An Immunodominant Membrane Protein (Imp) of ‘*Candidatus Phytoplasma mali*’ Binds to Plant Actin

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The phytopathogenic, cell-wall-less phytoplasmas exhibit a dual life cycle: they multiply in the phloem of their host plant and in the body of their insect vector. Their membrane proteins are in direct contact with both hosts and are supposed to play a crucial role in the phytoplasma spread within the plant as well as by the insect vector. Three types of nonhomologous but highly abundant and immunodominant membrane proteins (IDP) have been identified within the phytoplasmas: Amp, IdpA, and Imp. Although recent results indicate that Amp is involved in vector specificity interacting with insect proteins such as actin, little is known about the interaction of IDP with the plant. We could demonstrate that transiently expressed Imp of ‘*Candidatus Phytoplasma mali*’ as well as the Imp without transmembrane domain (Imp^{ΔTm}) bind with plant actins *in vivo*. Moreover, *in vitro* co-sediment and binding assays showed that *Escherichia coli*-expressed recombinant Imp^{ΔTm}-His binds to both G- and F-actins isolated from rabbit muscle. Transgenic plants expressing Imp- or Imp^{ΔTm}-green fluorescent protein did not exhibit any remarkable change of phenotype compared with the wild-type plant. These results indicate that Imp specifically binds to plant actin and a role of Imp-actin binding in phytoplasma motility is hypothesized.

Immunodominant membrane proteins (IDP) are very abundant among total cellular membrane proteins in most phytoplasmas. Genes encoding IDP were isolated from eight phytoplasmas (Kakizawa et al. 2004) which are enormously different in amino acid compositions and antigenicity. IDP are classified into three types: i) Imp, ii) IdpA (immunodominant membrane protein A), and iii) Amp (antigenic membrane protein) (Kakizawa et al. 2006; Morton et al. 2003). To date, only the possible biological function of Amp was studied. It was shown that Amp of onion yellows phytoplasma (OY) co-localized with the insect microfilaments of the visceral smooth muscle surrounding the intestinal tract (Suzuki et al. 2006). Moreover, the results of a pull-down assay revealed that Amp was co-precipitated with insect actin and myosin proteins. Interestingly, Amp-microfilament complexes were detected only in OY-transmitting leafhopper species. These results suggested that an interaction between the phytoplasma Amp and host proteins could be essential for phytoplasma transmission by insect vectors (Suzuki et al. 2006). Because phytoplasmas

can only survive in their insect vectors or in the phloem of their host plants, it would be interesting to investigate the biological function of IDP in sieve elements.

Imp is being considered to be a common ancestor of phytoplasma IDP (Kakizawa et al. 2009) because it was observed in the genomes of Western X (WX) (Liefting and Kirkpatrick 2003) and OY phytoplasma (Kakizawa et al. 2009) in addition to their predominant IDP genes. However, it is not known yet if Imp is expressed as a transmembrane protein in these phytoplasmas. In contrast, neither the ortholog of IdpA nor the ortholog of Amp was found in ‘*Candidatus Phytoplasma mali*’ (Kube et al. 2008). In addition to the difference in amino acid composition between Amp and Imp, it was shown that Amp was expressed and secreted out of the phytoplasma cells while Imp was trapped to the cell membrane (Barbara et al. 2002; Morton et al. 2003). According to previous studies, Amp and Imp are IDP; however, it is likely that Amp and Imp are different not only in gene composition but also at the protein level. In recently published results of preliminary experiments, it was shown that Imp without transmembrane domain of lime witches’ broom phytoplasma interacted *in vitro* with actin and ATP synthase of leafhopper vectors proteins *in vitro* (Siampour et al. 2011). However, biological studies of Imp in plants are missing. In this report, we analyzed the actin-binding activity of Imp *in planta* and *in vitro*.

RESULTS

Imp co-localizes with actin cytoskeleton in planta.

