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The plant nuclear envelope protein MAF1 has an additional location at the Golgi and binds to a novel Golgi-associated coiled-coil protein

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Abstract Tomato MAF1 (LeMAF1) is a plant-specific, nuclear envelope (NE)-associated protein. It is the founding member of a group of WPP domain-containing, NE-associated proteins. This group includes the Arabidopsis WPP family, which is involved in cell division, as well as plant RanGAPs. In addition to its NE localization, LeMAF1 accumulates in speckles in the cytoplasm. Here, we show that the LeMAF1-containing speckles are components of the Golgi apparatus. A novel tomato coiled-coil protein was identified that specifically binds to LeMAF1. Tomato WPP domain-associated protein (LeWAP) interacts in yeast and in vitro through its coiled-coil domain with several WPP-domain containing proteins, including AtRanGAP1 and the WPP family (LeMAF, WPP1 and WPP2). Like LeMAF1, LeWAP is localized at the Golgi. Moreover, we present data showing that Arabidopsis WAP is necessary for the existence of a multi-protein complex containing WPP2.

Keywords WPP domain · WAP · MAF1 · Coiled-coil · Golgi · Golgin · Blue-native gel electrophoresis

Abbreviations BN: blue native · CFP: cyan fluorescent protein · GFP: green fluorescent protein · MAF1: MFP attachment factor · Nag: N-acetylglucosaminyl

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transferase I · NE: nuclear envelope · ST: sialyl transferase · TGN: trans-Golgi network · WAP: WPP-domain-associated protein

Introduction

The nuclear envelope (NE) compartmentalizes the eukaryotic cell into a genomic and a metabolic compartment. While NE composition and function in metazoan cells is a highly active research field, in part motivated by the recent recognition of nuclear envelopathies (Somech et al. 2005), the plant NE is barely understood. Only few endogenous plant NE proteins have been characterized. Those include nuclear matrix constituent protein 1 (NMCP1), MFP1 attachment factor 1 (MAF1), Ran GTPase activating protein (RanGAP), and the microtubule nucleation factor Spc98p (Erhardt et al. 2002; Gindullis et al. 1999; Patel et al. 2004; Masuda et al. 1997; Pay et al. 2002; Rose and Meier 2001). Of these, NMCP1 and MAF1 appear to be plant-specific proteins with no non-plant homologs currently known.

Tomato MFP1 attachment factor 1 (LeMAF1) is a member of a family of 15–20 kDa proteins containing the conserved WPP domain (WPP family), which are well represented in green plants, but are apparently absent from non-plant organisms (Gindullis et al. 1999). LeMAF1 and the Arabidopsis homologs WPP1 and WPP2 are located at the NE and in cytoplasmic speckles. LeMAF1 accumulates at the cell plate during cytokinesis and WPP1 and WPP2 are NE-located specifically in undifferentiated cells and are involved in cell division (Patel et al. 2004). Here, we demonstrate that the LeMAF1-containing cytoplasmic speckles are part of the Golgi, suggesting a structural or functional connection between the plant NE and Golgi.

Although the plant and the animal Golgi apparatus are similar in that they consist of the Golgi stacks and the *trans*-Golgi network (TGN), several unique features of the plant Golgi have been described in recent years

(Staelin and Moore 1995; Jurgens and Geldner 2002; Neumann et al. 2003). Plant cells, in contrast to animal cells, contain Golgi stacks and the TGN scattered ubiquitously in the cytoplasm, which might be instrumental in reducing the travel distance of Golgi vesicles (Nebenfuhr et al. 1999). The plant Golgi appears smaller and more mobile than the animal Golgi (reviewed by Saint-Jore-Dupas et al. 2004; Nebenfuhr et al. 1999). This mobility depends on intact actin filaments and a close spatial association between actin filaments and the Golgi has been demonstrated in plants. In contrast, microtubule de-polymerization has practically no effect on the plant Golgi, while intact microtubules are necessary for the structural integrity of the animal Golgi apparatus (Boevink et al. 1998; Brandizzi et al. 2003; Nebenfuhr et al. 1999). These observed differences suggest differences in the protein composition of the plant and animal Golgi apparatus.

Besides proteins present in the Golgi lumen, a population of proteins has been described in animals that are located at the cytoplasmic side of the Golgi membranes. Some of these proteins are proposed to form a filamentous matrix surrounding the Golgi, possibly involved in interactions with the cytoskeleton (Munro 1998). Recent studies in animals have identified several proteins with long coiled-coil domains as members of the Golgi matrix, named golgins (Gough et al. 2003; Diao et al. 2003; Munro 1998). They have been shown to be necessary for Golgi structure and for tethering events during membrane fusion (Barr and Short 2003). Animal golgins often contain a C-terminal trans-membrane domain or another conserved C-terminal domain, e.g., the GRIP domain, which is necessary and sufficient for Golgi targeting (Barr and Short 2003; Brown et al. 2001). Except for one Arabidopsis Golgi-localized GRIP-domain protein (AtGRIP; Gilson et al. 2004), no plant golgins are currently known.

Here, we have identified a novel long coiled-coil protein (tomato WPP-domain associated protein, LeWAP) that interacts with LeMAF1 as well as Arabidopsis WPP-domain proteins and is associated with the Golgi. We have mapped the interaction domains of WPP-domain proteins and LeWAP and have demonstrated that Arabidopsis WAP (AtWAP) is required for in vivo complex formation of WPP-domain proteins. We suggest that WAP is a plant golgin-like protein, which functionally interacts with WPP family proteins at the Golgi, but not at the NE or the cell plate.

Materials and methods

Yeast two-hybrid screen

The open reading frame of LeMAF1 (Gindullis et al. 1999) was cloned into the yeast GAL4 binding domain vector, pBD (Stratagene, La Jolla, CA) to create pBD-

MAF1. pBD-MAF1 was co-transformed with a tomato leaf cDNA library in the GAL4 activation domain vector, pAD (Stratagene, La Jolla, CA), and 2.3 x 10⁵ co-transformed colonies were screened on histidine dropout plates for interaction. Six cDNAs were identified that code for four different proteins. The longest open reading frame (2953 bp) was sequenced and found to encode a novel protein with a C-terminal coiled-coil domain and an N-terminal domain having no similarity to known proteins. The protein encoded by the cDNA (GenBank accession number: AY917129) was named LeWAP for tomato WPP-domain associated protein. The yeast reporter strains YRG-2 (Stratagene, La Jolla, CA) and PJ69-4A (James et al. 1996) were used for all yeast two-hybrid assays. A synthetic complete medium lacking leucine and tryptophan was used for the selection of co-transformants, and the same medium lacking histidine (for YRG2 and PJ69-4A strains) and adenine (only for the PJ69-4A strain) was used to detect protein-protein interaction.

Cloning

The coiled-coil domain of LeWAP was cloned into the *EcoRI* site of the yeast GAL4 activation domain vector pAD (Stratagene, La Jolla, CA) and the *E. coli* protein expression plasmid pRSET B (Invitrogen, Carlsbad, CA) using primers 5'-TTGAATTCAAGATTCC AGAAATTATA-3' and 5'-TTGAATTCAATCCCTT TTGATCAGCT-3'. The non-coiled-coil domain of LeWAP was cloned into the *SalI* site of the yeast activation domain containing plasmid pAD (Stratagene, LaJolla, CA) using primers 5'-TTTGTGCGACGCAC-GAGGGAAAATTTG-3' and 5'-TTTGTGCGACTTCC TTAGAATGTC-3'. It was cloned into the *PstI* site of the *E. coli* protein expression plasmid pRSET B (Invitrogen, Carlsbad, CA) by using primers 5'-TTTC TGCAGAAAATGAGAATTTG-3' and 5'-TTTCTG CAGTTCCTTAGAATGTCA-3'. The forward primer 5'-CACCATGGAATTCGGCAGGAGG-3' and the reverse primer 5'-AGAATCTCCATAATCCCCAGG-3' were used to clone LeWAP into pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA). The conserved C-terminal domain of LeWAP was cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA) using the forward primer 5'-CACCATGTATCAACAAAGGCTTGA-3' and same reverse primer mentioned earlier for LeWAP. The deletion construct of LeWAP that lacked the conserved C-terminal domain was cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA) using the same forward primer mentioned earlier for LeWAP and the reverse primer 5'-TAGTGTTGTTCTTCTCAGCAA ATTG-3'. The constructs from the pENTR/D-TOPO (Invitrogen, Carlsbad, CA) vector were transferred by LR recombination using the LR recombination kit (Invitrogen, Carlsbad, CA) to the destination vectors pK7FWG2 (<http://www.psb.rug.ac.be/gateway/>) and pDEST22 (Invitrogen, Carlsbad, CA).

