Visualizing microtubule dynamics and membrane trafficking in live and dividing plant cells
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Citation for published version (APA):

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Arabidopsis Tubulin Folding Cofactor B
Interacts with α Tubulin \textit{in vivo} Revealed by Multi-Mode-FRET-Microscopy

Chapter 3

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Under Review
ABSTRACT

Microtubule biogenesis requires assembly competent α and β tubulin that are posttranslationally modified by five tubulin folding cofactors (TFCs) namely TFCA, TFCB, TFCC, TFCD and TFCE together with one small G-protein Arl2. Here we report the isolation and characterization of Arabidopsis TFCB (AtTFCB). AtTFCB contains a Cytoskeletal Associated Protein-Glycine rich (CAP-Gly) and is found to be ubiquitously expressed in all organs of Arabidopsis. The AtTFCB-Yellow Fluorescent Protein (YFP) fusion protein remains cytosolic. AtTFCB-overexpression results in cell death that can be rescued by simultaneous overexpression of α tubulin, indicating their functional interaction. The microtubules visualized with Cyan Fluorescent Protein (CFP) labeled α tubulin are less in number in AtTFCB-YFP coexpressing cells as compared to the controls. Multi-mode-Fluorescence Resonance Energy Transfer (FRET)-microscopy for the first time reveals the physical interaction of TFCB with α tubulin inside live cells. These findings suggest that the excess AtTFCB sequesters available α tubulin and causes an imbalance between α/β monomers. This results into protoplast lethality which can be counterbalanced by elevated production of α tubulin which is similar in yeast cells.
INTRODUCTION

Microtubules are essential in plants and play vital roles in many processes including embryo development (Steinborn et al., 2002), organ formation (Whittington et al., 2001), organ twisting (Thitamadee et al., 2002), maintaining growth direction (Bibikova et al., 1999) and establishing spatial organization of cells (Mathur, 2004). The dynamic microtubule polymers are made of α and β tubulin heterodimers. Microtubule biogenesis and dynamics mainly depend on the presence of assembly competent tubulin-dimers and various microtubule associated proteins (Mayer and Jurgens, 2002; Szymanski, 2002). Unlike actin and γ tubulin (Melki et al., 1993), α and β tubulin require posttranslational modifications for their transformation into functional αβ-heterodimers. These modifications are carried out by chaperonins and tubulin folding cofactors acting in a stepwise concert to manufacture the functional heterodimer (Lopez-Fanarraga et al., 2001). α tubulin is processed by Tubulin Folding Cofactor B (TFCB) and Tubulin Folding Cofactor F (TFCE) while β tubulin is modified by Tubulin Folding Cofactor A (TFCA) and Tubulin Folding Cofactor D (TFCD) sequentially. Finally, Tubulin Folding Cofactor C (TFCC) plays a role producing a functional heterodimer from the α and β tubulin monomers (Tian et al., 1996; Tian et al., 1997). This model of tubulin folding pathway is based on yeast mutant and mammalian in vitro system analyses. It is difficult to purify α and β tubulin subunits from cell extracts and in vitro translated α and β tubulins fail to incorporate into functional microtubules showing the requirement of TFCs.

The Arabidopsis PILZ group mutants KIESEL, PORCINO, CHAMPIGNON, PFIFFERLING, and HALLIMASCH displaying embryo lethality (Mayer et al., 1999), were found to encode Arabidopsis TFCs (AtTFCs) A, C, D, E and Arl2, respectively. Independently isolated TITAN mutants encode Arl2 (TITAN3) and AtTFCD (TITAN1) show cell division defects during endosperm and embryo development (Tzafrir et al., 2002). The PILZ group mutants lack microtubules whereas the organization of actin filaments and the localization of the actin-dependent plasma membrane cell polarity marker PIN1 are normal in them (Steinborn et al., 2002). The KIESEL displays microtubular defects (Kirik et al., 2002b; Steinborn et al., 2002). These TFCs mutants show that their lethal effects are mediated through the absence or malformations of microtubules that are vital for all dividing cells. The loss of PORCINO and KIESEL functions could be rescued by overexpression of either the endogenous gene or a vertebrate homologue showing their functional similarities (Kirik et al., 2002b; Kirik et al., 2002a).

