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PART OF A SPECIAL ISSUE ON THE PLANT CELL CYCLE

# Interaction of a 14-3-3 protein with the plant microtubule-associated protein EDE1

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• *Background and Aims* The cell cycle-regulated protein ENDOSPERM DEFECTIVE 1 (EDE1) is a novel plant microtubule-associated protein essential for plant cell division and for microtubule organization in endosperm. EDE1 is only present on microtubules at mitosis and its expression is highly cell cycle regulated both at the protein and the transcript levels.
• *Methods* To search for EDE1-interacting proteins, a yeast two-hybrid screen was used in which EDE1 was fused with GAL4 DNA binding domain and expressed in a yeast strain that was then mated with a strain carrying a cDNA library fused to the GAL4 transactivation domain. Candidate interacting proteins were identified and confirmed *in vitro*.
• *Key Results* 14-3-3 upsilon was isolated several times from the library screen. In *in vitro* tests, it also interacted with EDE1: 14-3-3 upsilon most strongly associates with EDE1 in its free form, but also weakly when EDE1 is bound to microtubules. This study shows that EDE1 is a cyclin-dependent kinase substrate but that phosphorylation is not required for interaction with 14-3-3 upsilon.
• *Conclusions* The results suggest that 14-3-3 proteins may play a role in cytoskeletal organization of plant cells. The potential role of this interaction in the dynamics of EDE1 during the cell cycle is discussed.

**Key words:** 14-3-3, microtubules, EDE1, cell cycle, cytoskeleton.

## INTRODUCTION

ENDOSPERM DEFECTIVE 1 (EDE1) is a novel microtubule-associated protein (MAP) essential for microtubule function in the *Arabidopsis* endosperm and embryo (Pignocchi *et al.*, 2009). When EDE1 is mutated, cytokinesis defects occur in developing embryos and endosperm lacks organized microtubule structures, resulting in mitotic failure and seed abortion. Subcellular localization of green fluorescent protein (GFP)–EDE1 fusions on the microtubules of the spindle and spindle poles during mitosis and on the phragmoplast during cytokinesis indicates that EDE1 has a key role in microtubule function during mitosis. Also, its expression pattern is strictly regulated by the cell cycle, being expressed only during G2 and M phase (Pignocchi *et al.*, 2009).

14-3-3 proteins, identified in all eukaryotic organisms from yeast to humans, have been assigned roles in many cellular processes, from metabolism to protein trafficking, signal transduction, apoptosis and cell-cycle regulation (Dougherty and Morrison, 2004). An increasing number of proteins have been found to be regulated by 14-3-3 binding. In *Arabidopsis* alone, in which the 14-3-3 family consists of 13 members (Ferl, 2004), as many as 20 % of the total proteins have been identified as potential 14-3-3 targets (Sehnke *et al.*, 2002). Although phosphorylation of the target sequence is often a requirement for 14-3-3 binding (Mackintosh, 2004), this is not a strict rule and some 14-3-3 interactions are independent of phosphorylation (Henriksson *et al.*, 2002). The wide distribution of 14-3-3 across all eukaryotes suggests that key biological functions are conserved amongst members of the family. However, differential tissue-specific and subcellular localization patterns seem to point to a degree of specialization among isoforms (Ferl *et al.*, 2002).

To identify EDE1 interactors that could provide insight into the mechanisms regulating EDE1 abundance and turnover during the cell cycle, we undertook a yeast two-hybrid approach using EDE1 as bait versus an *Arabidopsis* cDNA library. A member of the 14-3-3 family, namely 14-3-3 upsilon ($\upsilon$), was found to bind strongly to EDE1 both *in vivo* and *in vitro*. We discuss how this interaction might be important in the regulation of EDE1 activity during the cell cycle and unveil new roles for the 14-3-3 family of proteins in the dynamics of the plant cytoskeleton.