Cloning of WPP1, WPP2 and WPP3 into the *E. coli* expression vector pRSET B (Invitrogen, Carlsbad, CA) is described in Patel et al. (2004). These three constructs were further sub-cloned into the yeast GAL4 binding domain vector pBD (Stratagene, La Jolla, CA) using the *EcoRI* site. Cloning of AtRanGAP1 along with its deletion and point-mutated constructs is described in Rose and Meier (2001). The deletion constructs and the full length AtRanGAP1 were sub-cloned into the *E. coli* expression vector pRSET B (Invitrogen, Carlsbad, CA) and the yeast GAL4 binding domain vector pBD (Stratagene, La Jolla, CA) using the *EcoRI* site.

Protein purification from *E. coli*

The open reading frames of WPP1, WPP2, WPP3, AtRanGAP1ΔC and AtRanGAP1 were cloned into pRSET B and were each expressed separately as recombinant proteins with “His” and “Xpress” epitope tags in BL21 DE3 cells. Protein purification was performed using Ni-NTA superflow column and the protocol given by Qiagen (Valencia, CA).

Sequence analysis

The homologs of LeWAP were identified by sequence similarity searches using BLAST (Altschul et al. 1997). Coiled-coil domain predictions were carried out using the PROTEAN software (DNASTAR, Madison, WI). MEGALIGN protein alignment software (DNASTAR, Madison, WI) was used for multiple sequence alignments using the CLUSTAL algorithm.

Yeast protein extracts

About 50 ml of YPD was inoculated with a yeast culture grown overnight under selection conditions. After growth had reached an O.D. of 0.6, the culture was quick-chilled by pouring it into a 100 ml pre-chilled centrifuge tube half filled with ice. After harvesting, the cells were washed with ice-cold water. About 100 μl of cracking buffer (8M Urea, 5% SDS, 40 mM Tris-Cl, pH 6.8, 0.1 mM EDTA, 0.4 mg/ml Bromophenol blue, 0.1 mg/ml pepstatin A, 0.03 mM leupeptin, 145 mM benzamidine, 0.37 mg/ml aprotinin, 1.4M 2-mercaptoethanol, 1 mM PMSF) and 80 μl of glass beads were added per 7.5 O.D. units of culture (total O.D. units = O.D. × total volume). After heating for 10 min at 70°C the sample was vortexed vigorously for 1 min. The supernatant containing total yeast proteins was recovered after centrifugation at 14,000 rpm at 4°C.

In vitro protein–protein binding assays

Approximately 50 ng of each of the two proteins were combined in a total of 100 μl binding buffer (10 mM

Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, 1 mM PMSF). The ³⁵S-labeled proteins were obtained by coupled in vitro transcription and translation using the TNT Coupled Reticulocyte Lysate Systems (Promega, Madison, WI). After 30 min of incubation at 4°C, 1–2 μl anti-Xpress antibody (Invitrogen, Carlsbad, CA) was added and the mixture was further incubated for 2 h. About 20 μl of 50% protein A sepharose equilibrated in binding buffer was added and the tubes were placed on a rotator overnight at 4°C. After washing four times in 250 μl buffer, the samples were re-suspended in 20 μl SDS-Sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS 1.4M 2-mercaptoethanol) and used for SDS-PAGE analysis.

Immunoblot analysis

Dilutions of 1:500 and 1:300 were used for mouse antibodies against the GAL4 DNA binding domain and the GAL4 activation domain (Molecular probes, Eugene, OR), respectively. A dilution of 1:4,000 was used for the mouse anti-GFP antibody (Clontech, Palo Alto, CA). A 1:20,000 dilution was used for the horseradish peroxidase-coupled sheep anti-mouse secondary antibody (Sigma, St Louis, MO). Immunoblot analysis was performed as described by Sambrook et al. (1989). ECL detection was performed as described by Amersham Pharmacia Biotech (Uppsala, Sweden).

Nicotiana benthamiana protoplast transformation

About 10–15 μg of plasmid DNA was used for electroporation into *Nicotiana benthamiana* protoplasts using the protocol described in Qi and Ding (2002).

Tobacco BY-2 transgenic lines

The LeWAP-pK7FWG2 (pK7FWG2 contains the *GFP* gene, <http://www.psb.rug.ac.be/gateway/>) vector was electroporated (using voltage of 1.44 kV, cuvette of 1 mm gap) into GV3101 *Agrobacterium* cells, which were used to stably transform BY-2 cells in a procedure described by Hong et al. (2001). Stably transformed tobacco cells were selected using 500 mg/l kanamycin, and individual calli were used to establish liquid cultures. Stably transformed LeMAF-GFP transgenic lines were previously described (Patel et al. 2004). The tobacco BY-2 transgenic line expressing WPP1-CFP, GFP-MBD (a microtubule marker), and Nag-RFP was a gift from Drs. Ram Dixit and Richard Cyr, Pennsylvania State University (Dixit and Cyr 2002).

Golgi staining

BODIPY TR C5 Ceramide (Molecular probes, Eugene, OR) was used to stain Golgi membranes. Four-day-old

BY-2 cells, *Nicotiana benthamiana* protoplasts, or 3-day-old Arabidopsis seedlings were incubated with 5 μ M BODIPY TR C5 Ceramide-BSA for 30 min. After rinsing the samples several times with MS medium, they were incubated at 37°C for 30 min. The samples were then used for microscopy.

Tobacco BY-2 cell synchronization

Seven-day-old BY-2 cells transferred to MS medium containing 5 μ g/ml aphidocolin (Sigma-Aldrich, St. Louis, MO) at a 1:10 dilution. After 24 h of incubation (with shaking) in the dark, cells were collected by filtering through miracloth and washed with 500 ml of MS medium. Cells were then cultured in MS medium and were examined after 8–10 h of release from aphidocolin (Sigma-Aldrich, St. Louis, MO). About 1 μ M of SYTO 82 orange (Molecular Probes, Eugene, OR) was used for nucleic acid staining as described in Rose and Meier (2001).

Confocal microscopy

Confocal microscopy images were obtained as described by Rose and Meier (2001). In brief, the 488 nm excitation line of an Argon laser and the 543 nm excitation line of a Helium-Neon laser were used to detect the green GFP and the red BODIPY signal, respectively. The SIMPLEPCI software (Compix Imaging systems, Cranberry Township, PA), which enables separation of green or red fluorescence, was used for image capture. Gain settings in the range of 2,000–2,400 were used for the red and green channels. Photomultiplier tube black levels of 350 and 600 were used for fluorescence and transmitted light signals. Under these conditions, no filter leakage was observed between GFP and BODIPY (see supplementary Fig. S1a). For further processing the Adobe PHOTOSHOP software (Adobe Systems, Mountain View, CA) was used.

