensure the absence of GFP fluorescence in nontransformed leaves. Secondly, it has been reported that expression of the marker gene is influenced by differences in leaf tissue, leaf developmental stage, and location within the plant body (Hraska et al. 2008). Different cytoplasmic density of cells in young and older leaves leads to the "dilution" of GFP in older tissues thereby weakening the fluorescence (Omar and Grosser 2008). Therefore, in our experiments, the

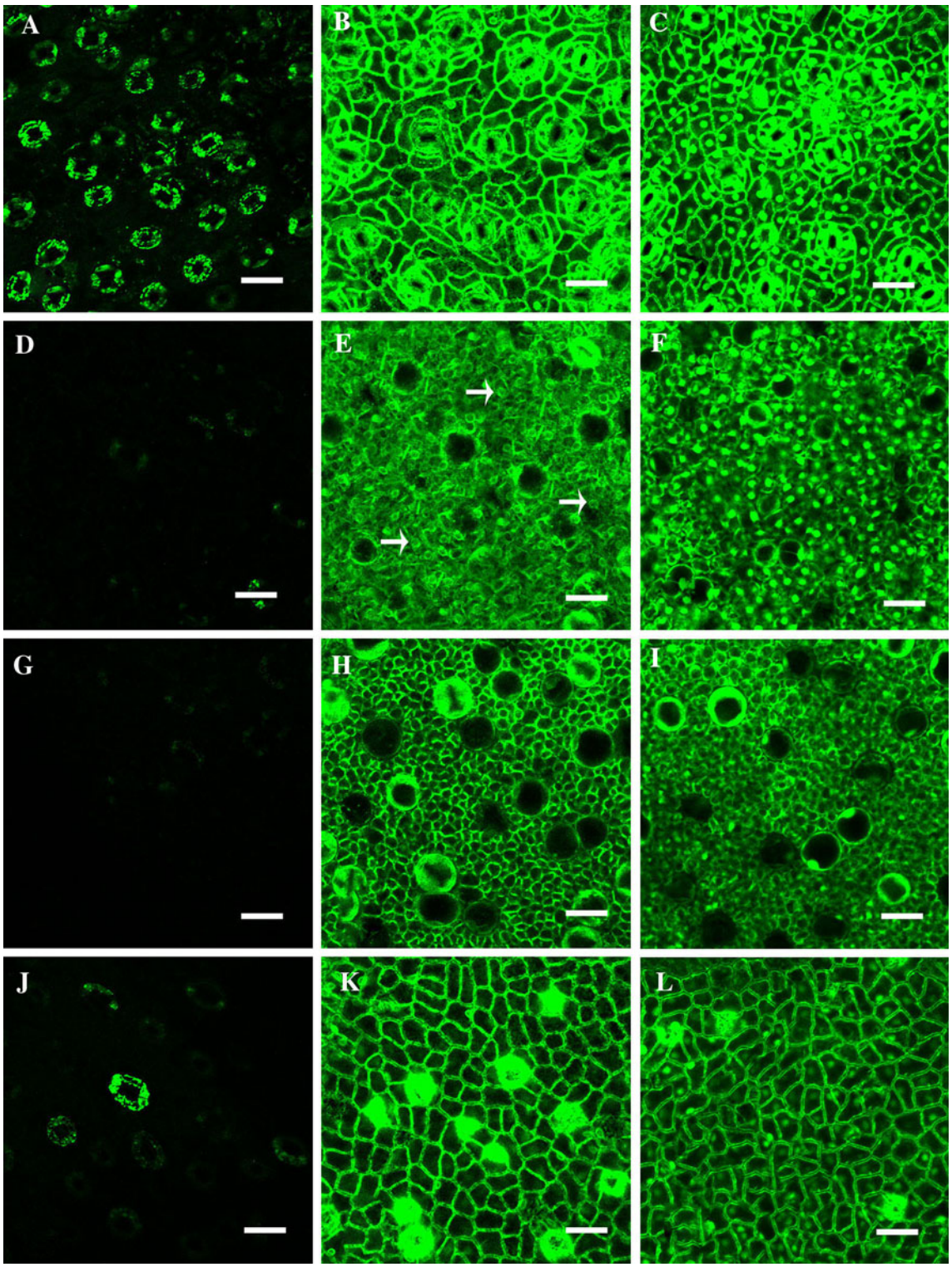

Fig. 3 Visualization of three different subcellular targeted GFP in citrus plant tissue in vivo. **a**, **d**, **g** and **j** the localization of Mt-GFP in lower epidermal cells, spongy parenchyma cells, palisade parenchyma cells and upper epidermal cells; **b**, **e**, **h**, **k** the localization of Er-GFP in lower epidermal cells, spongy parenchyma cells, palisade parenchyma cells and upper epidermal cells. The *arrow* highlights fluorescent hollow-rings observed specifically in the spongy tissue; **c**, **f**, **i** and **l** the localization of Cy-GFP in lower epidermal cells, spongy parenchyma cells, palisade parenchyma cells and upper epidermal cells. *Scale bar* 20 μm