## MATERIALS AND METHODS

### *Screening of the yeast two-hybrid library and interaction analysis*

EDE1 (At2g44190) cDNA was amplified from cDNA extracted from *Arabidopsis* Col-0 seedlings using primers pairs: EDE1-ATG (5′-ATGGAGGCGAGAATCGGCCGATC-3′) and EDE1-stop (5′-TCAAACAGAAGTTGTGCACTCTTG-3′), each containing respectively *att*B1 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTAT-3′) or *att*B2 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTC-3′) recombination sequences (Invitrogen, Paisley, UK) as adapter sites at the 5′ end, according to the manufacturer's instructions, and cloned into the Entry vector pDONR 207, via the BP reaction that allows for recombination between attB and attP sites, to generate pDONR207-EDE1. The insert was verified by

sequencing. LR Clonase mix (for recombination of attL sites with attR sites; Invitrogen) was then used to insert the DNA fragment into the destination vectors pGBKT7 and pGADT7 (Invitrogen). EDE1- pGBKT7 was introduced into the yeast strain Y187 and used as a bait to screen an *Arabidopsis* cDNA library (gift of the Hans Sommer Laboratory, Max Planck Institut, Germany), transformed into the yeast strain AH109. Y187 cells containing the bait were mated with the library (AH109) for 24 h and diploids were plated on high-stringency media lacking leucine, tryptophan, histidine, alanine (-LWHA). After 10 d, $1{\cdot}4 \times 10^7$ colonies were transferred onto master plates containing media – LWHA and the activity of α-galactosidase was assessed by x-α-gal agarose overlay assay (Rupp, 2002).

To test reciprocal interactions, 14-3-3 *υ* (At5g16050) cDNA was amplified from cDNA extracted from *Arabidopsis* Col-0 seedlings using primers pairs: 5-GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGTCTTCTGATTCGTCCCGGGAAG-3 and 5-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACTGCGAAGGTG GTGGTTGGGC-3 and cloned into pDONR207 first (pDONR207–14-3-3 *υ*) and then into both vectors pGBKT7 and pGADT7 (Invitrogen) and transformed into yeast strains Y187 and AH109.

### *Protein expression and antibody production*

EDE1 cDNA was amplified from *Arabidopsis* seedlings using primers 5-ACCAGGATCCATGGAGGCGAGAATCGGC-3 and 5-ACCAGCGGCCGCAACAG AAGTTGTGCACTCTTGCTG-3, which add *Bam*H1 and *Not*1 restriction sites respectively at the 5′ and 3′ ends of EDE1 and cloned into *Bam*H1 and *Not*1 sites of pGEX-4T-3 (GE Healthcare, Chalfont St Giles, UK) and transformed into BL21 *Escherichia coli* strain BL21 cells (Invitrogen). Upon induction with 0·5 mM IPTG (isopropyl β-D-thiogalactopyranoside) at 37 °C for 3·5 h, GST–EDE1 was found to be present in the insoluble fraction and was purified and bound to Sepharose 4B gel (GE Healthcare) according to Frangioni and Neel (1993). Briefly, induced cells were resuspended in STE buffer (10 mM Tris-HCl, pH 8·0; 1 mM EDTA; 150 mM NaCl) and lysed with 0·1 mg $mL^{-1}$ lysozyme, 1 % sarkosyl and 10 mM dithiothreitol (DTT) and 1 min sonication. After centrifugation, 10 % Triton X-100 was added to the supernatant and the volume was adjusted with STE buffer to give final concentrations of sarkosyl and Triton X-100 of 0·7 and 2 %, respectively. After 30 min incubation at room temperature, the lysate was bound to Sepharose for 1 h with gentle agitation. EDE1 alone was cleaved from the GST tag and released from the glutathione gel by overnight incubation at 4 °C with 10 U thrombin (GE Healthcare). EDE1 protein was concentrated using a Centriprep filter unit with 30-kDa cut off (Millipore, Watford, UK), and 400 μg protein was used to immunize rabbits at 2-week intervals with four injections of 100 μg protein each (Eurogentec S.A., Seraing, Belgium). EDE1 serum was used in western blotting at 1 : 1000 dilution.

### *Kinase assays*

Kinase assays were performed using 50 μL of GST–EDE1- or GST- Sepharose beads, 0·25 mM ATP, 1 μCi γ-$^{32}$P-ATP and 10 U Cdc2-cyclin B in kinase buffer (New England Biolabs, Hitchin, UK). The samples were incubated for 30 min at 30 °C. The reaction was stopped by adding 5× SDS-PAGE loading buffer. The protein samples were separated by SDS-PAGE, stained with Coomassie staining and visualized by autoradiography.