The multi-step TFC system takes care of producing functional heterodimers and at the same time maintains a correct balance of monomers and dimers. In mammalian and
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yeast cells, overexpression of β tubulin is lethal (Burke et al., 1989; Katz et al., 1990; Gonzalez-Garay and Cabral, 1995) but not that of α tubulin (Weinstein and Solomon, 1990). In budding yeast the lethal effects of excess β tubulin can be rescued either by β tubulin interacting TFCA Rbl2p overexpression or by α tubulin overproduction (Archer et al., 1995). In mammalian cells, upon β tubulin overexpression the levels of endogenous α tubulin rise (Gonzalez-Garay and Cabral, 1995). In Arabidopsis weak allele kis-T1 KIESEL mutant which lacks sufficient β tubulin folding AtTFCA, plants become dwarf because of severe cell division and expansion defects (Kirik et al., 2002b; Steinborn et al., 2002). The kis-T1 mutant can be rescued by 35S-driven KIS and mouse TFCA, but also by overproduction of α tubulin (Kirik et al., 2002b). In addition there was no deleterious effect of overexpression of AtTFCA (Kirik et al., 2002b). Together these findings suggest that the correct balance between tubulin subunits is essential for normal cell functioning.

Consistent with the notion that TFCs function in a common pathway, double and triple combinations of yeast mutants for tubulin folding cofactors do not cause more severe phenotypes (Archer et al., 1995). Overexpression of β tubulin in AtTFCA mutant fails to make the phenotype more severe (Kirik et al., 2002b). The genetic analyses in yeast has identified the functional hierarchy among tubulin folding cofactors and overexpression studies showed differences between various TFCs in their ability to bypass the requirement for each other (Radcliffe et al., 1999). In plants quite a lot is known about AtTFCA and AtTFCC, even (indirect) subcellular localization studies have been performed (Kirik et al., 2002b; Kirik et al., 2002a). However, information on AtTFCB is still lacking since no mutants encoding AtTFCB have been isolated even after completely saturated mutant screens (Steinborn et al., 2002). Importantly, AtTFCB contains a CAP-Gly domain with remarkable homology to the CAP-Gly domains of CLIP170 indicating its plant microtubule binding capability (Dhonukshe and Gadella, 2003). Together, this prompted us to analyze the subcellular distribution of AtTFCB and its overexpression effects. In addition, we incorporated Fluorescence Resonance Energy Transfer (FRET) analysis (Gadella et al., 1999; Immink et al., 2002; Vermeer et al., 2004) for monitoring molecular interactions between AtTFCB and α tubulin. FRET occurs if an energy quantum is transferred from a donor fluorophore to an acceptor fluorophore (or chromophore) that are in close proximity of 2-6 nm (within the range of protein dimensions). The prerequisites for this phenomenon are that i) the donor fluorescence emission spectrum overlaps with the absorption spectrum of the acceptor fluorophore ii) that the transition moments of donor and acceptor are not perpendicular and iii) that the distance between the donor and acceptor is less than the 1.5 R₀ units with R₀ being the Forster Radius (Gadella et al., 1999).
RESULTS