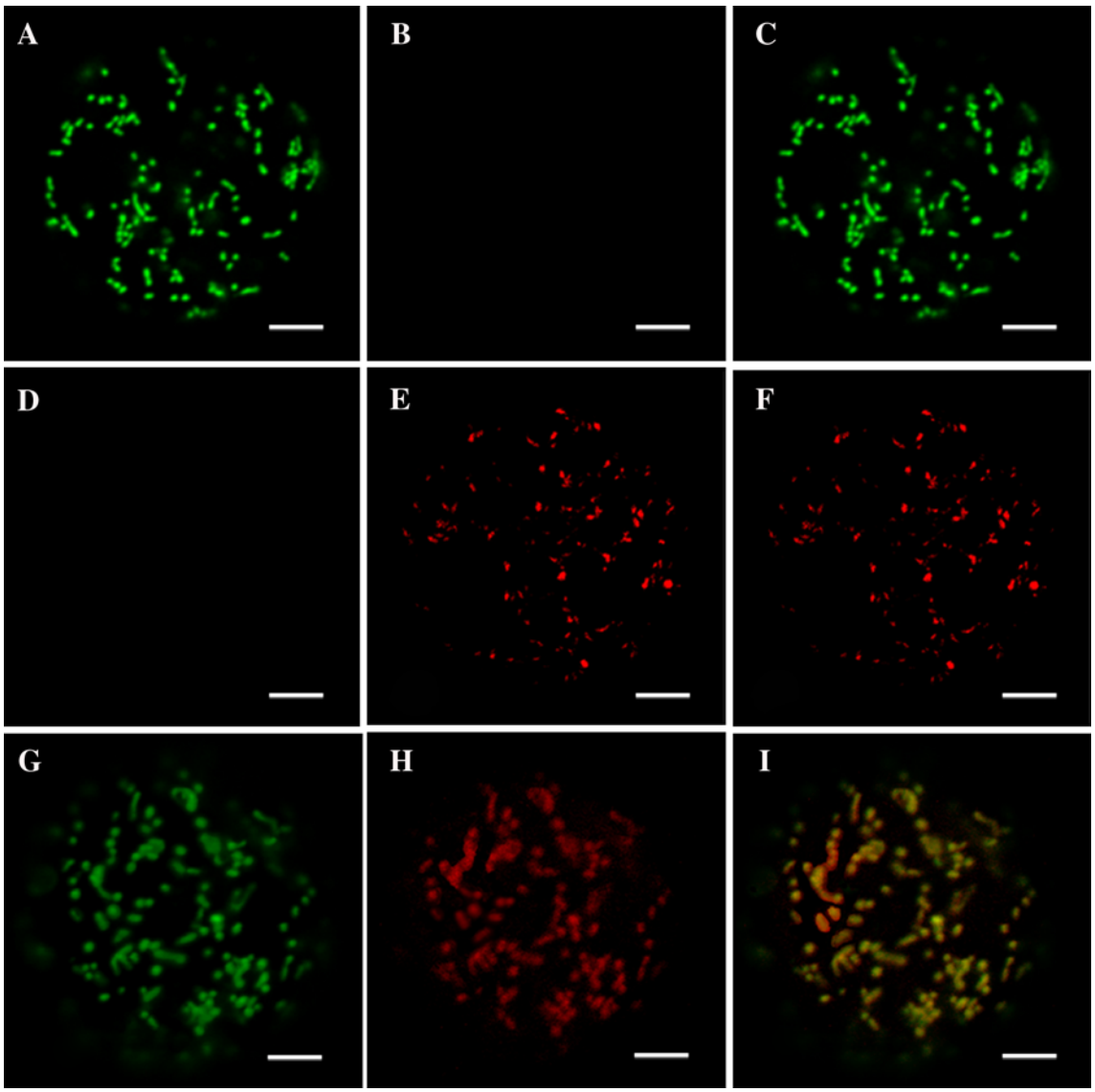

Fig. 4 The Mt-GFP fusion protein co-localizes with a mitochondrial-specific dye. **a**, **b** Transformed protoplasts without MitoTracker at 530 and 615 nm, respectively; **d**, **e** Nontransformed protoplasts stained with MitoTracker at 530 and 615 nm, respectively; **g**, **h** Transformed protoplasts stained with MitoTracker at 530 and 615 nm, respectively; **c**, **f**, **i** Merge of the GFP fluorescence and MitoTracker fluorescence of **a** and **b**, **d** and **e**, **g** and **h**, respectively. *Scale bar* 5 μm

tested leaves were of similar physiological age, and leaf tissues were sampled from the same location on individual leaves.