To study the subcellular localization of Imp, a recombinant Imp with green fluorescent protein (GFP) fused to its C terminus was transiently expressed under the control of a 35S promoter in *Nicotiana benthamiana* using an *Agrobacterium*-mediated expression system. The protoplasts were prepared from the infiltrated leaves and GFP fluorescence was visualized under a microscope using a GFP filter. The results showed that Imp-GFP was detected in the cytoplasm associated with a filamentous network (Fig. 1, upper panel). To ensure that this filamentous network was the actin cytoskeleton, we co-expressed Imp-GFP and the actin binding domain of the mouse Talin (mTalin) fused to red fluorescent protein (mTalin-DsRed2) from the same vector, which would allow the two proteins to be expressed in the same cells and at the same time point. The mTalin is known to decorate the filamentous actin (Kost et al. 1998). The merge of Imp-GFP and mTalin-DsRed2 signal clearly indicated that Imp co-localized with the actin cytoskeleton. It must be noted that the co-expression of Imp without a transmembrane domain (Imp^{ΔTm})-GFP and mTalin-DsRed2 gave the same result as shown by Imp-GFP (data not shown). To further confirm the actin-binding ability of Imp, we applied latrunculin B (LatB, an actin-disrupting drug) treatments to leaves co-infiltrated with Imp-GFP and mTalin-

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DsRed2. As a result, the protoplasts prepared from the LatB-treated leaves showed that the actin cytoskeleton was destroyed and that the expressed Imp-GFP as well as the mTalin-DsRed2 were dispersed in the cytoplasm (Fig. 1, lower panel). This result demonstrated that Imp did, indeed, bind actin filaments.

Recombinant Imp^{ΔTm} protein binds directly to G-actin in vitro.

The association of Imp with networks of actin filaments was further investigated in an attempt to elucidate the possible role of Imp in modulating the interactions of these cytoskeleton components. We preferred to use Imp^{ΔTm} for these studies because Imp^{ΔTm} exhibited binding with actin similar to that of Imp (shown in transient expression assay). Furthermore, previous reports indicated that the Imp protein was trapped in the cell membrane and the C-terminal part behind the transmembrane domain that was exposed outside of the cell membrane. To obtain high amounts of protein, the Imp^{ΔTm}-His was expressed in *Escherichia coli* and purified (Fig. 2A and B). The actin binding ability of Imp^{ΔTm}-His was then assayed by an enzyme-linked immunosorbent assay (ELISA) and in co-sedimentation assays. The ELISA was performed to evaluate the interaction of Imp^{ΔTm}-His with G-actin. The result revealed that Imp^{ΔTm}-His binds to G-actin from rabbit muscle, which was coated on a microtiter plate in a concentration-dependent fashion (Fig. 2C). Moreover, bovine serum albumen (BSA), a negative control, did not bind to actin, thus indicating the specificity of Imp^{ΔTm}-His-actin binding.

Co-sedimentation assay.

The actin-binding of Imp was further investigated in vitro. In order to obtain further evidence for a direct interaction, a co-sedimentation assay was performed. The binding ability of Imp^{ΔTm}-His on F-actin was studied in low-speed co-sedimentation assays. The purified recombinant Imp^{ΔTm}-His was incubated for 1 h with polymerized F-actin, and the mixture was centrifuged at low speed. The pellets were then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE). The result in Figure 3 clearly showed that Imp^{ΔTm}-His was found enriched in the pellet together with F-actin, and no trace of aggregates of Imp^{ΔTm}-His was detected (data not shown), whereas the control BSA did not co-precipitate with F-actin. These data demonstrate that Imp^{ΔTm}-His interacts directly with F-actin in vitro.

Our results showed that Imp^{ΔTm}-His binds G- and F-actin in vitro; therefore, we queried whether the binding of Imp^{ΔTm}-His to G-actin could inhibit the F-actin formation during polymerization. To elucidate this, Imp^{ΔTm}-His was preincubated with G-actin prior to induction of polymerization. After centrifugation, the co-sediment pellets were analyzed in SDS-PAGE. The result indicated that the amount of F-actin was not decreased by increasing amounts of Imp^{ΔTm}-His, thus demonstrating that Imp^{ΔTm}-His did not inhibit the polymerization of actin (Fig. 4). Furthermore, the increasing amount of Imp^{ΔTm}-His found in the co-sediment revealed that the binding of Imp^{ΔTm}-His to F-actin occurs in a dosage-dependent manner (Fig. 4).

Stably expressed Imp and Imp^{ΔTm} in transgenic plants do not affect plant development.