Arabidopsis ubiquitously express a CAP-Gly domain containing TFCB

The remarkable sub-cellular localization of YFP-CLIP170 decorating the plus ends of plant microtubules (Dhonukshe and Gadella, 2003) prompted us to investigate whether plants also express CLIP170-like proteins. The most significant and conserved domain for the interaction of CLIP170 with microtubules is the CAP-Gly domain. From a BLAST sequence analysis we found two CAP-Gly domain containing ORFs present in Arabidopsis thaliana genome which turned out to be the human TFCB and TFCE orthologues. Out of the two, cloning of AtTFCE has been reported recently (Steinborn et al., 2002). AtTFCB that has not yet been reported shares high sequence conservation with human (36%), budding yeast (21%) and fission yeast (31%) cofactor B proteins (Figure 1a-d). In view of the high degree of sequence homology observed in these cofactors (suggesting a conserved critical cellular function) and failure to isolate a mutant for AtTFCB, we undertook the PCR-based cloning and expression analysis of AtTFCB. First we successfully isolated the full-length cDNA of AtTFCB from Arabidopsis cDNA library and confirmed its sequence by DNA sequencing. To find out the expression pattern of AtTFCB in different organs of Arabidopsis we prepared cDNAs from total RNA isolated from them and performed an RT-PCR analysis. By RT-PCR we could amplify equivalent amounts of AtTFCB from the different organs of Arabidopsis (Figure 1e) implicating ubiquitous expression.

Expression of AtTFCB fused to YFP remains cytosolic and causes protoplast lethality

To find out the sub-cellular localization and effects of AtTFCB expression in live plant cells we made YFP fusions to both the ‘N’ and the ‘C’ terminus of AtTFCB and introduced them into protoplasts. Both ‘N’ and the ‘C’ terminal fusion-proteins were expressed as inferred from the bright YFP fluorescence and they remained cytosolic without any microtubular labeling (Figure 2b). Surprisingly, within a short period after transfection (< 16 hrs) 40% of the protoplasts expressing the fusion protein at relatively high levels showed altered cell shapes (Figure 2a). This was never observed in protoplasts transfected with either non-targeted YFP (Figure 2a) or any other constructs tested so far (data not shown). Closer observations of these protoplasts revealed that the chloroplasts (in negative stain) together with the cytoplasm had bulged out completely (Figure 2c) which was in strong contrast to the controls (Figure 2d).
Figure 1. AtTFCB sequence homology and its organ specific expression

Sequence comparisons (a) and Phylogenetic relationships (b) between TFCB from Arabidopsis thaliana (AtTFCB), Homo sapiens (Human Cofactor B), Saccaromyces cerevisiae (Alf1p) and Saccaromyces pombe (Alp11). Sequence comparisons (c) and Phylogenetic relationships (d) between CAP-Gly domains from CLIP170 and from TFCB from Arabidopsis thaliana (AtTFCB), Homo sapiens (Human Cofactor B), Saccaromyces cerevisiae (Alf1p) and Saccaromyces pombe (Alp11). CLIP170 contains two such domains located at the ‘N’ terminus denoted here by CLIP170one and CLIP170two while tubulin-folding cofactors from Arabidopsis (AtTFCB), human, budding yeast (Alf1p) and fission yeast (Alp11) contain one CAP-Gly domain each at the ‘C’ terminus. Identical amino acids are highlighted by black, similar ones by gray shading and less homologous by faint letters. (e) AtTFCB expression analysis by RT-PCR in different organs.

Co-overexpression of α tubulin rescues the AtTFC expression defects

TFCB has been shown to functionally interact with α tubulin in yeast and mammalian in vitro systems (Tian et al., 1997; Feierbach et al., 1999). Overexpression phenotypes of AtTFCB prompted us to further analyze its predicted functional interaction with α tubulin. Therefore, we made a CFP-α tubulin 6 (CFP-TUA6) fusion and tested it in protoplasts. CFP-TUA6 was efficiently incorporated into protoplast microtubules (Figure 2h). This enabled us to study a possible in vivo interaction between AtTFCB and α tubulin by performing protoplasts co-transfection with CFP-TUA6 and YFP-AtTFCB (or AtTFCB-YFP). Strikingly, protoplasts expressing both CFP-TUA6 and YFP-AtTFCB (or
Figure 2. Cellular localization and (over) expression effects of AtTFCB and its functional interaction with α tubulin.