Identification of AtWAP T-DNA mutant

The T-DNA insertion line SALK_067822 was identified as the Arabidopsis homolog of LeWAP, AtWAP (At2g34730). The forward primer 5'-AAGAAGCCGTGAAGGAAGCTCA-3', the reverse primer 5'-ACA-CAATTCGCAAACGCCTGT-3' and the left border T-DNA primer (Lba1) 5'-TGGTTCACGTAGTGGCCATCG-3' were used to identify homozygous AtWAP T-DNA mutants, which showed PCR bands with only the forward and Lba1 primer pairs. While wild type plants showed PCR bands with only forward and reverse primers, heterozygous T-DNA mutant plants showed bands with both forward and reverse primers, and with forward and Lba1 primer pairs.

Arabidopsis growth conditions and transformation

Arabidopsis seedlings and plants were grown according to Patel et al. (2004). AtWAP T-DNA mutants were transformed with WPP1-GFP and WPP2-GFP binary vectors (Patel et al. 2004) according to the protocol described by Clough and Bent (1998).

Blue native (BN) PAGE

Sample preparation was performed as described in Camacho-Carvajal et al. (2004), with some modifications. Tissues from 10-day-old Arabidopsis seedlings expressing WPP1-GFP or WPP2-GFP fusion proteins (Patel et al. 2004) in both wild type and AtWAP T-DNA insertion mutant (SALK_067822) background were used. Seedling tissue was ground in liquid nitrogen and ice-cold extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) supplemented with protease inhibitor cocktail (Sigma, St Louis, MO) was added. After incubation on ice for 30 min, with occasional vortexing, lysates were centrifuged at 15,000 \times g at 4°C for 20 min. An aliquot of the supernatant was subjected to SDS-PAGE and immunoblot analysis to confirm equal loading. About 500 μ l of the supernatant was placed into Centricon YM-30 centrifugal filter unit (Amicon bioseparations; Millipore, Bedford, MA) and 1.5 ml of ice-cold BN buffer was added (500 mM 6-aminohexanoic acid, 20 mM bis-Tris, pH 7.0, 2 mM EDTA, 12 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM PMSF). The sample was centrifuged at 5,000 \times g at 4°C for 60 min. The flow-through was discarded and centrifugation step was repeated four times, each time adding 1.5 ml of fresh BN buffer. Samples were either directly loaded on the BN gel or kept in aliquots at -80°C for further use.

BN gels were prepared as described (Camacho-Carvajal et al. 2004; Schagger et al. 1994; Brookes et al. 2002). 5–15% gradient gels and Bio-Rad Mini-PROTEAN 3 (Bio-Rad, Hercules, CA) were used. About 50 μ g of high molecular weight calibration kit (Amersham Pharmacia Biotech, Uppsala, Sweden) dissolved in BN buffer was used as a marker. For further separation in a second-dimension SDS-PAGE, the bands from BN gel were excised and frozen at -20°C.

Results

Identification of a coiled-coil WPP-domain associated protein

The Arabidopsis homologs of LeMAF1 associate with the NE in a developmentally regulated manner linked to mitotic activity (Patel et al. 2004). Both for localization and for a function in cell division, WPP family proteins most likely need protein interaction partners.

The lack of a trans-membrane domain in WPP family proteins further suggests that their localization depends on protein-protein interactions. To identify LeMAF1-interacting proteins, a yeast two-hybrid screen was performed with a tomato leaf library as prey. This screen yielded the coiled-coil protein LeWAP, encoded by a 2953 bp cDNA, which interacted specifically with LeMAF1 (Fig. 1a, sectors 1–8). Northern blot analysis showed the length of the LeWAP mRNA to be approximately 3100 bp (data not shown), indicating that cDNA clone was almost of full length. Further, LeWAP was expressed in most tomato tissues, including fruit, leaves and seedlings (data not shown). Using the LeWAP amino acid sequence to query BLAST, no homologs with e -values less than $2e^{-36}$ were found outside the plant kingdom. Plant homologs were identified on the basis of sequence similarity and coiled-coil domain organization. The Arabidopsis genome has one homolog of LeWAP with coiled-coil regions in the C-terminal half of the protein, similar to the domain organization of the tomato protein (Fig. 1b). The rice genome has several putative homologs of this protein, as indicated in Fig. 1b. Interest-

ingly, the very C-terminal regions (approximately 70 residues) of all proteins shown in Fig. 1b have a high degree of similarity, indicating a functional conservation (box in Fig. 1b; sequence shown in Fig. 1c). The amino acid identity in this domain varies from 37.1 to 75.4% between the different proteins, with Arabidopsis and tomato WAP showing highest degree of amino acid identity (75.4%). This approximately 70 aa-conserved domain was not found in any other protein and its function is currently unknown (see below). Although one of the putative rice homologs, XP_473220, has a low e -value, it was still chosen because of the presence of the conserved C-terminal domain. One EST (GenBank accession number: AV549106) was found for AtWAP, but no ESTs are known so far for the rice WAPs.

Arabidopsis contains five WPP-domain containing proteins: WPP1, WPP2, WPP3, AtRanGAP1 and AtRanGAP2 (Patel et al. 2004). RanGAPs are GTPase-activating proteins instrumental in nucleocytoplasmic transport and AtRanGAPs contain an N-terminal WPP domain that is necessary and sufficient for NE targeting (Rose and Meier 2001). To date,

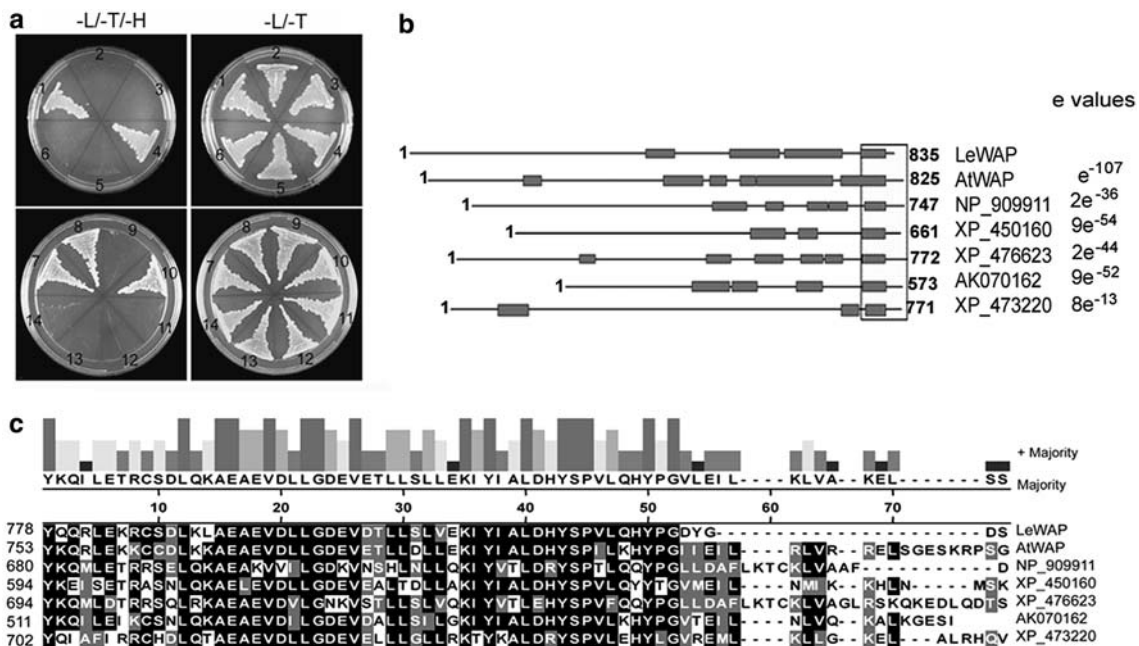


Fig. 1 a Yeast two-hybrid analysis with the following sets of bait/prey constructs: p53/SV40 (1), pLaminC/SV40 (2), pBD-LeMAF1/pAD-GAL4 (3), pBD-LeMAF1/pAD-LeWAP (4), pBD-GAL4/pAD-LeWAP (5), pLaminC/pAD-LeWAP (6), pBD-WPP1/pAD-LeWAP (7), pBD-WPP2/pAD-LeWAP (8), pBD-WPP3/pAD-LeWAP (9), pBD-AtRanGAP1/pAD-LeWAP (10), pBD-AtRanGAP1/pAD-GAL4 (11), pBD-WPP1/pAD-GAL4 (12), pBD-WPP2/pAD-GAL4 (13), pBD-WPP3/pAD-GAL4 (14). ‘-L’, ‘-T’ and ‘-H’ indicate the absence of the amino acids leucine (L), tryptophan (T) and histidine (H), respectively from the yeast minimal growth medium. **b** a cartoon indicating size and location of the coiled-coil domains in the different plant homologs of WAP, which are aligned at the C-