Although the result of in vitro binding of Imp to actin clearly indicated that Imp^{ΔTm}-His did not inhibit F-actin polymerization, other possible effects related to this binding could not be

Fig. 1. Co-expression of Imp-green fluorescent protein (GFP) and mTalin-DsRed2 fusion proteins in *Nicotiana benthamiana* and latrunculin B (LatB) treatment. *Agrobacterium* suspension carrying a double cassette of Imp-GFP and mTalin-DsRED2 was infiltrated into *N. benthamiana* plants. Two days post *Agrobacterium* spp.-mediated infiltration, the protoplasts were prepared from the infiltrated leaves and the subcellular localization of the fusion proteins was visualized by fluorescence microscopy. The co-localization of expressed Imp-GFP and mTalin-DsRED2 was judged by the presence of yellow filaments in the merge (upper panel). Imp-GFP and mTalin-DsRED2-labeled microfilaments are degraded in tissue treated for 24 h with 10 mM LatB (lower panel).

Fig. 2. Enzyme-linked immunosorbent assay experiment showing *Escherichia coli*-expressed Imp^{ΔTm} bound to G-actin. Imp^{ΔTm}-His was expressed in *E. coli* (BL21 codon+). After purification, the purified protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The purity and specificity of expressed Imp^{ΔTm}-His were determined by **A**, Coomassie Brilliant blue G staining and **B**, Western blot analysis using anti-His antibody. G-actin from rabbit muscle (1 and 2 μg) was coated on a microtiter plate. After blocking, 2 μg of purified Imp^{ΔTm}-His was added. Binding of Imp^{ΔTm}-His was measured using anti-His antibody (Qiagen) followed by anti-mouse-conjugated alkaline phosphatase (Sigma). After adding substrate, the signal was read with the absorbance at 405 nm. **C**, Bovine serum albumin was used as negative control.

excluded. In order to investigate the effect of expressed Imp on plant development, we produced transgenic plants expressing Imp- and Imp^{ΔTm}-fused GFP. Because the transformation of apple, the main host of 'Ca. P. mali', has not yet sufficiently established, we used transgenic *N. benthamiana* as the model system because it has been demonstrated previously that *Nicotiana* spp. can be experimentally inoculated with 'Ca. P. mali' (Kube et al. 2008). As a control, we produced 'Ca. P. mali'-infected *N. benthamiana* plants by grafting a shoot of phytoplasma-infected *N. occidentalis* that showed very strong symptoms (provided by B. Schneider, Julius-Kühn-Institut, Dossenheim, Germany) to wild-type *N. benthamiana*. The successfully grafted *N. benthamiana* plants were analyzed by polymerase chain reaction (PCR) for phytoplasma infection and the phytoplasma symptoms were monitored. The results showed that the PCR-positive grafted plants (data not shown) exhibited typical symptoms of phytoplasma infection, including shoot proliferation, deformation of leaves, necrosis, and seedlessness (Fig. 5A to C). This result indicated that *N. benthamiana* could be used as a model plant for studying the effect of phytoplasma proteins on plant development.

The transgenic *N. benthamiana* plants expressing Imp and Imp^{ΔTm}-GFP were then established and the expression of Imp-GFP and Imp^{ΔTm}-GFP of T1 generation from different lines was analyzed by Western blot analysis. The result showed that the transgenic plant lines expressed Imp-GFP and Imp^{ΔTm}-GFP (Fig. 6A and B). The protoplasts from the transgenic plants were isolated and the GFP fluorescence was visualized under the microscope using GFP filter. The result in Figure 6C showed networks of expressed Imp-GFP- and Imp^{ΔTm}-GFP-labeled microfilaments while the expression of a single-chain variable fragment fused with GFP as a negative control was accumulated in the cytoplasm. The monitoring of these plants from germination till flowering was compared with 'Ca. P. mali'-infected *N. benthamiana*. The Imp-expressing plants did not exhibit any remarkable change in phenotype or symptoms typical for phytoplasma infection (Fig. 5D), thus indicating that binding of Imp to plant actin did not affect plant development.

