The role of MAP65-1 in microtubule bundling during *Zinnia* tracheary element formation

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Summary

The MAP65 family of microtubule-associated proteins performs various functions at different stages of the cell cycle and differentiation. In this study, we have investigated the synchronous transdifferentiation of *Zinnia* mesophyll cells into tracheary elements in vitro. This allowed us to examine the role of the microtubule-associated protein MAP65 during the characteristic bunching of cortical microtubules that underlie the developing ribs of secondarily thickened cell wall. Immunofluorescence confirmed the microtubule bundles to be decorated with anti-MAP65 antibodies. Three *Zinnia MAP65* genes were examined; the expression of *ZeMAP65-1* was found to match that of the differentiation marker *TED2* and both were found to be upregulated upon addition of inductive hormones. We cloned the full-length sequence of *ZeMAP65-1* and found it to be most similar to other MAP65 isoforms known to bundle microtubules in other plant species. However, not all MAP65 proteins crosslink cortical microtubules and so, to confirm its potential bundling capacity, ZeMAP65-1 was transiently overexpressed in *Arabidopsis* suspension cells. This resulted in the super-bundling of microtubules in patterns resembling those in differentiating xylem cells. These findings establish that the MAP65-1 group of proteins is responsible for the bundling of cortical microtubules during secondary cell wall formation of xylogenesis as well as during the expansion of primary cell walls.

Key words: Microtubule-associated protein, MAP65, Microtubule, *Zinnia*, Xylem differentiation

Introduction

Zinnia mesophyll cells cultured in vitro undergo a remarkable transdifferentiation into xylem tracheary elements when appropriate hormones are added (Fukuda, 1980; McCann et al., 2001; Ye, 2002). The cells transform into xylem elements with secondarily thickened walls that reproduce the characteristic banded patterns seen in plants. As they transdifferentiate, the cortical microtubules begin to bunch into highly characteristic transverse/helical groups that predict the thick, sculptured cell walls that will form above them (Falconer, 1985; Fukuda, 1989). There is now good evidence that the 65 kDa microtubule-associated protein MAP65 is involved in the bundling of cortical microtubules. First discovered in tobacco BY-2 suspension cells (Jiang, 1993), carrot MAP65 (Chan et al., 1996) was subsequently shown to bundle microtubules in vitro (Chan et al., 1999) with 25-30 nm cross-bridges that reproduced the intermicrotubule spacing seen in planta (Lancelle, 1986). In cell-cycle-arrested cells undergoing cell elongation, the protein coded by carrot *DcMAP65-1* (Chan et al., 2003b) was shown to be the predominant form; of the nine MAP65 isoforms encoded in the *Arabidopsis* genome, the carrot sequence was found to be most closely related to AtMAP65-1 (Hussey and Hawkins, 2001). Bacterially expressed recombinant AtMAP65-1 was then formally demonstrated to bundle microtubules in vitro, probably by a process of dimerisation (Smertenko, 2004), reproducing the 25-30 nm cross-bridges observed with the carrot MAP65 fraction (Chan et al., 1999). This suggested that a subgroup of related MAP65 proteins is responsible for the parallel grouping of microtubules. Confirmation of this role has been provided for tobacco NtMAP65-1b (Wicker-Planquart et al., 2004) and for *Arabidopsis* AtMAP65-1 in vitro (Mao et al., 2005a; Mao et al., 2005b) and in vivo (Mao et al., 2005b; Van Damme et al., 2004). As the AtMAP65-1 isoform is expressed ubiquitously in all *Arabidopsis* organs and tissues, with the exception of anthers and petals, it is reasonable to expect it to have a bundling role throughout development (Smertenko, 2004). However, not all MAP65 isoforms behave in this way. Neither AtMAP65-3/PLE (Muller et al., 2004) nor AtMAP65-4 (Van Damme et al., 2004) decorates cortical microtubules, and AtMAP65-6 seems to be associated with mitochondria in cells (Mao et al., 2005b).