terminal end of the predicted coiled-coil domain; the last five sequences are rice open reading frames, accession numbers are shown. Grey bars represent the coiled-coil regions as defined by PROTEAN. The box delineates the conserved C-terminal regions and is enlarged in **c**. Numbers indicate first and last amino acid of each protein. e -values of WAP homologs to LeWAP are indicated on the right. **c** sequence alignment of the C-terminal conserved regions, each ending in the last amino acid of the proteins depicted in **b**. Amino acid positions are indicated on the left. Majority: consensus strength (height of bars) and sequence based on majority of at least four out of seven. Black shading indicates amino acids that are identical with the majority. Grey shading indicates amino acids that are similar to the majority

WPP3 is the only WPP-domain-containing protein known not to be located at the NE (Patel et al. 2004). We tested WPP1, WPP2, WPP3 and AtRanGAP1 for interaction with LeWAP in a yeast-two hybrid analysis. As shown in Fig. 1a (sectors 7–14), WPP1, WPP2 and AtRanGAP1, but not WPP3, interact with LeWAP. Together, these data indicate that LeWAP interacts specifically with WPP-domain containing proteins from different higher plant species, and that it discriminates between WPP-domain proteins that show specific sub-cellular targeting and those that don't.

Mapping of the LeWAP and AtRanGAP1 protein–protein interaction domains

We wished to determine which domain of LeWAP is responsible for interacting with the WPP-domain containing proteins. LeWAP can be divided roughly into an N-terminal half with no recognizable sequence or structural similarity and a C-terminal half consisting of coiled-coil domains and the 70 aa conserved domain (see Fig. 1). Accordingly, two LeWAP fragments were generated: the non-coiled-coil N-terminus (NC-LeWAP) (aa 1–387) and the coiled-coil C-terminus (CC-LeWAP) (aa 388–834) (Fig. 2a). In addition, we questioned whether the N-terminal WPP domain of AtRanGAP1 is indeed the domain responsible for the LeWAP–AtRanGAP1 interaction. Two sub-fragments of AtRanGAP1 were generated (Fig. 2a): the N-terminal WPP domain (AtRanGAP1ΔC) (aa 1–119) and the portion of the protein from aa 120 to the C-terminus (AtRanGAP1ΔN) (aa 120–535). Further, a mutant version of the AtRanGAP1 WPP-domain, in which the conserved WPP motif was replaced with AAP, was tested. Figure 2b shows the position of this mutation within an alignment of the five Arabidopsis WPP-domain containing proteins and LeMAF1.

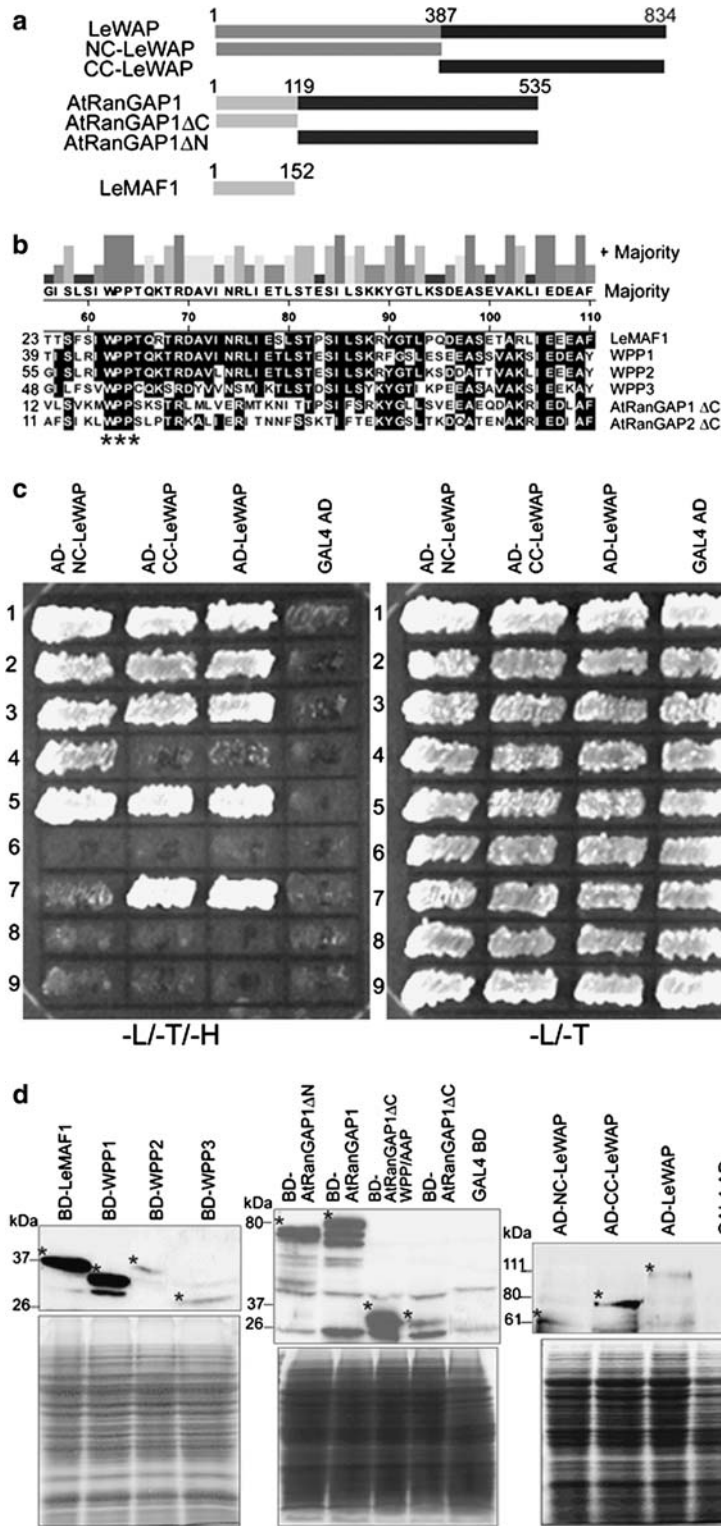
Figure 2c shows the results of the crosswise interaction analysis of LeWAP and WPP-domain containing proteins. LeMAF1, WPP1 and WPP2 interact with both the non-coiled-coil and the coiled-coil regions of LeWAP (Fig. 2c, lanes 1, 2, 3). WPP3 interacts with the non-coiled-coil region of LeWAP, but not with the whole protein, suggesting that this interaction might be spurious (Fig. 2c, lane 4). AtRanGAP1 interacts with the CC-LeWAP but not with NC-LeWAP (Fig. 2c, lane 7). AtRanGAP1ΔC (WPP domain) is responsible for interaction of AtRanGAP1 with LeWAP, as AtRanGAP1ΔN does not interact with LeWAP or its deletions (Fig. 2c, lane 6). In contrast, AtRanGAP1ΔC interacts with both NC-LeWAP and the CC-LeWAP (Fig. 2c, lane 5). The WPP to AAP mutant of AtRanGAP1ΔC (indicated in Fig. 2b) ceases to interact with LeWAP, CC-LeWAP and NC-WAP (Fig. 2c, lane 8). Immunoblot analysis on yeast protein extracts indicated that all fusion proteins were expressed (Fig. 2d). These data indicate that the coiled-coil portion of LeWAP is sufficient for binding WPP domains and that LeWAP, expectedly, interacts with the WPP domain of AtRanGAP1.