DISCUSSION

Imp was proposed to be an ancestor of phytoplasma membrane proteins. However, the biological meaning of this pro-

tein was not known. Although Amp of OY phytoplasma was shown to bind to microfilaments of transmitting insects, its function in plants has not yet been described. Because phytoplasmas propagate in both insect and plant hosts, the study of the function of phytoplasma proteins expressed in plants is required. The result of transient expression of Imp-GFP and Imp^{ΔTm}-GFP in *N. benthamiana* revealed that Imp and Imp^{ΔTm} localized along with the actin filament, which was confirmed by co-expression with actin-decorated protein, mTalin-DsRed2. These results indicated that the actin-binding domain of Imp was located at the C terminus of the protein behind the transmembrane region. This finding supports the result of protein prediction that the C-terminal part of Imp is located outside of the phytoplasma membrane and, thus, is supposed to bind to host (plant) proteins.

The reaction of the plant to phytoplasma infection often involves growth deformations such as stunting, proliferation, or leaf distortion, in which cytoskeleton remodeling might be involved. Recently, it was shown that the transient expression of tobacco WLIM1 (an actin-binding protein [ABP] with an LIM domain related to animal Cys-rich proteins) in *N. benthamiana* plants induced a dramatic decrease of the overall number of actin filaments or bundles and a concomitant thickening of actin bundles (Thomas et al. 2006). However this phenomenon was not observed in *N. benthamiana* plants expressing Imp and Imp^{ΔTm} suggesting that the actin-binding of Imp did not induce actin cytoskeleton remodeling.

ABP are essential players in cytoskeleton dynamics by acting in cooperation to define the spatial separation and organization of filament branching, elongation, and nucleation, as well as actin assembly and disassembly processes (dos Remedios et al. 2003). This prompted us to further closely investigate the actin-binding function of Imp. The binding ability of Imp^{ΔTm}-His to G-actin (result of ELISA) and F-actin (result of co-sediment assay) confirmed the result of the transient expression of Imp- and Imp^{ΔTm}-GFP that Imp does, indeed, bind to actin filaments. Because binding of Imp^{ΔTm}-His to G-actin (in ELISA) did not inhibit the F-actin polymerization (in co-sedimentation assay), it is suggested that the actin-binding activity of Imp is a specific feature necessary for phytoplasma survival and is not involved in phytoplasma pathogenesis in plants. In plant cells,

Fig. 3. Imp^{ΔTm} bound directly to F-actin in co-sedimentation assay. Imp^{ΔTm} (1 μg) or a negative control (bovine serum albumin [BSA], 1 μg) were incubated with F-actin (2 μg). Mixtures were centrifuged at 15,000 rpm (low-speed co-sedimentation) for 1 h. Subsequently, pellets were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Coomassie Brilliant blue G staining. The presence of Imp^{ΔTm}-His in the actin pellet after centrifugation indicated its binding to the actin while a negative control (BSA) did not co-sediment with F-actin. Lanes 1 is F-actin alone, lane 2 is the mixture of Imp^{ΔTm}-His and F-actin, and lane 3 is the mixture of BSA and F-actin.

Fig. 4. Imp^{ΔTm} did not inhibit F-actin formation. Increasing concentrations of Imp^{ΔTm}-His (0.5 to 1 μg) or a negative control (bovine serum albumin [BSA], 0.5 to 1 μg) were incubated with G-actin (0.5 μg) prior to induce actin polymerization. After inducing polymerization, the reactions were centrifuged at 15,000 rpm and the pellets were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Coomassie Brilliant blue G staining. Lane 1 is polymerized actin as a negative control; lanes 2, 3, and 4 are 0.5, 0.75, and 1 μg, respectively, of Imp^{ΔTm}-His; and lanes 5, 6, and 7 are 0.5, 0.75, and 1 μg, respectively, of BSA.

the formation of higher-order actin structures is crucial to stabilize the organization of transvacuolar strands and maintain the overall cellular architecture (Shimmen et al. 1995). Therefore, inhibiting actin polymerization can change cellular morphology, inhibit cellular processes such as cell division, and even cause cells to undergo apoptosis (Haidle and Myers 2004). Moreover, cellular disruption of the equilibrium between actin monomers and actin filaments by overexpression of ABP or using actin cytoskeleton drugs (e.g., latrunculin or phalloidin) induces morphological and cytoarchitectural defects in plant cells (Gibbon et al. 1999; Staiger et al. 1994; Szymanski et al. 1999; Vidali et al. 2001). The result of transgenic plants expressing Imp- and Imp^{ΔTm}-GFP showed that expressed Imp- and Imp^{ΔTm}-GFP bound to actin filament but did not induce phytoplasma-like symptoms or defections of the plant phenotype. This result clearly indicated that the actin-binding activity of Imp neither interfered with actin polymerization nor disrupted the equilibrium between actin monomers and actin filaments. Moreover, possible detrimental effects of Imp on other plant proteins which could lead to phytoplasma-like symptoms could be excluded.

