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 reproduce 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 bundling 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 bundling. 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.

**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
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 numberAY786506). 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. 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.
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 (Fu et al., 1989). In this paper, we have used the synchronisation of Zinnia tracheary element formation in vitro (Milion et al., 2002) to examine the role of MAP65 in the bundling process. MAP65 is known to form 25-30 nm cross-bridges between 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 (Milion 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-Planguart 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

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 (At1g27920), AtMAP65-7 (At1g14690), AtMAP65-8 (At1g27920) and AtMAP65-9 (At5g62250). The Phylogram was created with the ClustalW software (Thompson, 1994).

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.
Zinnia MAP65 and tracheary elements

**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 naphthyl acetic acid.

**Immunofluorescence**

Zinnia cells were fixed in 50 mM PIPES, pH 6.5, 8.5 mM MgSO4, 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 YOL1/34 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-ot 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://nrg.psc.riken.go.jp/). We used the following RT-PCR primers for Zinnia MAP65: ZJ2F 5'-CCGGATCCTAGGACAAACATCTGTGG-3' and ZJ2R 5'-CGGATCCCGAGTACTGAGCAAG-3'. The PCR product was digested with *SacI* and cloned into pBluescript. Several independent cDNA clones were sequenced. The ZmMAP65-1 sequence was excised from pBluescript and cloned into a modified pBlm19 vector containing an expression cassette for GFP-coupled expression. The resulting vector enables expression of GFP fused to the N-terminus of ZmMAP65-1 driven by the 3SS promoter in plants. *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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