Fig. 2 a deletion constructs used in the following yeast two-hybrid analysis. Numbers indicate positions of amino acids. **b** Alignment of the N-terminal region of LeMAF1 with the five Arabidopsis WPP-domain containing proteins. Majority: consensus strength (*height of bars*) and sequence based on majority of at least four out of seven. *Black shading* indicates amino acids that match the majority. *Asterisks* indicate the positions of point mutations of the construct pBD-AtRanGAP1ΔC WPP/AAP, which was used for yeast two-hybrid analysis in **c**. **c** yeast two-hybrid analysis with pBD-LeMAF1 (1), pBD-WPP1 (2), pBD-WPP2 (3), pBD-WPP3 (4), pBD-AtRanGAP1ΔC (5), pBD-AtRanGAP1ΔN (6), pBD-AtRanGAP1 (7), pBD-AtRanGAP1ΔCWPP/AAP (8) and pBD-GAL4 (9), each of them indicated by their corresponding numbers on the left, along with pAD-NC-LeWAP, pAD-CC-LeWAP, pAD-LeWAP and pAD-GAL4, each of them indicated on the top. ‘-L’, ‘-T’ and ‘-H’ indicate the absence of the amino acids leucine (L), tryptophan (T) and histidine (H), respectively from the yeast minimal growth medium. **d** western analysis of yeast protein extracts. Anti-GAL4 binding domain anti-serum was used to detect GAL4 BD-LeMAF1, GAL4 BD-WPP1, GAL4 BD-WPP2, GAL4 BD-WPP3, GAL4 BD-AtRanGAP1ΔN, GAL4 BD-AtRanGAP1, GAL4 BD-AtRanGAP1ΔCWPP/AAP, GAL4 BD-AtRanGAP1ΔC proteins; the bands corresponding to the expected size of the fusion proteins (seen in multiple independent immunoblot assays) are indicated on the blot with an *asterisk*. Two background protein bands are also present in the control yeast strain expressing only the GAL4 binding domain. The remaining bands with molecular weights lower than the fusion proteins are likely degradation products. Anti-GAL4 activation domain anti-serum was used to detect GAL4 AD-NC-LeWAP, GAL4 AD-CC-LeWAP and GAL4 AD-LeWAP; each of the proteins is indicated on the blot with *asterisks*. Strains expressing only GAL4 BD or GAL4 AD were used as negative controls. Coomassie Blue-stained replica gels are shown below the blots as loading control

LeWAP interacts in vitro with WPP-domains

To confirm the results of the yeast two-hybrid analysis, in vitro co-immunoprecipitation assays were performed. NC-LeWAP and CC-LeWAP were ³⁵S labeled during in vitro translation. LeMAF1, WPP1, WPP2, WPP3, AtRanGAP1 and AtRanGAP1ΔC were purified along with their Xpress tags from *E. coli*. Figure 3a shows that all six proteins can be immunoprecipitated with the anti-Xpress antiserum.

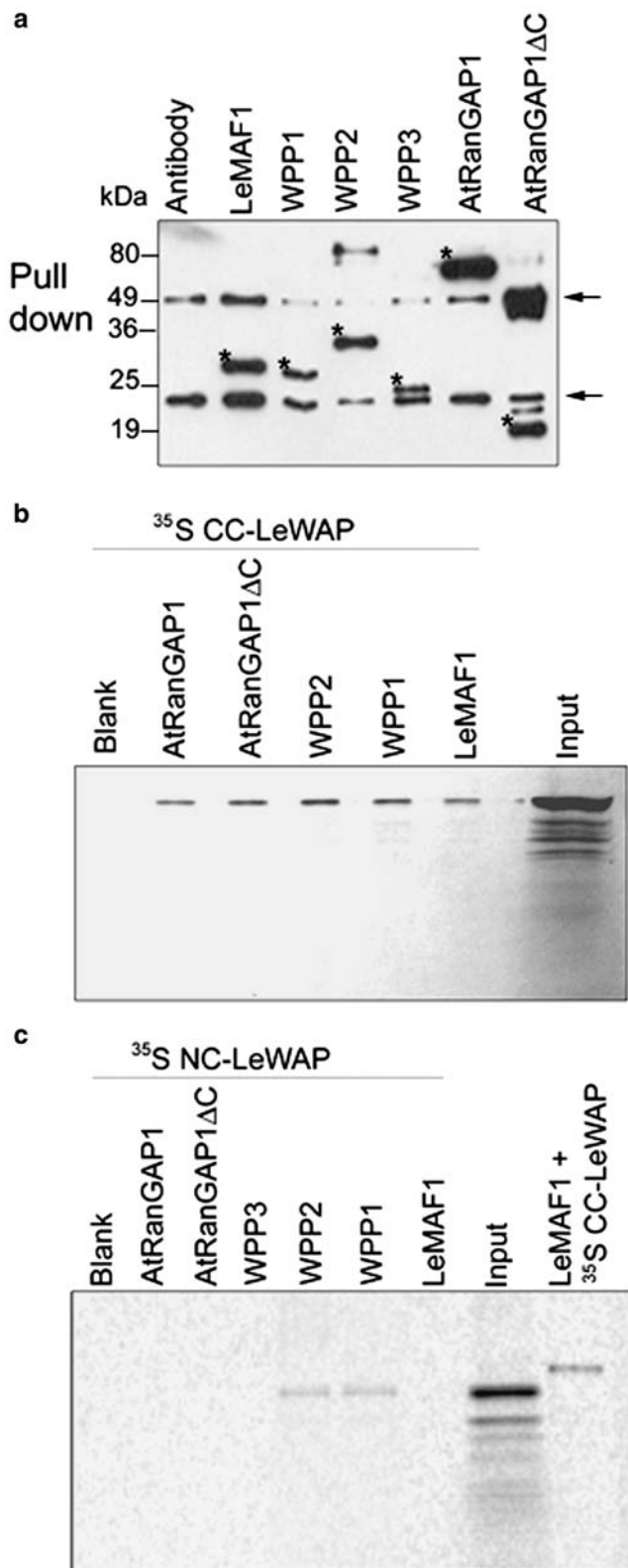
CC-LeWAP was co-precipitated with LeMAF1, WPP1, WPP2, AtRanGAP1 and AtRanGAP1ΔC, consistent with the results obtained from the yeast two-hybrid analysis (Fig. 3b). NC-LeWAP was weakly co-precipitated with WPP1 and WPP2, but not with LeMAF1 or WPP3 (Fig. 3c), indicating that the coiled-coil domain of LeWAP is the primary interaction domain with WPP-domain containing proteins. NC-LeWAP interacted neither with AtRanGAP1 nor with AtRanGAP1ΔC (Fig. 3c), further demonstrating that the primary domain of interaction with the WPP-domain resides in the coiled-coil domain of LeWAP. These data indicate that the coiled-coil domain of LeWAP directly interacts with WPP domains.