Although Amp was shown to bind to insect actin (Galetto et al. 2011; Suzuki et al. 2006), this binding was not reported to exhibit any negative effect on the life cycle of the host insect. Therefore, the results of our study support the hypothesis that binding of immunodominant protein to host actins could be a benefit for phytoplasma survival (probably for colonization, infection, and transmission) rather than the cause of its pathogenicity (Bertaccini and Duduk 2009).

Fig. 6. Transgenic plants expressing Imp- and Imp^{ΔTm}-green fluorescent protein (GFP). **A**, A leaf of transgenic plants expressing Imp- (**A**) and Imp^{ΔTm}-GFP (**B**) from two independent transgenic lines was extracted and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by Western blotting. The presence of Imp- and Imp^{ΔTm}-GFP was detected by using monoclonal anti-GFP-POD. Wt = wild type *Nicotiana benthamiana* as a negative control. Lanes 1 and 2 = transgenic *N. benthamiana* plant lines 1 and 2, respectively. **B**, Protoplasts were prepared from transgenic plants expressing Imp- and Imp^{ΔTm}-GFP and visualized under fluorescence microscopy. **C**, The protoplast prepared from a transgenic plant expressing a single-chain variable fragment (scFv) fused with GFP (cytoplasmic expression) (K. Boonrod, unpublished data) was used as a negative control.

Fig. 5. Establishment of phytoplasma-infected *Nicotiana benthamiana* plants by grafting and transgenic *N. benthamiana* plants Imp- and Imp^{ΔTm}-green fluorescent protein (GFP). Shoots of phytoplasma-infected *N. occidentalis* were grafted on wild-type (Wt) healthy *N. benthamiana*. The successfully infected plants were analyzed by polymerase chain reaction as described. Phytoplasma-infected *N. benthamiana* plants exhibited phytoplasma typical symptoms: **A**, proliferation; **B**, chlorosis; and **C**, lack of seed. **D**, T1 generation of transgenic *N. benthamiana* plants expressing Imp- and Imp^{ΔTm}-GFP.

Some intracellular bacteria stimulate actin to drive the rocketing motility by its polymerization (Borisy and Svitkina 2000; Haglund and Welch 2011). In plants, inhibiting actin polymerization by treatment with either actin polymerization inhibitors or actin silencing (Liu et al. 2005) reduces both the movement protein (MP) virus particle trafficking of *Tobacco mosaic virus* (Sambade et al. 2008) and the efficiency by which MP accumulates in plasmodesmata (Kawakami et al. 2004; Wright et al. 2007). Proteomic analysis of phloem exudates of different plant species showed that levels of actin were found within the phloem sap of all species tested. Interestingly, the actin content in the phloem sap of *Ricinus* spp. hypocotyls was much higher than in a total tissue extract (Schobert et al. 1998). Moreover, a high amount of profilin, a potent regulator of actin polymerization, was found in the assimilate stream along with actin. It was suggested that this high profilin content prevented microfilament formation within the assimilate stream by producing monomeric G-actin. Therefore, plants may use the phloem system to deliver this key cytoskeleton protein to sink tissue (Schobert et al. 2000). Chaffey and Barlow (2002) reported that actin microfilaments were found alongside the cell plate and postulated that an actin-myosin contractile system is present at cell plates and sieve pores. We showed that Imp of '*Ca. P. mali*' binds to both G- and F-actin. Because phytoplasmas lack genes coding for movement, this feature could indicate a role of actin binding for the phytoplasma transport within the sieve elements and through the sieve plates. The binding of IDP to actin could support the movement of the phytoplasmas in the phloem and, thus, in the colonization of the plant host.

MATERIALS AND METHODS

Nucleic acid extraction and PCR.