### In vitro *GST-binding assays*

14-3-3 *υ* was cloned into the Gateway vector pDEST14 via the LR reaction from pDONR207–14-3-3 *υ* according to the manufacturer's instructions and $^{35}$S-methionine-labelled protein was produced using TnT Quick Coupled Transcription/Translation System (Promega, Southampton, UK). Briefly, 40 μL of T7 TnT master mix was incubated with 1 μg of vector and 2 μl of [$^{35}$S] methionine (1000 Ci $mmol^{-1}$ at 10 mCi $mL^{-1}$; GE Healthcare) in a total volume of 50 μL at 30 °C for 90 min. The interaction assay was performed by incubating for 1 h at 4 °C the $^{35}$S-labelled 14-3-3 *υ* with 50 μL of Sepharose beads to which purified GST alone or GST–EDE1 had been absorbed, in NETN buffer (20 mM Tris-HCl, pH 8·0; 0·1 M NaCl, 1 mM EDTA, 0·5 % NP40). The beads were pelleted by centrifugation and washed three times with 1 mL of NETN buffer. Bound proteins were resolved by SDS-PAGE on a 10 % polyacrylamide gel and visualized by radiography. Phosphorylation-dependent binding was tested by treating GST–EDE1 beads with 40 U Cdc2-cyclin B (New England Biolabs) at 30 °C for 40 min or 20 U alkaline phosphatase (New England Biolabs) at 37 °C for 1 h prior to binding.

### *Microtubule pull-down assays*

Microtubules were obtained by incubating 5 mg $mL^{-1}$ bovine brain tubulin (Cytoskeleton Inc., Denver, CO, USA) in GTB buffer (80 mM PIPES, pH 6·9, 2 mM $MgCl_2$, 0·5 mM EGTA) with 30 % (v/v) glycerol, 1 mM GTP and 20 μM Taxol (Sigma, Dorset, UK) for 40 min at 37 °C. EDE1 was cloned into pDEST14 (Invitrogen) through the LR reaction according to the manufacturer's instructions and both EDE1 and 14-3-3 *υ* were *in vitro* translated as described above, using either $^{35}$S methionine or 'cold' methionine, according to the manufacturer's instructions. Twenty-five microlitres of *in vitro* translated [$^{35}$S] methionine-labelled proteins were diluted in 200 μL GTB buffer containing complete protease inhibitor mixture (Roche, Welwyn Garden City, UK) and spun at 16 000***g*** for 1 h at 4 °C. One hundred microlitres of supernatant was incubated with 50 μg of microtubules and 20 μM Taxol and with or without 'cold' EDE1 or 14-3-3 *υ*, for 30 min at 37 °C. The microtubule and radiolabelled proteins mixtures were layered over 1 mL of 15 % (w/v) sucrose in GTB buffer and spun at 16 000***g*** for 30 min. Pellets were analysed for the presence of radiolabeled EDE1 or 14-3-3 *υ* by SDS-PAGE followed by autoradiography. Equal loading of the gel was verified by Coomassie Blue staining.

*Plant material and transformation*

To generate GFP–14-3-3 υ *Arabidopsis* lines, 14-3-3 υ cDNA was cloned into the Gateway vector pGFP-N-Bin (Invitrogen) through the LR reaction, according to the manufacturer's instructions. 14-3-3 υ–pGFP-N-Bin was transformed into *Agrobacterium tumefaciens* cells (strain LBA4404.pBBR1MCSvirGN54D) and *Arabidopsis* Col-0 cells were transiently transformed as previously described (Koroleva *et al.*, 2005). Transformed cells were observed after 3 d and images were recorded using a Nikon E600 (Nikon UK Ltd, Kingston upon Thames, UK) with a Hamamatsu Orca CCD camera (Hamamatsu Photonics K.K., Tokyo, Japan) and Metamorph image software (Molecular Devices, Sunnyvale, CA, USA). Images were processed using ImageJ software (http://rsb.info.nih.gov/ij/).

*Immunoprecipitation assays*

Wild-type and GFP–14-3-3 υ *Arabidopsis* soluble cell extracts were obtained from grinding frozen 3-d-old cultures in extraction buffer containing 100 mM HEPES, pH 7·5, 5 % glycerol, 50 mM KCl, 5 mM EDTA, 5 mM NaF, 0·1 % Triton X-100, 15 mM Na-β-glycerophosphate, 0·5 mM sodium orthovanadate, 1 mM DTT and protease inhibitor cocktail (Roche). After centrifugation, 8 mL of soluble cell extract was incubated with 0·4 mL Protein A beads (Sigma) and 160 μL anti-GFP antibodies (G 1544; Sigma) overnight at 4 °C with gentle agitation. Beads were washed three times with extraction buffer and bead-bound proteins were resolved on SDS-PAGE. EDE1 was detected by western blotting using anti-EDE1 antibodies at 1 : 1000 dilution.