The transdifferentiation of *Zinnia* tracheary elements forms an excellent system for studying the role of MAP65 since microtubule bunching observed underneath these secondary wall thickenings occurs to a far greater extent than in the more dispersed groups of microtubules that underlie primary cell walls. An added advantage is that bunching can be examined in vitro, in a single cell type, in a highly synchronous manner. Originally developed by Fukuda (Fukuda, 1980), Milioni et al. found that, by delaying the hormonal induction until 48 hours after the mesophyll cells are released from leaves, thereby allowing the major wounding responses to occur, transdifferentiation proceeds with a high efficiency (Milioni et al., 2002).

Whereas MAP65-1 proteins are known to support the

parallel grouping of cortical microtubules beneath primary cell walls, the present study now establishes that ZeMAP65-1 is involved in the more accentuated bundling of microtubules that occurs beneath the thickenings of differentiating secondary cell walls.

Results

Microtubule bunching during differentiation

Using the conditions described by Milioni et al. (Milioni et al., 2002), isolated mesophyll cells are allowed to recover by pre-incubating for 48 hours before the zero time addition of the inductive auxin and cytokinin. First, we performed pilot studies to determine the course of microtubule bunching. Anti-tubulin immunofluorescence showed that, after addition of inductive hormones, cells containing highly bundled microtubules increased to a maximum 60±9.3% (average of three experiments) by 48 hours. Although no further increase then occurred, by 72 hours the cell wall thickenings characteristic of xylem tracheary elements could be easily discerned under bright-field optics. Fig. 1 illustrates the coincidence between the anti-tubulin staining pattern and the Calcofluor pattern for the cellulose-rich wall thickenings.

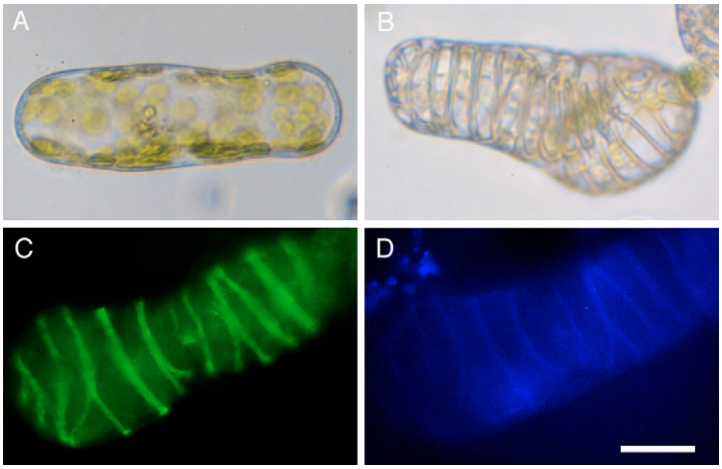

Fig. 1. Microtubule bunching during *Zinnia* tracheary element differentiation. (A) Isolated mesophyll cell before addition of inductive hormones. (B) A tracheary element 72 hours post-induction, with cell wall thickenings visible under bright-field optics. (C) Bundles of cortical microtubules labelled by anti-tubulin immunofluorescence. (D) The same cell with coincident cellulose-rich secondary thickenings labelled with Calcofluor White. Bar, 10 μm.

A MAP65 isoform is upregulated upon induction

To investigate the expression of MAP65 during the transdifferentiation process, we used a peptide antibody raised against the CEEESWLEDYNR peptide. This peptide is well conserved in the MAP65-1 subgroup of the MAP65 family, to which carrot DcMAP65-1 (Chan et al., 2003b), *Arabidopsis* AtMAP65-1 (Hussey and Hawkins, 2001) and tobacco NtMAP65-1b (Wicker-Planquart et al., 2004) belong. This antibody has been shown to recognise MAP65 isoforms in tobacco and *Arabidopsis* (Mao et al., 2005a). Whereas MAP65 is barely detectable on 1D immunoblots of undifferentiated cells at zero time (Fig. 2A), at 48 hours post-induction (Fig. 2B) there are two heavy bands in the size range expected for the MAP65 family, together with a lower band, which may be a breakdown product. Fig. 2C illustrates that the antibody against MAP65 peptide recognises bunched microtubules in transdifferentiating cells at this 48 hour stage.