LeWAP-GFP and LeMAF1-GFP are located at the Golgi

The subcellular localization of LeWAP was investigated by expressing LeWAP as a GFP-fusion protein under the control of the 35S promoter in transiently transformed *N. benthamiana* protoplasts and stably trans-

formed *Nicotiana tabacum* BY-2 cells. LeWAP-GFP accumulated in speckles in the cytoplasm (Fig. 4a). BODIPY TR C5 Ceramide (Molecular probes, Eugene, OR) is a commonly used Golgi membrane marker in animal cells, which incorporates into the sphingolipids of the Golgi (e.g., Haeggström et al. 2004; Karan et al. 2004; Valetti et al. 1999). The BODIPY TR fluorophore



exhibits a red fluorescence (617 nm) compatible for dual color imaging with GFP (see supplementary Fig. S1a). BODIPY TR C5 ceramide co-localizes in Arabidopsis cells with rat sialyl transferase (ST)-GFP, which was previously established to be Golgi-located in plants

Fig. 3 **a** pull-down analysis with anti-Xpress antibody for each of the proteins used in the following in vitro binding assay. The predicted molecular weights of LeMAF1, WPP1, WPP2, WPP3, AtRanGAP1 and AtRanGAP1ΔC are 16 kDa, 16 kDa, 19 kDa, 16 kDa, 58 kDa and 13 kDa, respectively. As shown previously (Patel et al. 2004), all WPP-domain proteins have a higher perceived than predicted molecular weight in SDS PAGE. The proteins with the correct perceived molecular weight are indicated with an *asterisk*. The antibody bands are indicated by *arrows*. In the lane containing AtRanGAP1ΔC, two antibody degradation products are also seen. **b** in vitro binding analysis with the ³⁵S-labeled coiled-coil region of LeWAP. The proteins used are indicated in the figure. The protein complex was pulled down with anti-Xpress antibody and ³⁵S-labeled-CC-LeWAP (51 kDa) was detected. Input: Labeled ³⁵S CC-LeWAP used in the analysis. **c** in vitro binding analysis with the ³⁵S-labeled non-coiled-coil region of LeWAP. The proteins used are indicated. The protein complex was pulled down with the anti-Xpress antibody and the ³⁵S-labeled NC-LeWAP (44 kDa) was detected. Input: Labeled ³⁵S NC-LeWAP used in the analysis. ³⁵S-labeled CC-LeWAP pulled down by LeMAF1 in **b** was used as a positive control

(Saint-Jore et al. 2002). This indicates that BODIPY TR C5 Ceramide faithfully labels the plant Golgi too (see supplementary Fig. S1b). When LeWAP-GFP-expressing cells were co-stained with BODIPY TR C5 Ceramide, a majority of speckles were found to co-localize with the red BODIPY TR fluorescence (Fig. 4b), indicating that most of the LeWAP-GFP fusion protein is associated with the Golgi.

Along with their previously described NE association, tomato MAF1, and Arabidopsis WPP1 and WPP2 had been found to also localize in speckles in the cytoplasm (Gindullis et al. 1999; Patel et al. 2004). These speckles do not coincide with MitoTracker Red staining, confirming that they are neither mitochondria nor chloroplasts (data not shown). Figure 4b shows that most LeMAF1-GFP speckles also co-localized with BODIPY TR C5 Ceramide, indicating that both LeMAF1 and LeWAP are associated with the Golgi. In contrast to the Golgi, the NE was only labeled by LeMAF1-GFP, but not by LeWAP-GFP (Fig. 4b). Supplementary Fig. S1c shows an image of a BY2 cell line expressing both WPP1-CFP and N-acetylglucosaminyl transferase I (Nag)-RFP, which is Golgi-located (Dixit and Cyr 2002). The majority of cytoplasmic speckles labeled by WPP1-CFP are also labeled by Nag-RFP, providing further evidence for WPP-protein association with the Golgi. Together with the yeast and in vitro binding data, these data suggest that LeMAF1 and LeWAP are interacting in the vicinity of the Golgi apparatus, but not at the nuclear envelope.

The conserved domain of LeWAP is not required for Golgi targeting

Recently, a family of Golgi-localized, coiled-coil proteins containing a 40 aa conserved C-terminal domain, called GRIP domain, was characterized (Barr and Short 2003). A GRIP domain-containing protein has been

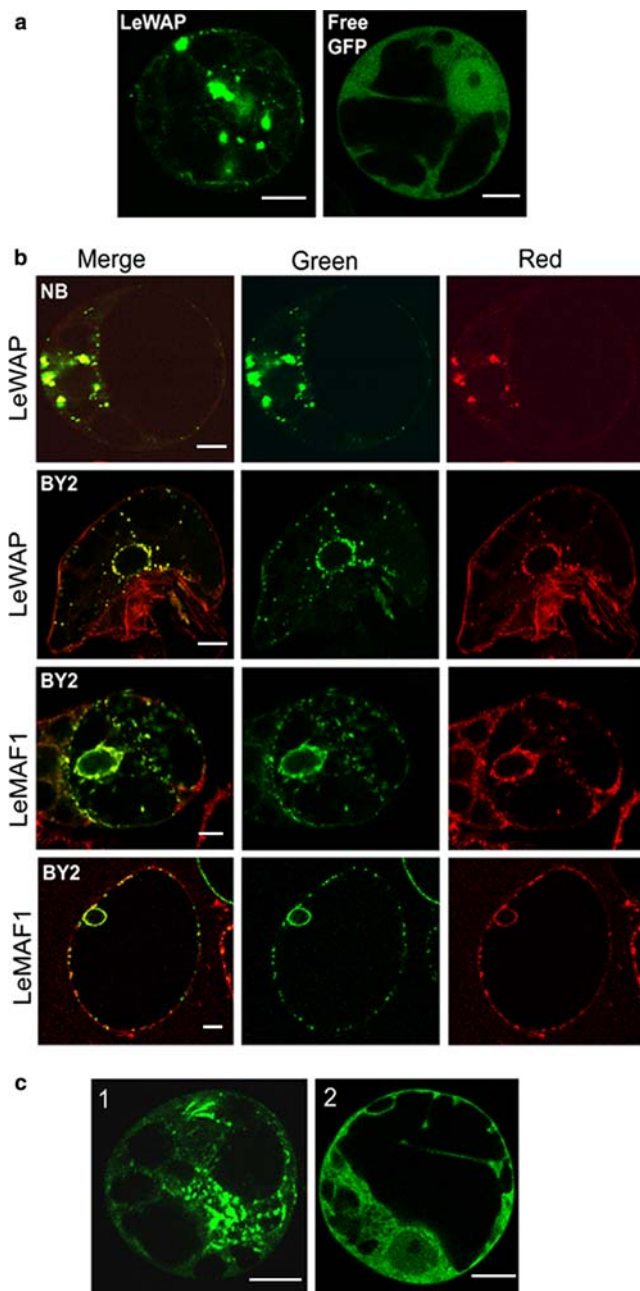


Fig. 4 Sub-cellular localization of LeWAP-GFP and LeMAF1-GFP. **a** GFP fluorescence of *N. benthamiana* protoplasts transiently expressing LeWAP-GFP or free GFP. **b** green GFP fluorescence and red BODIPY TR C5 ceramide fluorescence of *N. benthamiana* protoplasts (indicated as NB) transiently expressing LeWAP-GFP and transgenic BY-2 cells (indicated as BY2) expressing LeWAP-GFP or LeMAF1-GFP. **c** GFP fluorescence of LeWAP-GFP without the conserved C terminal domain (1) or with the conserved C-terminal domain only (2). Size bars: 10 μ m

identified recently in Arabidopsis, indicating its conservation in plants, and the GRIP domain in that protein is sufficient for targeting to the Golgi (Gilson et al. 2004). Because the WAP family shares a 70 aa conserved C-terminal domain, we wanted to determine whether this domain is involved in Golgi targeting. Figure 4c shows

that LeWAP-GFP lacking the conserved C-terminal domain is still targeted to the Golgi (Fig. 4c-1), while the conserved C-terminal domain alone does not target GFP to the Golgi (Fig. 4c-2). Therefore, the 70 aa conserved C-terminal domain is neither necessary nor sufficient for Golgi targeting. We also tested the influence of the conserved domain on binding to WPP-domain containing proteins in yeast two-hybrid assays using the same two deletion constructs. The results showed that the 70 aa conserved C-terminal domain is neither necessary nor sufficient for binding to the WPP-domain containing proteins (data not shown). Its function in the context of LeWAP is therefore currently not known.