*Microtubule co-sedimentation assay*

Microtubules were isolated from wild-type *Arabidopsis* cells essentially as previously described (Weingartner *et al.*, 2001). Briefly, 5 g frozen cells was ground in extraction buffer [50 mM Tris-HCl (pH 8·0), 150 mM NaCl, 5 mM EDTA, 5 mM NaF, 0·1 % (v/v) Triton-X 100] containing plant protease inhibitor cocktail (Roche). After centrifugation at 20 000 r.p.m. for 1 h at 4 °C, the supernatant was divided into two aliquots. GTP (2 mM) and Taxol (20 μM) were added to one aliquot only (+) and both aliquots (+ and −) were incubated at 37 °C for 30 min. Following incubation, each aliquot was spun at 20 000 r.p.m. for 40 min at room temperature on 1 mL of 40 % sucrose cushion (in extraction buffer). Supernatants and pellets were analysed by SDS-PAGE and western blotting using anti-β-tubulin (Sigma) and anti-14-3-3 antibodies (kind gift of Carol Mackintosh, MRC, Dundee, UK).

*Bioinformatic analysis*

Protein sequence motifs were identified using the Scansite Motif Scanner software (http://scansite.mit.edu/motifscan_seq.phtml)

## RESULTS

*Interaction between EDE1 and a member of the 14-3-3 family (upsilon)*

The *Arabidopsis EDE1* cDNA was used as the bait to screen an *Arabidopsis* library made from RNA isolated from shoot apices (gift of Hans Sommer, Max Planck Institut, Germany). More than $1 \times 10^7$ clones were screened and eight confirmed positive clones were obtained, all showing strong expression of β-galactosidase. Seven of these were different clones encoding the 14-3-3 upsilon (υ) protein, a member of the *Arabidopsis* 14-3-3 family of proteins. Figure 1A shows the β-galactosidase activity of the diploid yeast clones tested by X-Gal agarose overlay assay: neither EDE1 nor 14-3-3 υ interacts with itself but they strongly interact with each other in reciprocal tests.

A bioinformatics approach using the Scansite Motif Scanner sotware (http://scansite.mit.edu/) was adopted to identify potential 14-3-3 sequence binding motifs within the EDE1 protein sequence. Four binding motifs similar to the Mode 1 type (RSXpSXP; Mackintosh, 2004) were identified and corresponded to T159, S80, S219 and S221 of the EDE1 protein sequence (Fig. 1B).

14-3-3 binding is primarily, but not exclusively, phosphorylation-dependent. We therefore tested the ability of EDE1 to be phosphorylated *in vitro*, by the cyclin-dependent kinase Cdc2-cyclin B, as this is the major kinase responsible for mitosis-specific phosphorylation of structural proteins in eukaryotic cells (Ookata *et al.*, 1995). In an *in vitro* kinase assay, the bacterial GST–EDE1 fusion protein was a substrate of Cdc2-cyclin B, while GST alone was not (Fig. 1C). Scansite Motif Scanner software identified a single high-affinity target of Cdc2 kinase in S66 of the EDE1 sequence (Fig. 1B).

*EDE1 binds 14-3-3 υ in a phosphorylation-independent manner*

To confirm the direct interaction between EDE1 and 14-3-3 υ, co-immunoprecipitation experiments were performed using either bacterially expressed or native *Arabidopsis* EDE1 (Fig. 2). EDE1 was expressed in *Escherichia coli* as a GST fusion and its ability to bind to a radiolabelled 14-3-3 υ was tested. When expressed in a rabbit reticulocyte lysate system and run in an SDS-PAGE gel, 14-3-3 υ protein appears as two closely migrating bands of approx 30 kDa molecular weight. 14-3-3 υ bound to GST–EDE1 agarose beads but not to GST beads alone (Fig. 2A). Moreover, when GST–EDE1 was either phospory-lated with Cdc2-cyclin B or de-phosphorylated by treatment with alkaline phosphatase, the binding of 14-3-3 υ remained unchanged (Fig. 2B).