Gene expression of MAP65 isoforms during transdifferentiation

To study the expression of MAP65 isoforms over the 48-hour microtubule-bundling period, we designed degenerate primers based on the highly conserved domains (Hussey and Hawkins, 2001; Hussey et al., 2002) LQKEK and CEEESW. [The CEEESW sequence occurs in the microtubule-binding region (Smertenko, 2004) and in the CEEESWLEDYNR peptide against which the peptide antibody used in Fig. 2C was made.] Sequencing the obtained PCR products led to the identification of a novel *MAP65* isoform from *Zinnia*, which we named *ZeMAP65-2*. The primers G2_4F and G2_4R were designed specifically to amplify *ZeMAP65-2*. In addition, we designed primers based on sequences for another two MAP65 isoforms (*ZeMAP65-1* and *-3*) obtained from two *Zinnia* expressed sequence tags (ESTs), z8121f1 and z5715f1, by using BLAST to search the RIKEN database (http://mrg.psc.riken.go.jp) with *AtMAP65* sequences. The gene-specific primers for *ZeMAP65-2* and *ZeMAP65-3* were Zj2F/Zj2R and Zj3F/Zj3R. These three pairs of primers were used in RT-PCR reactions to examine the pattern of gene expression and compared against the expression pattern of the tracheary element differentiation marker *TED2*. TED2 is a ζ-crystallin orthologue expressed in *Zinnia* at an early stage of the differentiation of procambial cells both to immature xylem and phloem cells (Demura and Fukuda, 1994).

Fig. 3 shows that *TED2* was not expressed in uninduced cells (zero time, before the addition of inductive hormones), but was expressed from 2 hours through to 40 hours, with diminished expression at 48 hours. *ZeMAP65-1* showed the identical expression pattern to *TED2*, with transcripts being detected from 2 hours, diminishing after 40 hours but still detected at 48 hours. *ZeMAP65-2* showed a similar pattern of expression to actin, being detected at a more or less constant level in

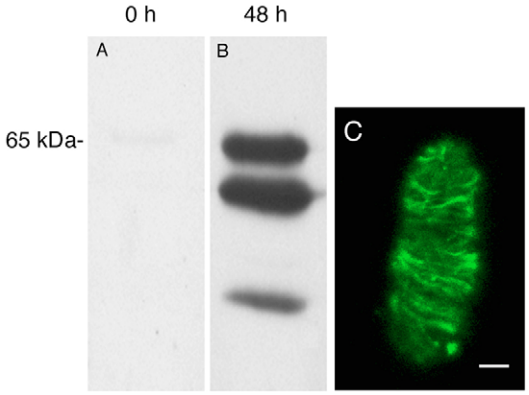

Fig. 2. Immunoblotting and immunofluorescence with an antibody against MAP65 peptide. Samples at equal protein loading immunoblotted with the antibody against the CEEESWLEDYNR peptide. (A) Immunoblot of *Zinnia* suspension cells at 0 hours (pre-induction); (B) differentiating cell culture at 48 hours. (C) Immunofluorescence of induced microtubule bundles at 48 hours. Bar, 10 μm.