LeWAP-GFP does not associate with the cell plate during cell division

AtRanGAP1 and LeMAF1 localize in the vicinity of the immature cell plate during cell division (Jeong et al. 2005; Patel et al. 2004; Pay et al. 2002). Because Golgi-mediated vesicles are involved in cell-plate formation (Segui-Simarro et al. 2004), we investigated the localization of LeWAP during cell division, too. A LeWAP-GFP transgenic tobacco BY-2 cell line was created and GFP fluorescence was imaged in synchronized cells at different stages of cell division (Fig. 5). SYTO 82 orange was used for nucleic acid staining in live cells. While SYTO 82 orange, unlike DAPI, does not affect cell viability, nuclear DNA is somewhat understained by this dye in comparison to organelle DNA (red cytoplasmic speckles in Fig. 5). However, mitotic chromatin is well labeled by SYTO 82 orange (Fig. 5). Throughout cell division, LeWAP was found diffusely located in the cytoplasm and in occasional cytoplasmic speckles (Fig. 5, Metaphase, Anaphase and Telophase). No association with the cell plate was seen (Fig. 5). These data show that, unlike LeMAF1, LeWAP does not accumulate at the immature cell plate.

Arabidopsis WAP is required for a multi-protein complex that contains WPP2

A T-DNA insertion in the Arabidopsis homolog of LeWAP (At2g34730) was identified (SALK_067822), in which the T-DNA was inserted in the first annotated intron. A line homozygous for the insertion was identified and the absence of the corresponding mRNA was shown by RT-PCR (data not shown). The AtWAP mutant plants did not show any macroscopic differences in comparison to wild-type Columbia plants. The localization profiles of WPP1-GFP and WPP2-GFP were not changed when the corresponding constructs were introduced into the mutant background. Both fusion proteins were still targeted to the NE and Golgi speckles (data not shown), indicating that LeWAP is not required for WPP1 and WPP2 targeting.

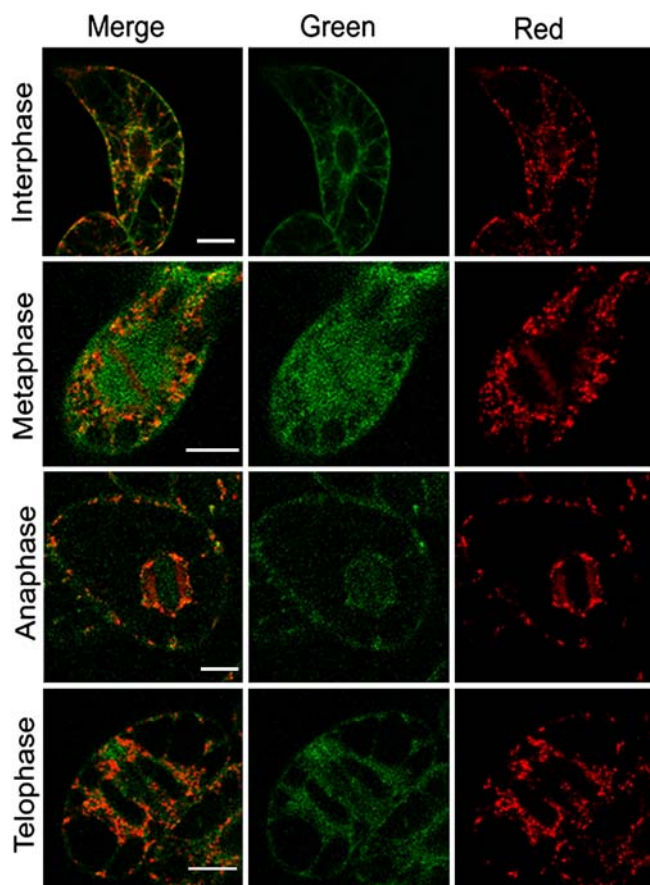


Fig. 5 Localization of LeWAP-GFP during cell division. *Green* GFP fluorescence and *red* SYTO 82 orange fluorescence (labeling nucleic acids) of transgenic BY-2 cells expressing LeWAP-GFP during the stages — interphase, metaphase, anaphase and late telophase. Size bars: 10 μ m

In order to investigate whether the lack of AtWAP would alter WPP1-GFP or WPP2-GFP *in vivo* complex formation, BN-PAGE followed by immunoblots with the anti-GFP antibody was performed. In WPP2-GFP transgenic plants, at least three WPP2-GFP-containing complexes were detected (Fig. 6a, lane 3, complexes I, II and III). In contrast, only the lowest molecular weight WPP2-GFP-containing complex was present in AtWAP T-DNA mutant plants, which were transformed with the same WPP2-GFP fusion construct (Fig. 6a, lane 4, complex I). The molecular weights of the complexes I, II and III were estimated to be approximately 75–80 kDa, 120 kDa and 190 kDa, respectively. Protein isolation and BN-PAGE were repeated several times and in some cases the complex II band was absent, suggesting that it might represent a degradation product of complex III (data not shown).

While complex I was present in WPP1-GFP plants as well, only very weak signs of complexes II and III were seen and no difference was observed between wild type and AtWAP-mutant backgrounds (Fig. 6a, lanes 1, 2). SDS-PAGE and immunoblot analysis of the protein extracts of all the plants mentioned before were per-

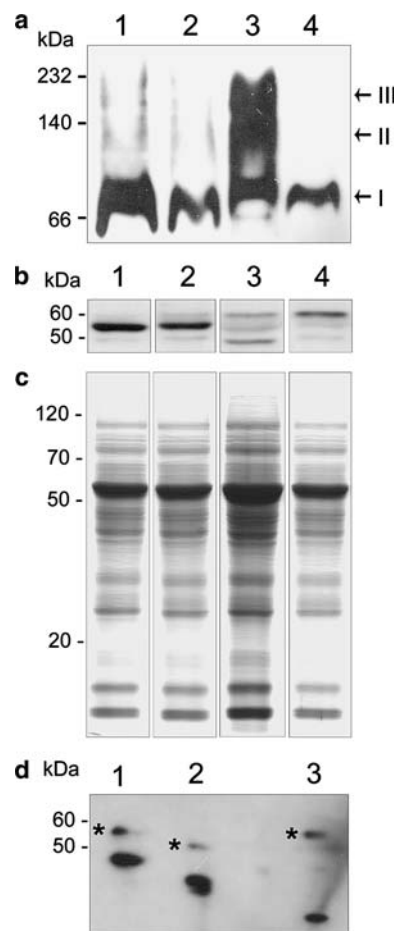


Fig. 6 Identification of the multi-protein complexes that contain Arabidopsis WPP-domain containing proteins. **a** western analysis of whole cellular lysates of Arabidopsis seedlings (a Triton X-100:protein ratio of 0.4 was used to solubilize the membranes) separated by BN-PAGE. Anti-GFP antibody was used to detect proteins from Arabidopsis transgenic lines expressing WPP1-GFP in the wild-type background (*lane 1*); WPP1-GFP in the AtWAP T-DNA mutant background (*lane 2*); WPP2-GFP in the wild-type background (*lane 3*) and WPP2-GFP in the AtWAP T-DNA mutant background (*lane 4*). **b** western analysis of whole cellular lysates of Arabidopsis seedlings separated by SDS-PAGE. Anti-GFP antibody was used to detect proteins from the transgenic lines: WPP1-GFP in the wild-type background (*lane 1*); WPP1-GFP in the AtWAP T-DNA mutant background (*lane 2*); WPP2-GFP in the wild-type background (*lane 3*) and WPP2-GFP in the AtWAP T-DNA mutant background (*lane 4*). A comassie Blue-stained replica gel is shown below the blot. **c** western analysis, using the anti-GFP antisera, of BN-PAGE-obtained complexes I and III from WPP2-GFP in the wild-type background (*lanes 1, 3*, respectively), and complex I obtained from WPP1-GFP in the wild-type background (*lane 2*), separated by second-dimension SDS-PAGE. Full-length fusion proteins are indicated by *asterisks*. Lower molecular weight bands are likely degradation products

formed in order to confirm that approximately equal amounts of total and fusion proteins were present in all cases (Fig. 6b). Although slightly more total protein was detected for WPP2-GFP plants, slightly less fusion protein was present, allowing comparison between all samples. An immunoblot of a second-dimension SDS gel of the isolated bands representing complexes I and

III from WPP2-GFP plants showed that both complexes contain the WPP2-GFP fusion protein (Fig. 6c, lanes 1, 3), while complex I from a WPP1-GFP plant contained the slightly smaller WPP1-GFP (Fig. 6c, lane 2). Together, these data suggest that AtWAP is required for the formation of complexes II and III but not for the formation of complex I. Because the localization patterns of WPP1-GFP and WPP2-GFP are not altered in the AtWAP knockout mutant, we conclude further that complexes II and III are not required for subcellular targeting of WPP2.