Total nucleic acids from plant tissue (*Malus* and *Nicotiana* spp.) were extracted with a modified cetyltrimethylammonium bromide-based protocol as described by Jarausch and associates (2011). '*Ca. P. mali*' was detected by PCR with standard procedures as referenced in Jarausch and associates (2011). The *imp* gene of '*Ca. P. mali*' PM19, a strain recently transmitted from field-collected *Cacopsylla picta* to healthy test plants of *Malus × domestica* (Jarausch et al. 2011), was amplified from the total DNA extract with primers f318A/r318B as reported by Berg and associates (1999). PCR products were cloned into the pGEMT-easy vector according to the manufacturer's instructions (Promega, Madison, WI, U.S.A.), resulting in pGEMT-Imp.

Plasmid constructs.

NcoI restriction sites were introduced at the 5' and 3' terminus of the Imp gene via PCR using primers 5'-GATAGCCAT GGAAGCAAATC-3' and 5'-CCCATGGGTTTCAAATC-3' and pGEM-T-Imp as a template. For Imp^{ΔTm}, the first 96 nucleotides (code for first 32 amino acid residues) of Imp gene were deleted via PCR using primer 5'-GATAGCCATGGG TTCAA AAAG-3' (Supplementary Fig. 1). The purified PCR products were digested and cloned upstream fused to the GFP gene in pPZP200:GFP binary vector (Hajdukiewicz et al. 1994) for plant transformation, resulting in pPZP200:Imp-GFP and pPZP200:Imp^{ΔTm}-GFP. In order to co-express Imp with an actin-binding mTalin protein (Kost et al. 1998), the whole expression cassette of a kanamycin-resistant gene (NPTII) at the left border of the pPZP200:Imp-GFP and pPZP200:Imp^{ΔTm}-GFP plasmids was removed by digestion with *SmaI* and *KpnI* restriction enzymes. The expressed cassette of mTalin-DsRED2 (35S:Promoter-mTalin-DsRED2:Nos-terminator) was amplified from pCambia1390:mTalin-DsRed2 (kindly provided by E.

Blancaflor, The Samuel Roberts Noble Foundation) by a PCR, and *SmaI* and *KpnI* restriction sites were introduced at the 5' and 3' terminus. The PCR products were digested and then subcloned into the left border of pPZP200:Imp-GFP and pPZP200:Imp^{ΔTm}-GFP, resulting in pPZP200:Imp-GFP/mTalin-DsRED2 and pPZP200:Imp^{ΔTm}-GFP/mTalin-DsRED2.

Plant transformation.

The plasmid was transformed into *Agrobacterium tumefaciens* ATHV by electroporation. The bacterial cell suspension was used for leaf disc transformation of *N. benthamiana* plants. Transgenic *N. benthamiana* plants were generated and maintained as described by Horsch and associates (1985).

Transient protein expression in planta and protoplast preparation.

Agroinfiltration was performed as previously described (Schöb et al. 1997). Briefly, a single colony of transformed agrobacteria was grown at 28°C. Bacteria were centrifuged and the pellet was resuspended in induction media and grown overnight at 28°C. The pellet of this overnight culture was resuspended in infiltration media up to an optical density at 600 nm (OD₆₀₀) of 2.4 and was incubated at room temperature for at least 2 h. The bacterial suspension was then infiltrated into leaves of each *N. benthamiana* plant using a 1-ml syringe. The infiltrated plants were incubated in the greenhouse for 2 days. The infiltrated leaves were collected 2 days after *Agrobacterium*-mediated infiltration. Two infiltrated leaves were cut in small strips (approximately 1 mm) and placed in a 100-ml flask containing 10 ml of 1% cellulase (Onozuka R-10; Serva) and 0.2% macerozyme (Onozuka R-10; Serva, Heidelberg, Germany) in Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 0.4 M sucrose at pH 5.8. The mixture was incubated at room temperature for 2 h on a gyratory shaker (20 rpm) to allow cell wall maceration. Intact protoplasts floating to the liquid surface were recovered with a Pasteur pipette and placed on a glass slide for fluorescence microscopy. Protoplasts were examined using a Zeiss Observer Z1 with LSM510 confocal laser-scanning head.

LatB treatment.