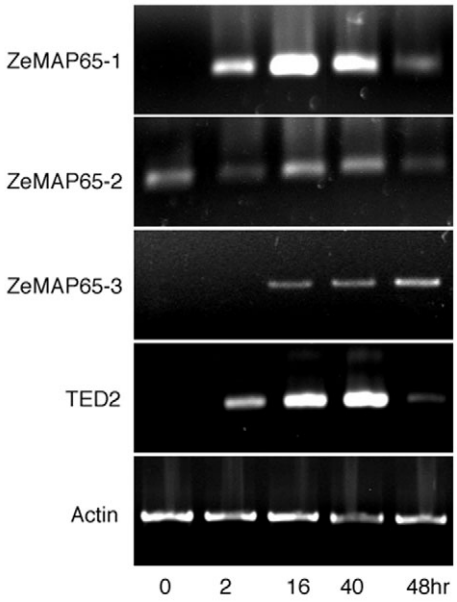

Fig. 3. Expression of *ZeMAP65* isoforms during *Zinnia* cell differentiation. After a delay of 2 hours, *ZeMAP65-1* was shown by RT-PCR to be expressed from 2-40 hours after induction, and decreased at 48 hours, similar to the pattern for the tracheary element differentiation marker *TED2* (Demura and Fukuda, 1994).

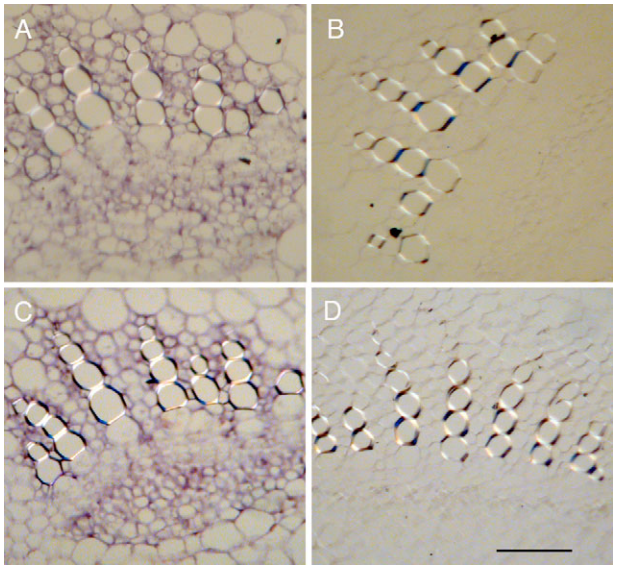

Fig. 4. Localisation of *TED2* and *ZeMAP65-1* transcripts in *Zinnia*. In situ hybridisations using cross-sections of 14-day-old *Zinnia* stems showed a similar pattern of expression in vascular bundles for *ZeMAP65-1* (A) and *TED2* (C), but this signal was not observed in the respective sense controls (B,D). Bar, 0.1 mm.

induced and uninduced cells alike. By contrast, *ZeMAP65-3* expression was upregulated over the period that microtubules were seen to form bundles; there was no signal at 0 and 2 hours, but signal could be detected from 16-48 hours.

In order to differentiate between the contributions of *ZeMAP65-1* and *ZeMAP65-3* to the differentiation process, we induced tracheary element formation in the presence of the DNA synthesis inhibitor aphidicolin. Aphidicolin inhibits the background mitosis that occurs in some of the cells in this system but it does not affect tracheary element differentiation (Mourelatou et al., 2004). The expression pattern of *ZeMAP65-1* was unaffected by this drug but no signal could be detected for *ZeMAP65-3* (not shown), indicating that it is not required for differentiation. Therefore, its presence in non-drug-treated cells can be attributed to aphidicolin-sensitive cells going through the division cycle (Mourelatou et al., 2004).

In situ hybridisation

To confirm that *ZeMAP65-1* is expressed in xylem cells in plants, we used *ZeMAP65-1* sense and anti-sense probes to perform mRNA *in situ* hybridisation of *Zinnia* stems. *TED2* was used as a control since Demura and Fukuda (Demura and Fukuda, 1994) have established that its transcripts are present in the xylem and phloem of *Zinnia* stems. Fig. 4 (B,D) shows that the sense probes produced no specific labelling. However, the anti-sense probes for *TED2* and *ZeMAP65-1* were found to produce very similar patterns of labelling (compare Fig. 4A and C), showing that transcripts for *ZeMAP65-1* occur in xylem tissue.