Yellow is YFP (non targeted YFP or AtTFCB-YFP) and cyan is CFP (CFP-TUA6) Protoplasts transfected with non-targeted YFP (a, d; 36 hrs after transfection) and with AtTFCB-YFP (b, c; 14 hrs after transfection). Note in case of AtTFCB-YFP transfected protoplasts, the morphology defects are related to the expression levels (YFP fluorescence intensity). This is in contrast to protoplasts expressing non-targeted YFP (where protoplasts with higher YFP expression also maintain their spherical shapes). In (a) and (b) arrowheads and arrows indicate
protoplasts with high and low expression levels, respectively. AtTFCB-YFP remains cytosolic without any microtubular labeling.

Protoplasts transfected with CFP-TUA6 (h) and cotransfected with CFP-TUA6 and AtTFCB-YFP (e-g) and i: 36 hrs after transfection. (e) shows YFP fluorescence (depicting AtTFCB-YFP), (f) shows CFP fluorescence (depicting CFP-TUA6) and (g) depicts merged image. Note that in (e-g) and (i) even after 36 hrs after transfection the protoplasts maintain their spherical shapes. Comparison of (h) and (i) shows that microtubule (labeled by CFP-TUA6) numbers in (i) are far less than in (h). Bars 10 μm.

AtTFCB-YFP) maintained their normal spherical shape even >36 hrs after transfection (Figure 2e-g) irrespective of the AtTFCB expression levels suggesting their functional in vivo interaction. In addition, the CFP and YFP fluorescent signals were co-localized (in the cytosol) and a higher fraction of CFP-TUA6 remained cytosolic exhibiting reduced number of microtubules as compared to the control (Figure 2h, i).

**Multi-mode FRET microscopy reveals physical interaction of AtTFCB with α tubulin in vivo**

There are various methods for determining FRET (Gadella et al., 1999; Jares-Erijman and Jovin, 2003; Vermeer et al., 2004) with different levels of analytical capabilities. We utilized two independent methods called as Fluorescence Spectral Imaging Microscopy (FSPIM) and Fluorescence Lifetime Imaging Microscopy (FLIM). FSPIM provides both spatial and spectral resolution and records both donor quenching and acceptor sensitization due to FRET (Gadella et al., 1999; Shah et al., 2001; Immink et al., 2002; Vermeer et al., 2004). This provides an elegant and fast technique for screening the physical interactions. FLIM is a quantitative technique for determining FRET in which the excited-state decay kinetics of the donor fluorescence is measured. In case of FRET the donor fluorescence lifetime decreases which can be measured with FLIM.

First, fluorescence emission spectra were taken to determine if there exists sensitized emission in protoplasts co-expressing YFP-AtTFCB (or AtTFCB-YFP) and CFP-TUA6 by inclusion of all possible combinations as controls. Figure 3 shows the average spectra of the protoplasts co-expressing YFP-AtTFCB (or AtTFCB-YFP) and CFP-TUA6 together with those expressing other combinations. All spectra are normalized to the fluorescence observed at 475 nm (which is the first emission band of CFP). Figure 3 shows that upon 435 nm excitation (of CFP) there was a strong fluorescence emission of YFP only in protoplasts coexpressing YFP-AtTFCB (or AtTFCB-YFP) and CFP-TUA6. The strong YFP-emission likely reflects YFP-sensitization due to FRET from CFP to YFP. Such a strong YFP-emission was not present in all other
FRET Reveals AtTFCB-α Tubulin Interaction

combinations. FRET between YFP-AtTFCB (or AtTFCB-YFP) and CFP-TUA6 suggests their physical interactions in vivo.

To confirm that the results of FSPIM analysis indeed reflect FRET and not directly excited overexpressed YFP, we employed FLIM microscopy with similar co-expression combinations. Figure 4 shows the average phase ($\tau_p$) and modulation ($\tau_m$) fluorescence lifetimes. The protoplasts coexpressing YFP-AtTFCB (or AtTFCB-YFP) and CFP-TUA6 show decreased donor (CFP) fluorescence lifetimes in comparison with all other combinations (compare Figure 4e, f with Figure 4a-d and compare Figure 4k, l with Fig 4g-i). The average phase and modulation fluorescence lifetimes are shown in Table 1. They clearly show the decreased donor (CFP) lifetimes only in case of YFP-AtTFCB + CFP-TUA6 and AtTFCB-YFP + CFP-TUA6 coexpressions displaying the in vivo physical interactions in live cells. This independently confirms the FSPIM results and shows for the first time the direct in vivo physical interaction of TFCB with α tubulin in living cells.