Discussion

LeWAP is an interaction partner of WPP-domain proteins

Previous studies have demonstrated the interaction of LeMAF1 with the tomato matrix attachment region binding protein, MFP1 (Gindullis et al. 1999). Apart from MFP1, there are no binding partners known for LeMAF1. The significance of the binding of MFP1 with LeMAF1 was questioned because the two proteins localize to different sub-cellular compartments. MFP1 is associated with the thylakoid membranes of the chloroplast (Jeong et al. 2003), while an onion homolog localizes in distinct foci of the nuclear matrix (Samaniego et al. 2001). LeMAF1, on the other hand, is associated with the outer NE and the Golgi. Further, binding analysis demonstrated that the Arabidopsis WPP family proteins neither interacted with tomato MFP1 nor with Arabidopsis MFP1 in yeast (data not shown), indicating that the LeMAF1–MFP1 interaction might not be biologically significant. Our data here illustrates the binding of LeWAP to WPP-domain proteins from both tomato and Arabidopsis in yeast two-hybrid as well as *in vitro*. Additionally, we show that LeWAP and LeMAF1 localize to the Golgi apparatus and that AtWAP is required for WPP2 complex formation *in vivo*, suggesting a functional significance for their interaction.

WAP domain structure and protein interactions

The proteins with highest degree of similarity to LeWAP were identified from Arabidopsis and rice. All WAP-like proteins have in common the combination of coiled-coil domains and a highly conserved C-terminal signature motif of approximately 70 aa. Except for XP_473220 from rice, all proteins containing the 70 aa motif have a similar domain structure: they are 700–800 aa long and contain almost continuous coiled-coil domains in the C-terminal half. XP_473220 is an exception, in that it has a high degree of similarity to the 70 aa motif, but fewer and shorter coiled-coil domains.

The deletion analysis showed that the C-terminal half of LeWAP is the domain primarily responsible

for binding WPP-domains. The 70 aa motif is not required for binding, suggesting that the interaction surface lies within the coiled-coil domains. This interaction requires the WPP motif and discriminates between WPP domains targeted to the NE and Golgi (WPP1 and WPP2) and those that are not (WPP3). This clear correlation between binding of LeWAP and specific subcellular location of WPP-domain proteins further suggests that the interaction between WAP and WPP-domain proteins is functionally relevant.

LeWAP and LeMAF1 are both located at the Golgi

Proteins containing long coiled-coil domains are involved in structural aspects of the cytoskeleton and the nuclear matrix (Strelkov et al. 2003). Similarly, the coiled-coil golgins are involved in tethering events at the Golgi and are important for Golgi structure (Barr and Short 2003). The only currently known plant golgin is a GRIP-domain protein from Arabidopsis (Gilson et al. 2004). Based on the localization of LeWAP, the presence of long coiled-coil domains, and the absence of an N-terminal signal peptide, we predict LeWAP too to be a constituent of the plant Golgi matrix.

Golgi-derived vesicles are transported via microtubules to the initial cell plate-forming membrane networks and are the building blocks of the immature cell plate during cytokinesis (Segui-Simarro et al. 2004). LeMAF1 associates with the immature cell plate during cell division (Patel et al. 2004) and the localization pattern coincides with the localization pattern of trafficking and fusing vesicles during phragmoplast formation (Mayer and Jürgens 2004; Segui-Simarro et al. 2004; Smith 2002; Staehelin and Hepler 1996). Taken together with the Golgi association of LeMAF1, WPP family proteins might have a function in labeling specific membrane compartments or in their trafficking during cell division. Since WPP family proteins lack a predicted transmembrane domain, their association with membranes most likely requires protein–protein interactions. In contrast, LeWAP localizes to the Golgi, but not to the cell plate or the NE. This indicates that WPP-domain proteins have more than one interaction partner in those different locations. Consistent with this assumption, another coiled-coil protein has been identified by yeast two-hybrid screening that co-localizes with WPP-domain proteins at the nuclear envelope, but not the Golgi (Xu and Meier, unpublished results).

AtWAP is required for a WPP2-containing multi-protein complex

To investigate whether WAP and WPP family proteins are involved in *in vivo* complex formation, BN-PAGE experiments were performed. Three complexes were rec-

ognized in the WPP2-GFP expressing transgenic plants using anti-GFP antisera. The absence of complexes II and III in AtWAP T-DNA plants containing WPP2-GFP protein demonstrates that AtWAP is necessary for their formation. Although no difference was seen between WPP1–LeWAP and WPP2–LeWAP interactions in yeast and in vitro, only WPP2 complexes were affected in the mutant in vivo. This might indicate that although both WPP1 and WPP2 can bind WAP, affinity differences or in vivo modifications make WPP2 its primary in vivo target. Alternatively, affinity differences between LeWAP and AtWAP might explain these results.

The finding that WPP2-GFP is still targeted to the speckles in the absence of AtWAP protein (data not shown) indicates that the AtWAP-requiring complexes are not necessary for WPP2 localization. Instead, a functional complex containing one or more WPP-domain proteins might assemble at the Golgi, with the primary anchoring protein that connects the complex to the Golgi still unknown. The AtWAP knockout mutant has no observable phenotype. This indicates that although no true family members of AtWAP based on sequence similarity or conservation of the C-terminal domain were found in Arabidopsis, unknown functional homologs might exist. Especially for coiled-coil proteins, examples are known where a protein takes over the function of another coiled-coil protein in a mutant background (e.g., Zhao et al. 2004).

LeWAP is the first protein shown to bind a WPP domain and the second golgin-like protein identified from plants. While both LeWAP and WPP family proteins localize at the Golgi, LeWAP was neither identified at the cell plate nor at the nuclear envelope, the other prominent location of WPP family proteins. The finding that both WAP and WPP family proteins localize at the Golgi together with the observed WAP requirement in vivo suggests a role for WAP in a currently unknown Golgi-associated function of WPP family proteins.

Is there a connection between the nuclear envelope and the Golgi in plants?

Our findings show that a group of WPP family proteins is located both at the nuclear pore (Patel et al. 2004) and at the Golgi (this study). While no connection between these two cellular compartments had been formerly anticipated, an exciting recent finding shows that the *Xenopus* COPI co-atomer complex, involved in vesicular trafficking, is targeted to the NE via the nucleoporin Nup 153. Further, COPI was shown to be involved in the disassembly of the *Xenopus* NE (Liu et al. 2003). These data for the first time link the machinery of vesicular trafficking to NE breakdown, through a connection to the nuclear pore. This suggests a greater complexity in this process in animals than previously anticipated. It is possible that such a connection also exists in plants, and that WPP proteins are involved in a

currently unknown process linking the plant Golgi and the plant nuclear pore.

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