Relationship of the *Zinnia* MAP65 orthologues within the MAP65 superfamily

In view of the potential relevance of *ZeMAP65-1* to the differentiation of *Zinnia* tracheary elements, we performed RACE-PCR to explore the 5′ and 3′ sequences of the *ZeMAP65-1* message. On the basis of these results, a full-length cDNA for *ZeMAP65-1* was cloned and the sequence was submitted to GenBank (accession number AY786506). The start of transcription was determined to be 144 nucleotides upstream of the *ZeMAP65-1* start of translation. Its open reading frame translates to 576 amino acids with a predicted molecular weight of 65.4 kDa. BLAST searches indicated that ZeMAP65-1 is most similar to MAP65-1 proteins from tobacco (69% identity), *Arabidopsis* (68%) and carrot (67%). The phylogenetic tree in Fig. 5, which includes the entire *Arabidopsis* MAP65 family, indicates that ZeMAP65-1 belongs to the MAP65-1 subgroup of proteins, with the other members from *Arabidopsis* (AtMAP65-3 to -9) being more distantly related. When compared with members of the MAP65-1 subgroup, high sequence homology is apparent along the entire amino acid sequence, with a somewhat reduced homology at the C-terminus. Many MAP65 proteins contain a destruction box motif at the C-terminus (R-x-x-l-x-x-x-x-N) (Hussey at al, 2002). In ZeMAP65-1, this motif is changed to RLSLNQNGT and is repeated almost perfectly three to four times. The epitope CEEESWLEDYNR, which is involved in microtubule binding and is present in MAP65-1 subgroup proteins, is fully conserved in ZeMAP65-1. ZeMAP65-1 is a typical MAP65 protein in that it shows several regions with high probability to form coiled coils (Lupas, 1997). The *ZeMAP65-2* and *ZeMAP65-3* genes are not fully sequenced and could therefore not be placed in the phylogram. In BLAST searches, the ZeMAP65-2 sequence was found to be most similar to AtMAP65-6, and ZeM65-3 to AtMAP65-3/PLE.

Overexpression of GFP–ZeMAP65-1 induces bundling of cortical microtubules

The upregulation of *ZeMAP65-1* during cell differentiation

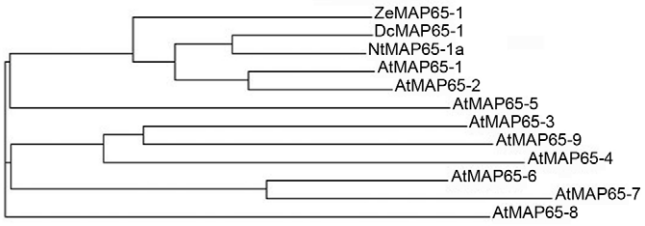

Fig. 5. Phylogram of ZeMAP65-1 and known MAP65 family proteins. The following sequences were analysed: tobacco NtMAP65-1a (CAC17794), carrot DcMAP65-1 (CAD58680) and *Arabidopsis* AtMAP65-1 (At5g55230), AtMAP65-2 (At4g26760), AtMAP65-3 (At5g51600), AtMAP65-4 (At3g60840), AtMAP65-5 (At2g38720), AtMAP65-6 (At2g01910), AtMAP65-7 (At1g14690), AtMAP65-8 (At1g27920) and AtMAP65-9 (At5g62250). The Phylogram was created with the ClustalW software (Thompson, 1994).

suggested that *ZeMAP65-1* could be involved in the characteristic microtubule bundling. Since not all MAP65 isoforms can bundle microtubules, we tested its ability by expressing it in cell culture. We were unable to transform *Zinnia* cells and instead used an *Arabidopsis* cell suspension that has been used for transformation studies in this laboratory (Mao et al., 2005a; Chan et al., 2003a). *ZeMAP65-1* was fused with the gene for green fluorescent protein (*GFP*) and then expressed under the 35S promoter in *Arabidopsis* suspension cells. Fig. 6 shows that overexpression of GFP–ZeMAP65-1 induced dramatic bundling of the cortical microtubules. The normally evenly distributed cortical microtubules were drawn into thick helical (Fig. 6A) or transverse (Fig. 6B) bundles.