This study showed the distinct subcellular distribution of fluorescence in different mature leaf cells expressing the Cy-GFP, Er-GFP or Mt-GFP. To our knowledge, the version of Mt-GFP was for the first time used in citrus transformation to produce transgenic Valencia orange lines. The Mt-GFP transgenic citrus leaves exhibited GFP fluorescence specifically in the guard cell of the stomata (Fig. 3a, j). No Mt-GFP expression was visualized in spongy parenchyma cells or palisade parenchyma cells (Fig. 3d, g). Our results differ from the observations in *A. thaliana* or tobacco, where GFP-tagged mitochondria are visible in most cells of leaf tissue (Kohler et al. 1997; Logan and Leaver 2000), but are not inconsistent to the mitochondrial heterogeneous morphology in roots (Lo et al. 2004). It was confirmed by the observation that a narrow band of green fluorescence within epidermal cells could be observed in longitudinal optical sections of a leaf (data not shown), and then by co-localizing the Mt-GFP fusion protein with a mitochondrial-specific dye in citrus protoplasts. Mitochondria are known to associate with energy-consuming organelles and structures, and particularly, in the guard cell of the stomata. The observations from this study of the association of mitochondria with

guard cells provide further evidence for this fact. In addition, plant mitochondria show cell-specific and tissue-specific changes in number, morphology, size and subcellular localization as well as association with other organelles or membrane systems (Okamoto and Shaw 2005; Logan 2010). The expression of Er-GFP was found mainly within the endomembrane system and a lot of fluorescing hollow-rings were observed (Fig. 3b, e). We presume that this is due to endoplasmic reticulum clustered around the nucleus, but we cannot exclude the possibility that they are some specialized forms of organelle (Herman 2008). Conley et al. (2009) observed that the endoplasmic reticulum possesses an intrinsic ability to form protein body-like accretions in eukaryotic cells when overexpressing particular proteins. The equal distribution of fluorescence throughout the cytoplasm and the nucleus in guard cells expressing untargeted GFP indicates that GFP has no inherent obvious affinity for subcellular structures (Fig. 3c and data not shown) that might interfere with attempts to target it to different subcellular locations in citrus tissues and cells. In contrast to its obvious exclusion from vacuoles, untargeted GFP accumulates within the nucleoplasm in the lower epidermis and spongy mesophyll cells (Fig. 3c, f). Due to its small size (less than 30 kDa), GFP probably enters the nucleoplasm by diffusion, since proteins smaller than 40–60 kDa are thought to diffuse through the nuclear pore (Forbes 1992; Raikhel 1992). Transgenic leaves expressing either the untargeted or targeted constructs generally fluoresced brightly, but we did not observe any toxic effects in the transgenic tissues through the use of strong promoter and highly expressed GFP gene, indicating that citrus tissues can tolerate high expression levels of GFP in the nucleus and the cytoplasm, as well as in mitochondria.

For citrus somatic hybridization, the fusion model of "diploid embryogenic callus protoplasts + diploid mesophyll derived protoplasts'' has been extensively used, where almost all recovered hybrids or cybrids plants from protoplast symmetric fusion usually received their mitochondrial DNA (mtDNA) from the embryogenic parents (Guo et al. 2004). To date, only citrus somatic hybrids and/or cybrids with the mtDNA from the corresponding embryogenic parent have been recovered, while mesophyll protoplasts without the mtDNA from the corresponding embryogenic parent do not divide and regenerate into plants in the current culture system (Olivares-Fuster et al. 2007). It was supposed that mtDNA from the corresponding embryogenic callus parent played an important role in both allotetraploid somatic hybrid and cybrid regeneration. Moreira et al. (2000) suggested that higher quantities of mitochondria in embryogenic protoplasts provide the energy required to regenerate diploid cybrid plants of the leaf parent, as well as allotetraploid somatic hybrids, but the role of mtDNA in citrus cybrid regeneration mechanism is still unknown. The availability of transgenic Mt-GFP lines and efficient visualization of Mt-GFP expression could be useful to reveal the mechanism in citrus, and this strategy has been well demonstrated in *Arabidopisis* and tobacco by Sheahan et al. (2005).

In conclusion, this study, by using powerful confocal laser scanning microscopy, revealed the distinct subcellular distribution of fluorescence in citrus tissues expressing the Cy-GFP, Er-GFP or Mt-GFP genes in citrus leaf cells. The result of this study will add another useful part to the mosaic of our knowledge about the relationship of the mitochondria and endoplasmic reticulum in different plant leaf cells and the stability of its expression pattern in different plant organs and tissues.

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