The interaction between EDE1 and 14-3-3 υ was confirmed in *Arabidopsis* suspension cells over-expressing 14-3-3 υ fused to a GFP (GFP–14-3-3 υ). Anti-GFP antibodies were used in immunoprecipitation experiments to pull down GFP–14-3-3 υ complexes and EDE1 was detected by western blotting using EDE1-specific antibodies. Figure 2C shows that EDE1 co-immunoprecipitated with GFP–14-3-3 υ in transgenic cell lines but not in wild-type cells.

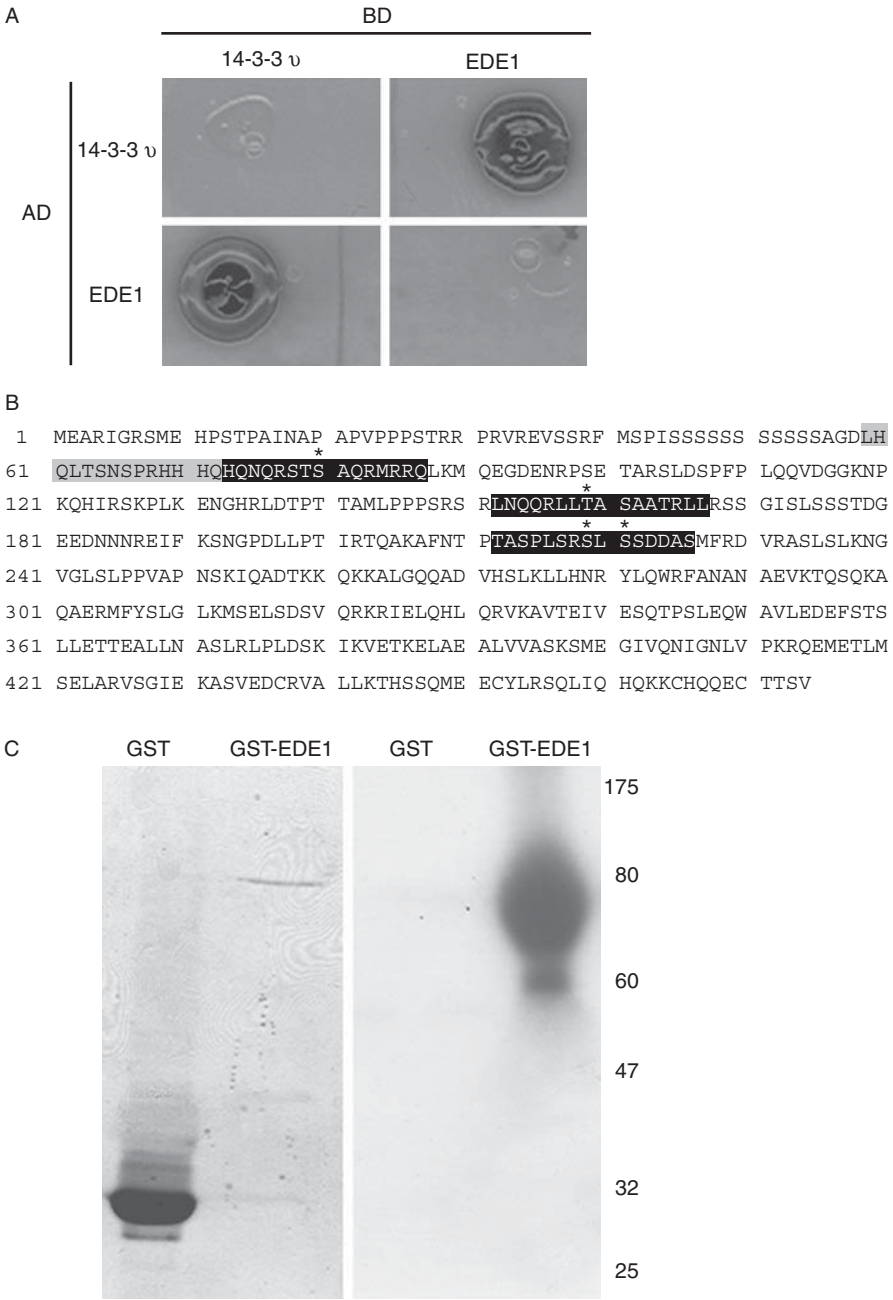


FIG. 1. Interaction between EDE1 and 14-3-3 *υ* and phosphorylation by Cdc2. (A) X-Gal agarose overlay assay of the diploid yeast clones EDE1 and 14-3-3 *υ*. β-galactosidase activity is observed only in reciprocal crosses and not in self crosses. (B) EDE1 protein sequence. Highlighted in black are the three putative 14-3-3 binding sites and in grey the single Cdc2 kinase target sequence. (C) Kinase assay of GST–EDE1 and GST alone (right panel) using Cdc2-cyclin B. Coomassie staining of the same gel (left panel).