Discussion

Cortical microtubules form localised parallel groups in interphase while remaining more-or-less evenly distributed over the cortex. However, in a few well-known cases, cortical microtubules form tight, restricted bundles: during preprophase band formation, and during the differentiation of mesophyll cells (Fu et al., 2005) and xylem tracheary elements (Fukuda, 1989). In this paper, we have used the synchronisation of *Zinnia* tracheary element formation in vitro (Milioni et al., 2002) to examine the role of MAP65 in the bundling process. MAP65 is known to form 25-30 nm cross-bridges between

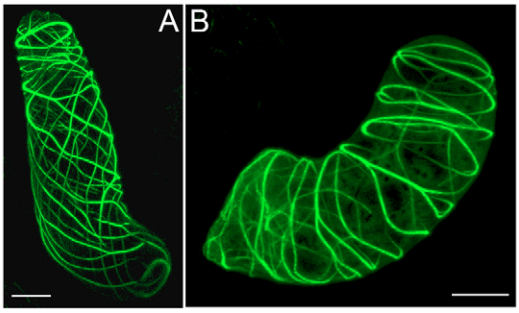

Fig. 6. GFP–ZeMAP65-1 induces microtubule bundles when expressed in *Arabidopsis* suspension cells. Massive bundling of microtubules still allows for the formation of ordered microtubule arrays, which may be helical (A) or transverse (B). Both pictures are z-projections of confocal sections. Bar, 8 μm.

microtubules (Chan et al., 1999; Smertenko, 2004) and can induce the bundling of microtubules in vitro (Chan et al., 1999; Smertenko, 2004) and in vivo (Chan et al., 2003b; Van Damme et al., 2004), and is therefore a prime candidate for the bundling of microtubules in tracheary element formation.

Fukuda first described the transdifferentiation of *Zinnia* mesophyll cells into xylem tracheary elements in vitro (Fukuda, 1980), and Milioni et al. showed that delaying the addition of inductive hormones until after a wound-recovery period resulted in improved synchrony (Milioni et al., 2002). In this system, we found that microtubules increasingly bundle to a peak at 48 hours after addition of hormones and we therefore examined the role of MAP65 over this period. In the initial part of the investigation, we used an antibody raised against the CEEESWLEDYNR peptide that is well conserved among the MAP65-1 subgroup of MAP65 proteins (Mao et al., 2005a). Members of this subgroup have been shown to bundle cortical microtubules in carrot (Chan et al., 1999), tobacco (Wicker-Planquart et al., 2004) and *Arabidopsis* (Mao et al., 2005a; Van Damme et al., 2004), and the sequence has been found to occur in the microtubule-binding domain of AtMAP65-1 (Smertenko, 2004). In *Zinnia* cells, the antibody could be shown to decorate bundled microtubules at the 48 hour stage; on immunoblots at the same stage, the antibody recognised two protein bands of a size appropriate to MAP65 isoforms, together with a lower band that is likely to be a breakdown product.