Figure 3. FSPIM analysis

Image spectra were taken using 435nm excitation light and are normalized to the intensity observed at 475 nm. Each curve represents the mean spectrum for eight individual living protoplasts.
DISCUSSION

Microtubule related genes cloned from Arabidopsis mutant screens for cell division defects and embryo lethality mainly belong to the microtubule ‘biogenesis class’ indicating their vital roles (Kirik et al., 2002b; Mayer and Jurgens, 2002; Steinborn et al., 2002). They represent the Arabidopsis orthologues for TFC A, C, D and E from yeast and mammals (Steinborn et al., 2002). Our AtTFCB analysis completes the TFC family from Arabidopsis.

We show that AtTFCB is ubiquitously expressed in the different organs of Arabidopsis similar to the expression pattern of AtTFCA (Kirik et al., 2002b), and AtTFCCC (Kirik et al., 2002a). Since all cells in all organs contain microtubules, a ubiquitous expression pattern of genes involved in tubulin biogenesis is expected. We observed that AtTFCB remains cytosolic. This is similar to the TFCB orthologues \( \text{Alf} \text{lp} \) in budding yeast (Feierbach et al., 1999) and \( \text{Alp} \text{llB} \) in fission yeast (Radcliffe et al., 1999). In addition, we saw that expression of AtTFCB affected microtubular organization by reducing their number, eventually causing drastic morphological defects. Very similar observations have been made for \( \text{Alf} \text{lp} \) and \( \text{Alp} \text{llB} \), the budding and fission yeast orthologues. These defects could be overcome by co-expression of \( \alpha \) tubulin in the protoplasts. Our cotransfection experiments showed that protoplasts co-expressing \( \alpha \) tubulin and AtTFCB maintain the spherical protoplast shape and viability for prolonged time. Consistently, an increased level of \( \alpha \) tubulin in \( \text{Alp} \text{llB} \)-overexpressing fission yeast cells rescues lethality (Radcliffe et al., 1999). Together these results indicate a functional conservation of plant and yeast TFCB.
Table 1. FLIM values and FRET efficiency and molecular distance calculations

<table>
<thead>
<tr>
<th>Co-expression</th>
<th>n</th>
<th>Phase (τ_d) Fluorescence Lifetime (ns)</th>
<th>Modulation (τ_m) Fluorescence Lifetime (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFP + YFP</td>
<td>33</td>
<td>2.32 ± 0.19</td>
<td>2.75 ± 0.12</td>
</tr>
<tr>
<td>CFP + YFP-AtTFCB</td>
<td>10</td>
<td>2.22 ± 0.26</td>
<td>2.74 ± 0.13</td>
</tr>
<tr>
<td>CFP + AtTFCB + YFP</td>
<td>10</td>
<td>2.12 ± 0.12</td>
<td>2.70 ± 0.12</td>
</tr>
<tr>
<td>CFP-TUA6 + YFP</td>
<td>17</td>
<td>2.27 ± 0.26</td>
<td>2.80 ± 0.11</td>
</tr>
<tr>
<td>CFP-TUA6 + YFP-AtTFCB</td>
<td>87</td>
<td>1.40 ± 0.24</td>
<td>2.26 ± 0.22</td>
</tr>
<tr>
<td>CFP-TUA6 + AtTFCB + YFP</td>
<td>31</td>
<td>1.31 ± 0.11</td>
<td>2.23 ± 0.16</td>
</tr>
</tbody>
</table>

\[
E = 1 - \frac{\bar{\tau}_{IM}}{\bar{\tau}_D} \quad (1)
\]

\[
E = \frac{1}{1 + \left(\frac{r}{R_n}\right)^6} \quad (2)
\]