### *14-3-3 υ localizes in the cytoplasm and nucleus of* Arabidopsis *suspension cells*

As EDE1 is a microtubule-binding protein, we tested whether 14-3-3 *υ* localizes to microtubules *in vivo* either by direct binding or indirectly through binding to EDE1. *Arabidopsis* suspension cells were transiently transformed with *14-3-3 υ* cDNA fused to GFP and under the control of the 35S promoter. GFP–14-3-3 *υ* localized diffusely within the cytoplasm and nucleus (Fig. 3A). Although GFP fluorescence on the microtubules was not detected, it is difficult to exclude the possibility that a small population of the GFP–14-3-3 *υ* was associated with microtubules.

To confirm these results, we performed *in vitro* microtubule co-sedimentation experiments using *in vitro* translated [$^{35}$S] methionine-labelled 14-3-3 *υ* and unlabelled EDE1 (Fig. 3B). 14-3-3 *υ* was incubated with Taxol-polymerized mammalian brain microtubules in the presence or absence of EDE1, microtubules were pelleted through a sucrose cushion, and both supernatants and pellets were analysed by

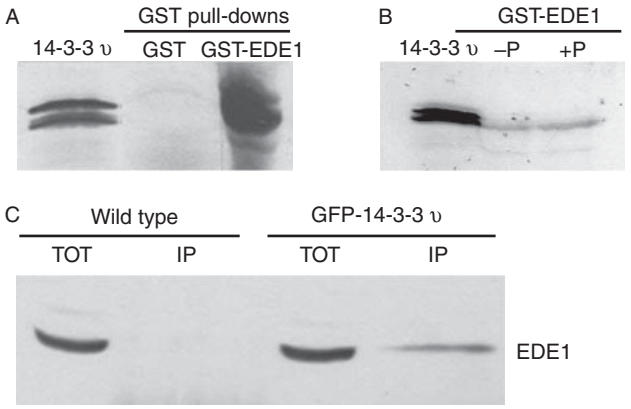


FIG. 2. *In vitro* and *in vivo* binding of EDE1 to 14-3-3 υ. (A) *In vitro* binding of radio-labelled 14-3-3 υ to GST–EDE1 and GST alone. (B) GST pull-down assays of radio-labelled 14-3-3 υ using phosphorylated (+P) and de-phosphorylated (-P) GST–EDE1. (C) Immunoprecipitation of 14-3-3 υ in wild-type and *Arabidopsis* cells expressing GFP-14-3-3 υ. EDE1 is detected only in the GFP–14-3-3 υ IP fraction. Tot, soluble protein extract; IP, GFP-immunoprecipitated fraction.