Next, we performed RT-PCR to check the expression patterns of putative *Zinnia MAP65* genes over the bundling period. One set of primers was based on conserved motifs (including the CEEE sequence present in the peptide used for making the antibody) and two others based on *MAP65* isoforms identified in a *Zinnia* EST library (Demura and Fukuda, 1994). Transcripts for putative *ZeMAP65-2* were present at equivalent levels in uninduced and induced cells over the bundling period and were therefore not upregulated by addition of the inductive hormones. Transcripts for *ZeMAP65-1* and *ZeMAP65-3* were both undetectable in uninduced cells but, after addition of hormones, appeared at the 2-hour and 16-hour time points, respectively. To distinguish between them, we added the DNA synthesis inhibitor aphidicolin, which has been shown to block division occurring in some cells in *Zinnia* cultures but has no effect on differentiation (Mourelatou et al., 2004). This treatment blocked expression of *ZeMAP65-3* transcripts but not those of *ZeMAP65-1*, so it would appear that *ZeMAP65-3* is more likely to be involved in division and not in the bundling of cortical microtubules. This is consistent with the known role of its nearest *Arabidopsis* homologue, *AtMAP65-3/PLE*, which has an essential role in cytokinesis and only occurs in dividing cells (Muller et al., 2004).

Of the three *MAP65* genes investigated in *Zinnia*, only *ZeMAP65-1* was upregulated upon addition of hormones and was not related to aphidicolin-sensitive cell division. This suggested that *ZeMAP65-1* could be involved in microtubule bundling during xylogenesis. However, not all MAP65 isoforms – such as AtMAP65-3/PLE (Muller et al., 2004) and AtMAP65-4 (Van Damme et al., 2004) – are capable of bundling microtubules. To confirm that ZeMAP65-1 was capable of bundling microtubules, we expressed the *ZeMAP65-1* gene as a *GFP* fusion under the 35S promoter in *Arabidopsis* suspension cells. This effectively gathered cortical

microtubules into thick bundles with clear areas between these accumulations. The heavily bundled patterns formed were variants of transverse hoop-like or helical arrays that typify the thickenings of xylem tracheary elements (Falconer, 1985).

Considered together, these findings are consistent with ZeMAP65-1 being involved in the induced bundling of microtubules during tracheary element formation in vitro.

In *Arabidopsis*, there are nine MAP65 genes but only three are known so far for *Zinnia* and so it is not possible to conclude whether *ZeMAP65-1* acts alone or in combination with other undescribed isoforms. However, the fact that bacterially expressed AtMAP65-1 is capable of forming 25 nm cross-bridges between microtubules in vitro, probably as homodimers, without the requirement for other isoforms, has established that a single isoform is sufficient to induce microtubule bundling (Smertenko, 2004).

Materials and Methods

Zinnia cell culture

Zinnia elegans cultivar '*Envy*' mesophyll cells were isolated and cultured as described by Domingo et al. (Domingo et al., 1998). Cells were cultured for 48 hours in noninductive medium to allow wound recovery, then transferred to inductive medium containing 1 mg/l benzylaminopurine and 1 mg/l naphtyl acetic acid.

Immunofluorescence

Zinnia cells were fixed in 50 mM PIPES, pH 6.8, 5 mM $MgSO_4$, 5 mM EGTA (PME buffer) containing 4% (w/v) formaldehyde. Cells were deposited on polylysine-coated slides, digested in PME with 0.5% (w/v) Onozuka R-10 cellulase and 0.05% (w/v) Pectolyase Y-23 for 30 minutes, washed in phosphate-buffered saline (PBS), then incubated with either YOL 1/34 anti-tubulin or an anti-MAP65 IgG raised against the peptide CEEESWLEDYNR (Mao et al., 2005a) in PBS with 3% (w/v) BSA at 37°C for 1 hour. Slides were then washed three times with PBS before incubating in secondary antibody (anti-rat FITC or anti-rabbit FITC) in PBS/BSA at 37°C for 1 hour. After washing and adding 0.2 μg/ml DAPI for 5 minutes in order to label the nuclei, slides were sealed with a coverslip. To stain the cellulosic cell wall, 0.01% (w/v) Calcofluor White was added for 5 minutes, cells were then washed with PME.