In equation (1) \( \bar{\tau}_{IM} \) and \( \bar{\tau}_D \) are the average fluorescence lifetimes of the donor in the presence and absence of the acceptor, respectively. The relationship between FRET efficiency (E) and the actual distance (r) between a single donor and a single acceptor molecule is given in the Förster equation (2), showing that FRET can also be used to estimate the approximate distances between the donor and the acceptor chromophores (Stryer, 1978). In equation (2) \( R_n \) is the Förster radius for FRET, which depends on the spectral properties and relative orientation of the donor and acceptor molecules (for reviews on FRET see (Clegg, 1996; Gadella et al., 1999; Selvin, 2000). For FRET between CFP and YFP \( R_n = 5.2 \) nm (Tsicn, 1998; Gadella et al., 1999).

According to equation (1) the calculated FRET efficiencies for CFP-TUA6 + YFP-AtTFCB and CFP-TUA6 + AtTFCB-YFP are 39 % and 42 % respectively. Assuming 100% association, the molecular distance between α tubulin and AtTFCB calculated according to equation (2) is approximately 5.18 nm. In case of an unassociated α tubulin part the same average FRET efficiency can only be explained by an even shorter intramolecular distance for the associated α tubulin part. Hence, the 5.18 nm distance can be regarded as the maximum distance (excluding orientation effects).

The observed effects of TFCB overexpression can be attributed to an excess of β tubulin in such cells. The model explaining this link assumes that excess TFCB sequesters α tubulin and thereby indirectly causes an excess of β tubulin which is toxic to cells. There are number of observations in support of this model. Overexpression of β tubulin and reduction of expressed α tubulin is lethal in yeast (Burke et al., 1989; Weinstein and Solomon, 1990; Archer et al., 1995; Gonzalez-Garay and Cabral, 1995). Balancing the excess of β tubulin by overexpression of α tubulin or TFCB rescues the lethal effects (Abruzzi et al., 2002). Inversely an excess α tubulin seems not to be toxic to yeast cells (Burke et al., 1989). Essentially similar observations have been made in plants where overexpression of α tubulin is not toxic (Ueda et al., 1999; Whittington et al., 2001), reduction of α tubulin expression by RNAi is toxic (Bao et al., 2001), and overexpression
of β tubulin (AtTUB1 or AtTUB3) results in slower plant growth (Shaw et al., 2003). We think our results fit with this model. An alternative explanation may be that overexpression of TFCB causes a reduction in the expression of α tubulin. Essential for the first model is showing that a physical interaction between α tubulin and TFCB does exist in co-overexpressing cells.

Indeed immunoprecipitation, yeast two hybrid and mutant complementation experiments have shown interactions of various TFC with α and β tubulins in different cell types. Our multi-mode FRET analysis shows for the first time the direct in vivo interaction of ATFCB with α tubulin in living cells. It even provides the approximate molecular distance of 5.1 nm between AtTFCB and α tubulin in the complex assuming 100% complex formation. Hereby our results provide strong support for the sequestration model.

In conclusion, our analysis of AtTFCB shows that like other cofactors it also influences tubulin levels and microtubule existence. Moreover, AtTFCB seems to have a dual function as it acts 1) in α tubulin processing to transform it into an assembly competent subunit and 2) as a reservoir of processed α tubulin, which in case of excess β tubulin (which is toxic) can release the required α tubulin to maintain the critical balance of α/β tubulin monomers. This may explain the reason for the failure to isolate the AtTFCB mutant. As in its absence either the α tubulin cannot be processed properly or the cell will have less availability of stored α tubulin when required that can cause the drastic effects.