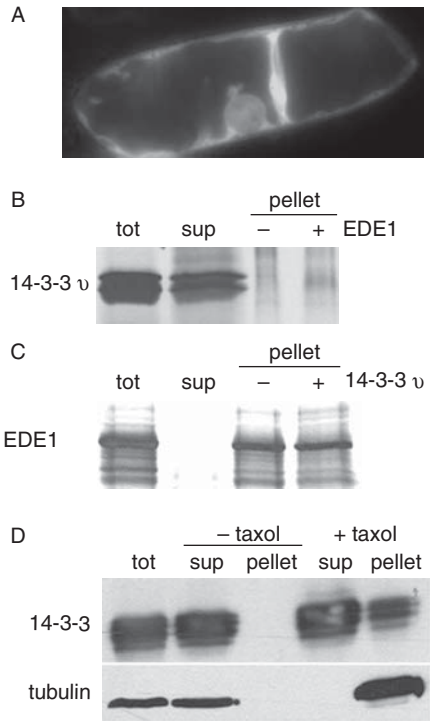


FIG. 3. Interaction between EDE1, 14-3-3 proteins and microtubules. (A) *Arabidopsis* cells transiently expressing GFP–14-3-3 υ in the cytoplasm and nucleus. (B) *In vitro* microtubule pull-downs with (+) and without (−) EDE1. Radio-labelled 14-3-3 υ (tot) was detected in the supernatant (sup) and weakly in the microtubule pellet in the presence of EDE1 (+). (C) *In vitro* translated radio-labelled EDE1 (tot) was detected in the microtubule pellets with (+) or without (−) 14-3-3 υ in microtubule pull-down assays. (D) Microtubule co-sedimentation assay in *Arabidopsis* cells extracts with (+) and without (−) Taxol. 14-3-3 proteins (tot) are present in the supernatant (sup) and microtubule fraction (pellet) of Taxol-treated cell extracts (upper panel); tubulin immunoblot of the same fractions (lower panel).

SDS-PAGE followed by autoradiography. Although the vast majority of the radio-labelled 14-3-3 υ remained in the supernatant, none was detected in the microtubule pellets in the absence of EDE1. However, a very small amount was present in the microtubule pellet in the presence of EDE1 (Fig. 3B). These results indicate that 14-3-3 υ could bind weakly to microtubules perhaps through interaction with EDE1, although most of the 14-3-3 υ does not associate with microtubules.

Next, we tested whether, *in vitro*, binding to 14-3-3 υ enhances or inhibits the microtubule binding properties of EDE1. *In vitro* translated [$^{35}$S] methionine-labelled EDE1 was incubated with Taxol-polymerized mammalian brain microtubules in the presence or absence of 14-3-3 υ, then microtubule pellets were analysed by autoradiography. EDE1 was absent from the supernatant but equally enriched in the microtubule pellets, regardless of the presence of 14-3-3 υ (Fig. 3C), indicating that EDE1 binding to microtubules occurs independently of 14-3-3 υ.

Finally, we evaluated the ability of *Arabidopsis* 14-3-3 proteins to bind microtubules by using soluble plant cell extracts and 14-3-3 antibodies (gift of C. Mackintosh). *Arabidopsis* cell extracts were treated (or not) with Taxol to polymerize the endogenous tubulin and then spun down to separate the microtubule pellet from the supernatant, and 14-3-3 and tubulin were detected by western blotting (Fig. 3D). As expected, a microtubule pellet was obtained only in the samples treated with Taxol, as shown by the tubulin immunoblot. Although most of the 14-3-3 signal was found in the supernatant, some was detected also in the microtubule pellet (Fig. 3D), indicating that *Arabidopsis* 14-3-3 isoforms can indeed bind microtubules, either directly or indirectly.

## DISCUSSION

14-3-3 proteins play diverse roles in cellular regulation, including shuttling of target proteins between different cellular locations, which in turn influences their activity and abundance through proteolysis (Gampala *et al.*, 2007). These mechanisms have been shown to occur mostly, but not exclusively, through phosphorylation (Dougherty and Morrison, 2004; Mackintosh, 2004). In mammalian cells, 14-3-3 proteins have been shown to interact during mitosis with a number of cytoskeletal proteins, including tubulin, dynein and actin (Meek *et al.*, 2004). Animal studies have shown that 14-3-3 proteins are involved in the phosphorylation and sub-cellular localization of human tau, an MAP involved in microtubule stabilization in neurons (Chun *et al.*, 2004), and bind to the c-kinesins, ATP-driven microtubule motors proteins (Ichimura *et al.*, 2002), in a phosphorylation-dependent manner. However in plants, the relationship between 14-3-3 and the plant microtubule cytoskeleton remains largely unexplored. This paper provides the first evidence for such a relationship.

We have recently reported the isolation and characterisation of a novel plant-specific MAP in *Arabidopsis*, EDE1, which is essential for microtubule organization in the endosperm (Pignocchi *et al.*, 2009). The expression of EDE1 is highly cell cycle-regulated, being present only during a small window in mitosis. This suggests that tight regulatory mechanisms are in place to control its function during cell division. Here we show that EDE1 is phosphorylated by Cdc2, the plant homologue of which is cyclin-dependent kinase A (CDKA). In plants, A-type CDKs associate physically

with mitotic microtubule structures and are involved in regulating the behaviour of specific microtubule arrays, such as the pre-prophase band and phragmoplast, throughout mitosis (Weingartner *et al.*, 2001). We speculate that the phosphorylation of EDE1 by CDKs could be responsible for modulating the interaction between EDE1 and microtubules, as reported for other MAPs such as MAP4 (Chang *et al.*, 2001).