LatB (Adipogen, Hamburg, Germany) solution in dimethyl sulfoxide was freshly prepared and mixed with infiltration media to a final concentration of 10 mM. The mixture was syringe infiltrated into the abaxial leaf side.

Cloning of Imp^{ΔTm} gene in pET23a⁺.

The Imp^{ΔTm} gene was amplified from pPZP:Imp^{ΔTm}-GFP by introduction of a *BamHI* site at the 5' terminus and an *XhoI* site at the 3' terminus using primers 5'-GCGGATCCCCCTTT TCATCAAAAAGTAAACAAC-3' and 5'-GGTGCTCGAGTT TCAAATCTAAAACA-3'. The PCR products were directly cloned into a pPCR vector (M. Wassenegger *unpublished data*), resulting in a pPCR:*BamHI*-Imp^{ΔTm}-*XhoI* plasmid. In order to fuse Imp^{ΔTm} with hexahistidine (6XHis), they were subcloned into an expression vector, pET23a⁺ (Novagen, Madison, WI, U.S.A.) at *BamHI* and *XhoI* sites, resulting in a pET23a⁺:Imp^{ΔTm}-His construct.

Protein expression and purification.

E. coli BL21 (DE3) *codon plus* (+) was transformed with pET23a⁺:Imp^{ΔTm}-His. Cells were grown in LB media supplemented with carbenicillin at 50 mg/liter as antibiotics. At an OD₆₀₀ = 0.6, protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. After incubation at 37°C for 2 h or 14°C overnight, cells

were harvested and resuspended in BugBuster Protein Extraction Reagent (Novagen; 10 ml/g of cells) containing 5 µl of Benzamide (25 U/µl), 10 mM dithiothreitol (DTT), and one tablet of complete protease inhibitor (EDTA-free; Roche Applied Science, Indianapolis, IN, U.S.A.). The resuspended cells were incubated for 1 h at 4°C under agitation. The lysate was centrifuged at 9,000 × g for 10 min and the soluble fraction was purified by Ni-NTA agarose according to the manufacturer's protocol (Qiagen, Basel, Switzerland). The purified proteins were analyzed by SDS-PAGE followed by Coomassie Brilliant blue G (Sigma-Aldrich, St. Louis) staining or Western blot analysis.

In vitro actin binding assay by ELISA.

Actin (rabbit skeletal muscle, 2 mg/ml; cytoskeleton) was prepared in low-salt buffer (5 mM Tris-HCl [pH 8.0], 0.2 mM CaCl₂, 0.2 mM ATP, and 0.5 mM DTT) and 1 or 2 µg of the actin solution was coated on a microtiter plate at 4°C overnight. Unspecific binding was blocked with 3% BSA in phosphate-buffered saline buffer for 1 h. Purified Imp^{ΔTm}-His (1 µg) was added and further incubated at room temperature for 1 h. The plate was then washed and 100 µl of primary anti-His antibodies (Qiagen) and anti-mouse conjugated with alkaline phosphatase (Sigma-Aldrich) were added. The protein binding was detected by adding freshly prepared substrate, p-nitrophenyl phosphate (1 mg/ml), and measuring the developed signal in a plate reader (Multiscan Ascent) at 405 nm.

Co-sedimentation assays.

Rabbit muscle (cytoskeleton) was diluted at 0.05 µg/µl in a general actin buffer (5 mM Tris-HCl [pH 8.0] and 0.2 mM CaCl₂). Polymerization was induced for 1 h at room temperature by the addition of actin polymerization inducer (50 mM KCl, 2 mM MgCl₂, and 1 mM ATP). Purified recombinant Imp^{ΔTm}-His protein or BSA was added to preassembled F-Actin and the mixture was incubated at 4°C for 1 h. Proteins were pelleted at 15,000 rpm for 1 h. The pellets were analyzed by a SDS-PAGE followed by Coomassie Brilliant blue G (Sigma-Aldrich) staining.

To determine the inhibiting effect of Imp to polymerization of actin, increasing amounts of Imp were added to the polymerization reaction of actin. The reaction mixture was incubated at room temperature for 1 h and centrifuged at 15,000 rpm for 1 h at 4°C. The pellets were analyzed by SDS-PAGE followed by Coomassie Brilliant blue staining.

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