**METHODS**

*Arabidopsis tubulin-folding cofactor B (AtTFCB) constructs*

AtTFCB was isolated from Arabidopsis cDNA library (cloned in the pGAD10 vector; Clontech Laboratories, PA, California) with a forward primer containing SalI site (AtTFCB forward; 5'-ACGCGTCGACATGGCAACTTCGCGTCTAC-3') and a reverse primer containing BamHI site (AtTFCB reverse; 5'-CGCGGATCCTTATATTTCATCTT CCTCG-3') and cloned into vector pEYFP-C1 (Clontech Laboratories) downstream of EYFP using the same restriction enzymes resulting into AtTFCB ‘N’ terminal fusion to EYFP (EYFP-AtTFCB) that was cloned into plant transfection vector pMON resulting into vector pMON-double 35S-EYFP-AtTFCB-tNOS. AtTFCB ‘C’ terminal fusion to EYFP was done by PCR based cloning.
**RT-PCR analysis**

The cDNAs were prepared from RNA isolated from different tissue sources of *Arabidopsis* as described before (Albrecht *et al.*, 1998). The amounts of cDNA prepared from different tissue sources were determined by spectrophotometer and agarose gel electrophoresis and equal amounts of synthesized cDNAs were used for PCR. The primers used were AtTFCB forward; 5'-ACGCGTCGACATGGCAACTTTACACCTGTTTAC-3' and AtTFCB reverse; 5'-CGCGGATCCTTATATTTCATCTTCTCG-3'. PCR amplified products were analyzed by agarose gel electrophoresis.

**Protoplasts preparation and transfection**

The lower epidermises of Cowpea (*Vigna unguiculata* L.) leaves were stripped and the stripped leaves were incubated with their stripped sides down in a petridish containing 50 ml freshly prepared enzyme solution (1% cellulase, 0.05% pectinase, 0.6 M mannitol and 10 mM CaCl₂) with slow shaking at 25°C for 3.5 hrs. The isolated protoplasts were filtered with sieve and collected in 50 ml Greiner tube and were centrifuged for 5 min at 600 rpm. The supernatant was removed and the remaining protoplasts were washed three times with 50 ml 0.6 M Ca-Mannitol (0.6 M mannitol, 10 mM CaCl₂; pH 5.6) by spinning them at 600 rpm for 5 min. After washing the protoplasts were re-suspended in 25 ml Ca-Mannitol. The protoplasts density was determined by counting the protoplasts in a Fuchs-Rosenthal chamber. 10⁶ protoplasts were transferred to 10 ml tubes and centrifuged for 5 min at 600 rpm. The supernatant was removed until 150 μl was left. The protoplasts were kept on ice until further processing. 10 μg of pure DNA (diluted in 30 μl MQ) was added to 150 μl protoplasts, mixed together and this mixture was immediately treated with freshly prepared 500 μl of Polyethylene Glycol (PEG) solution (40% PEG-6000, 10 mM Ca(NO₃)₂, and 0.5 M mannitol) by shaking for 15 s. 4.5 ml mannitol MES (0.6 M mannitol, 15 mM MgCl₂, 0.1% MES; pH 5.6) was added to this solution and mixed using parafilm to stop the PEG reaction. The mixture was incubated at RT for 15-20 min before washing 3 times with 5 ml 0.6 M Ca-mannitol for 5 min at 600 rpm. Supernatant was discarded and the protoplasts were re-suspended in 1 ml protoplast medium (0.6 M mannitol, 10 mM CaCl₂, 1 mM KNO₃, 1 mM MgSO₄, 1 μM KI, 0.01 μM CuSO₄, 1 μg/ml 2,4 D, 25 μg/ml gentamycin; pH 5.4). The protoplast suspension was incubated at 25°C in continuous illumination.