Design of primers used in RT-PCR

Degenerate primers were designed according to conserved regions of the nine *Arabidopsis* MAP65 proteins, using the online program DODEHOP (http://blocks.fhcrc.org). Sequencing of PCR products led to the identification of the *MAP65-2* gene. Primers to amplify further *MAP65* genes (*MAP65-1* and *MAP65-3*) were designed based on *Zinnia* ESTs (http://mrg.psc.riken.go.jp/). We used the following primers for RT-PCR: ZJ2F 5′-CGTGGAGAAATTGCAGGGAGTA-3′ and ZJ2R 5′-CGAGTAACATGATCACACGAAGTC-3′ (*ZeMAP65-1*); G2_4F 5′-CAGTCAGAACGGCTTCATAAAGTT-3′ and G2_4R 5′-CTGTTGAATAAT-CTCCAAAGA-3′ (*ZeMAP65-2*); ZJ3F 5′-GACACCTCAGCCTCAACATAAT-3′ and ZJ3R 5′-ACGTCGAGTAAGAGTAGAGTGA-3′ (*ZeMAP65-3*); TED2F 5′-CGAAAGCCGGAGAAATCAAAGT-3′ and TED2R 5′-CATGCCAAAGACC-CAACACCTC-3′ (*TED2*); Act3F 5′-TGCGACAATGGAACTGGAATG-3′ and Act3R 5′-GGATAGCATGTGGAAGTGCATACC-3′ (*Actin*). For RT-PCR, mRNA was extracted on polyA beads (Dynal Beads) from *Zinnia* cells following the manufacturer's guidelines. Reverse transcription was carried out using a Sensiscript RT kit from Qiagen.

In situ hybridisation

Sections were prepared and hybridised with sense and anti-sense probes corresponding to *TED2* and *ZeMAP65-1*, as fully described by Drea et al. (Drea, 2005).

Protein separation and Western blotting

Zinnia cells were collected by centrifugation at 100 ***g*** and the pellet was ground with a pestle and mortar in extraction buffer (100 mM Tris-HCl, pH 7.4, plus proteinase inhibitor cocktail from Roche), then centrifuged at 13,000 ***g***, 4°C. The supernatant was run on SDS-PAGE gel and transferred to nitrocellulose for western blotting.

Cloning of a full-length ZeMAP65-1 cDNA and expression of GFP–ZeMAP65-1 in *Arabidopsis* cells

Total RNA was extracted from 30-hour-induced *Zinnia* cells by using a RNeasy plant mini kit (Qiagen) as described by the supplier. The GeneRacer system (Invitrogen) and gene-specific primers ZJ2F and ZJ2R were used to amplify 3′ and 5′ gene fragments. The 3′ and 5′ RACE products were transferred into the pGEM T-easy vector (Promega) for sequencing. This sequence information enabled us to amplify a full-length *ZeMAP65-1* cDNA using the following primers: ZeGTF 5′-CGGGATCCATGGCAGAACCAACCTGTGG-3′ and ZeGTR 5′-CGTCTAGAT-TAAGGTGTAAGTGGAGCAGC-3′. The PCR product was digested with *Bam*HI and *Xba*I and cloned into pBluescript. Several independent cDNA clones were sequenced. The *ZeMAP65-1* sequence was excised from pBluescript and cloned into a modified pBin19 vector containing an expression cassette for GFP-coupled expression. The resulting vector enables expression of GFP fused to the N-terminus of ZeMAP65-1 driven by the 35S promoter in planta. *Arabidopsis* Columbia-0 suspension cells were transiently transformed with *Agrobacterium* (Mathur, 1998).

Confocal microscopy

All microscopy was performed using the Leica SP2 confocal microscope. GFP and FITC were excited by the 488 nm argon laser and imaged using the 500-550 nm emission filter. Calcofluor White was excited by the 405 nm violet laser diode and imaged using a 420-490 nm filter. Maximum intensity projections were calculated from z-section series using Leica software.

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