EDE1 is expressed in a cell cycle-dependent manner during G2/M phase that may be regulated, at least in part, by protein stability (Pignocchi *et al.*, 2009). Other proteins involved in the G2/M also interact with, and perhaps are regulated by, 14-3-3 proteins. One of the first examples were the rad23 and rad24 proteins (Ford *et al.*, 1994) that respond to DNA damage. Mammalian CDC25C phosphatase, a likely target of DNA damage pathway in G2, also binds to a 14-3-3 protein, which leads to cytoplasmic sequestration of the phosphatase blocking its access to mitotic CDK (Donzelli and Draetta, 2003). Although higher plants do not contain a CDC25 orthologue, plant 14-3-3 proteins have the ability to bind hetrologous Cdc25 proteins in yeast two-hybrid assays (Sorrell *et al.*, 2003). Binding of 14-3-3 proteins is thought to modulate the ability of the target proteins to enter the nucleus and thereby delay its effect on key cell cycle transitions. The 14-3-3/EDE1 interaction may have a similar role in that EDE1 accumulates on spindle microtubules to a greater extent than cytoplasmic microtubules and it may be important to sequester it in an inactive form during the early stages of mitosis. In support of this, our data suggest that 14-3-3 can bind to soluble (i.e. not bound to microtubules) EDE1 *in vitro* and its association with microtubule-bound EDE1 is weak.

The interaction of EDE1 with 14-3-3 $\upsilon$ is independent of phosphorylation. The lack of a requirement for phosphorylation is somewhat surprising as 14-3-3 proteins are thought to bind phosphorylated consensus motifs in target proteins (Muslin *et al.*, 1996) and e thereby nhance conformational changes, in effect amplifying the effect of phosphorylation. The protein was produced in *E. coli* so should not be phosphorylated, although we cannot exclude the possibility that partially folded or mis-folded protein might mimic the effect of phosphorylation and allow interaction. However, this is the first time that a member of the 14-3-3 family has been shown to interact with an MAP in plants, suggesting an involvement of the 14-3-3 family in the regulation of complex plant-specific microtubule dynamics associated with the cell cycle. *In vivo*, when fused to GFP, 14-3-3 $\upsilon$ accumulates in the cytoplasm and nucleus of *Arabidopsis* cells. *In vitro*, 14-3-3 $\upsilon$ does not bind microtubules directly but indirectly and weakly through interaction with EDE1. These results suggest that the strong interaction between 14-3-3 $\upsilon$ and EDE1, detected in the yeast two-hybrid system, does not occur on the microtubules, but rather when EDE1 is free in the nucleus and/or cytoplasm. EDE1 microtubule binding *in vitro* occurs independently of 14-3-3 $\upsilon$, suggesting that 14-3-3 $\upsilon$ is not involved in the EDE1–microtubule interaction or, alternatively, that it requires other cellular components to do so *in vitro*. Instead, 14-3-3 may participate in the regulation of the sub-cellular localization and abundance of EDE1 by promoting its translocation between the nucleus and the cytoplasm. GFP–EDE1 does not accumulate in the cytoplasm of *Arabidopsis* cells (Pignocchi *et al.*, 2009), and therefore its translocation into the cytoplasm could be important for its degradation. The association with 14-3-3 proteins has been shown to promote cytoplasmic as opposed to nuclear localization for a variety of target proteins, including Cdc25 (Kumagai and Dunphy, 1999).

Other mitosis-related functions include modulation of mitotic translation in mammalian cells (Wilker *et al.*, 2007), indicating that 14-3-3 proteins are potential regulators of diverse kinase-mediated processes. In conclusion, through yeast two-hybrid screening we have identified a novel interaction between a 14-3-3 protein and the cell-cycle-regulated microtubule binding protein EDE1. This interaction suggests a novel role for 14-3-3 $\upsilon$ in cytoskeletal re-organization as EDE1 is required for spindle function and links the *Arabidopsis* 14-3-3 family to the regulation and functions of the microtubule cytoskeleton in plants. However, 14-3-3 proteins are multifunctional regulatory proteins that interact with a broad spectrum of proteins both in animals and in plants (for a recent review, see Oecking and Jaspert, 2009) and understanding the role of any particular interaction and its physiological significance is a major challenge.

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