**Confocal Laser Scanning Microscopy**

Samples were prepared in NUNC chambers (Nunc Inc., Naperville, Illinois) especially available for microscopy. For acquiring images of transfected cowpea protoplasts and
transformed tobacco BY-2 cells with different fluorescent constructs we used confocal laser scanning microscopy (CLSM) based on Zeiss LSM 510 microscopy system (Zeiss Corp., Oberkochen, Germany) composed of axiovert inverted microscope equipped with an argon ion laser as excitation source. Protoplasts expressing EYFP were excited with 514 laser line and EYFP emission was detected with 535-590 nm band-pass filter. Protoplasts expressing ECFP were excited with 458 nm laser line and ECFP emission was captured with 470-500 band-pass filter. In co-transfection experiments both ECFP/EYFP were scanned simultaneously using a 458/514 main dichroic splitter and a 515 secondary dichroic splitter by operating in the multi-channel imaging mode. This procedure effectively eliminates bleed-through of CFP fluorescence into the YFP channel and vice versa. A 40× oil immersion objective (numerical aperture 1.3) was used for scanning samples. The images were captured using LSM510 image acquire software (Zeiss Corporation, Jena, Germany). For each sample at least 30-60 optical sections of 0.5 μm apart were taken to cover the entire protoplast sphere. Acquired images were processed using LSM510 image browser version 3.2 (Zeiss Corp.). Maximum projections were obtained from serial optical sections spaced by 0.5 μm along the optical axis and were exported as TIFF files. All the exported images were processed with Adobe Photoshop version 5.0 (Adobe Systems Inc., Mountain View, CA).

**Fluorescence Spectral Imaging Microscopy (FSPIM)**

For FSPIM measurements the same FLIM/SPIM setup was used as described before (Vermeer et al., 2004). A 100W Mercury-Arc lamp and a D436/20 nm excitation filter provided excitation. For spectral analysis a 20/80% reflection/transmission dichroic mirror and an HQ460LP emission filter (both from Chroma, USA) were used. On the side port of the microscope an imaging spectrograph (Imspecto V7, Specim, Finland) coupled to a CCD camera (ORCA ER, Hamamatsu, Japan) was mounted for spectral detection. For all experiments the Zeiss plan Neofluar 40× 1.3 N.A. oil objective was used. For this objective, the slit dimensions corresponded to 201 μm × 2 μm in the object plane. The wavelength axis of the image spectra was calibrated using the mercury lines of the excitation source. The response function of the spectrograph and camera was calibrated using a calibration source (QTH 20W, Oriel, U.S.A.). Software for control, acquisition, processing and analysis of the data was written in C++, using Matlab 6.1 (The Mathworks, USA) and the image-processing library DIPlib (Pattern Recognition Group, TU Delft, The Netherlands, http://www.ph.tn.tudelft.nl/DIPlib/).
Fluorescence Lifetime Imaging Microscopy (FLIM)

For frequency-domain wide field FLIM measurements, the FLIM/SPIM setup implemented on an inverted fluorescence microscope (Axiovert 200M, Zeiss Corp.) was used as described before (Van Munster and Gadella, 2004). For selective imaging of CFP, a Helium-Cadmium laser (442 nm, 125 mW, Melles-Griot, USA) for excitation, a 455DCLP dichroic mirror and a D480/40 bandpass emission filter (Chroma, USA) were used. The frequency of the modulation was 74.818 MHz. Reference phase and modulation were obtained using a reference filter cube reflecting 0.1 % of the excitation laser light directly onto the detector (Van Munster and Gadella, 2004). FLIM stacks of 8 phase images were acquired with 0.5-1 s exposure time each using a Zeiss plan Neofluar 40× 1.3 N.A. oil objective. Protoplasts were mounted in 8-chambered cover slides (Nunc Inc.).

ACKNOWLEDGEMENTS

We are grateful to E. B. van Munster (University of Amsterdam, The Netherlands) and J. F. M. Vermeer (University of Amsterdam, The Netherlands) for their suggestions and help for FSPIM and FLIM analysis. We thank Paul Passarinho (Wageningen University, The Netherlands) and Valeri Hecht (Wageningen University, The Netherlands) for suggestions on RT PCR and J. Carette ((Wageningen University, The Netherlands) for stimulating discussions. P.D. and T.W.J.G. were supported by NWO FOM-ALW 805.47.012 and by NWO van der Leeuw 835.25.004.

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