Arabidopsis tonoplast proteins TIP1 and TIP2 interact with the cucumber mosaic virus 1a replication protein

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The cucumber mosaic virus (CMV) replication complex has previously been shown to associate with cellular membranes. However, it remains unknown whether any host factors participate in this process. In this study, five groups of Arabidopsis tonoplast intrinsic protein (TIP) genes were isolated and the proteins they encoded were evaluated with regard to their interactions with CMV proteins. TIP1 and TIP2 were found to interact with the CMV 1a protein in the Sos recruitment system, whereas no interactions with the other three TIP subgroups were observed in this assay. The interaction of CMV 1a with the TIP1 and TIP2 proteins was confirmed via co-immunoprecipitation assays. Additionally, CMV 1a co-localized with TIP1 and TIP2 in transfected Arabidopsis protoplasts. The findings of this study suggest that members of two TIP subfamilies might affect CMV replication via interaction with CMV 1a in the tonoplasts.

Eukaryotic positive-strand RNA virus replication is known to occur in close association with intracellular membranes. Thus far, no exceptions to this phenomenon have been observed. This clearly indicates the pivotal role of membranes in the formation and operation of viral RNA replication complexes. Direct evidence for the importance of membranes in the formation and operation of viral RNA replication has been obtained in several cases (Barton & Flanagan, 1993; Molla et al., 1999; Schlegel et al., 1998). Despite these expectations, specific information regarding such host factors is currently limited to only a few examples.

Cucumber mosaic virus (CMV) replication occurs in association with the vacuolar membrane, also known as the tonoplast (Cillo et al., 2002). Protein 1a, encoded by RNA 1, has a putative methyltransferase domain and helicase domain, and has been recognized as a component of the isolated CMV replicase (Hayes & Buck, 1990). It co-localizes to the tonoplast with protein 2a which harbours an RNA-dependent RNA polymerase motif (Cillo et al., 2002).

The tobamovirus multiplication 1 (TOM1) protein and its homologues TOM3 and TOM2A are required for the efficient replication of Tobacco mosaic virus (TMV), but not for the multiplication of either CMV or Turnip crinkle virus in Arabidopsis. They are transmembrane proteins localized in the tonoplast, which interact with TMV virus replication factors, as well as with each other (Tsujimoto et al., 2003; Yamanaka et al., 2002). We decided to search for vacuolar transmembrane proteins interacting with CMV proteins which might be good candidates for the formation of the membrane-associated CMV replication complex.

Tonoplast intrinsic proteins (TIPs) are integral membrane proteins (Johanson et al., 2001) and represent a distinct group within the family of ubiquitous intracellular membrane proteins (Maurel, 1997). In Arabidopsis, the TIP family is divided into five distinct subfamilies, consisting of 10 proteins: TIP1;1, TIP1;2, TIP1;3, TIP2;1, TIP2;2, TIP2;3, TIP3;1, TIP3;2, TIP4;1 and TIP5;1 (Johanson et al., 2001). TIPs have been suggested to function as aquaporins, which

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Figures showing interaction analyses of Arabidopsis TIPs and CMV or TMV proteins using a yeast two-hybrid system, and the results of liquid assays for quantification of β-galactosidase activity are available as supplementary material in JGV Online.
regulate water transport in a variety of vacuolar activities (Chrispeels & Maurel, 1994; Maurel, 1997).

In this study, all 10 TIP genes were isolated from Arabidopsis by using PCR with TIP-specific primer sets, and the TIPs were tested with regard to their interaction with the CMV 1a protein using the yeast two-hybrid system (YTHS), the Sos-recruitment system (SRS) and co-immunoprecipitation assays. CDNA encoding CMV 1a was fused with the GAL4 activation domain in pGADT7 and different classes of cDNAs encoding TIPs were fused with the GAL4 DNA-binding domain in pGBK7T. The two fusion vectors were co-transformed into the Y187 yeast strain, and then subjected to colony-lift filter assays using X-Gal as a substrate. TIP1;1, TIP1;2, TIP1;3 (TIP1 subfamily) and TIP2;1, TIP2;2, TIP2;3 (TIP2 subfamily) interacted strongly with CMV 1a, but TIP3;1, TIP3;2, TIP4;1 and TIP5;1 barely interacted with the CMV 1a protein (see Fig. S1a, available as supplementary material in JGV Online). Additionally, these results were confirmed via quantitative liquid assays of β-galactosidase activity using a very sensitive luminescence-based detection system (see Fig. S2, available as supplementary material in JGV Online). The β-galactosidase activities of the TIP1 and TIP2 subfamilies were 23–35% of the positive control (pVA3-1/pTD1-1). TIP3;1, TIP3;2, TIP4;1 and TIP5;1 showed much lower activity than the TIP1 and TIP2 groups, but slightly above the negative control background level (unfused pGADT7/pGBK7T vectors).

Whereas the YTHS is currently the most powerful method for the study of protein–protein interactions, the analysis of interactions occurring between membrane proteins has remained a significant challenge primarily due to the hydrophobic nature of membrane proteins, as well as the fact that integral and membrane-associated proteins often undergo post-translational modifications or oligomerization via interactions between transmembrane domains. The SRS overcomes several of the limitations of the YTHS and thus serves as an attractive alternative for studying protein–protein interactions between known and novel proteins (Thaminy et al., 2003). To rule out any misinterpretation, we attempted to reconfirm the interaction between TIPs and CMV 1a further via an SRS (Clontech). This system employs the temperature-sensitive cdc25H mutant strain of Saccharomyces cerevisiae, which can grow at the permissive temperature (25°C) but not at the non-permissive temperature (37°C). The SRS is based on the ability of the human Sos (hSos) gene to complement the cdc25 (yeast homologue of hSos) defect, thereby activating the yeast Ras-signalling pathway. The expression of hSos and the subsequent localization of its product to the plasma membrane enables the cdc25H yeast strain to grow at 37°C. The localization of hSos to the plasma membrane occurs via interactions between two hybrid proteins. The CMV 1a cDNA clone was fused to sequences encoding the C terminus of hSos at the BamHI site in the pSos vector, and each of the TIP genes was cloned into the pMyc vector harbouring the myristylation signal. As is shown in Fig. 1(a), the co-expression of CMV 1a and all TIP1 and TIP2 subfamily members, but no other TIPs, suppressed the temperature sensitivity. Neither CMV 1a alone nor each TIP alone was able to suppress the temperature sensitivity. None of the TIPs interacted with other CMV-encoded proteins, that is 2a (Fig. 1b), 2b, MP or CP (data not shown). Two TIPs, TIP1;2 and TIP 2;1, were selected for further study as they showed the highest β-galactosidase activity level in each subfamily and exhibited strong positive interaction in the SRS. Additionally TIP1;2 and TIP 2;1 are known to have the highest basal gene expression level in groups 1 and 2, respectively (Alexandersson et al., 2005).

To confirm the interaction of TIPs and CMV 1a in vitro, we utilized the Binding Domain Matchmaker Co-IP kit (Clontech). The TIP1;2::c-Myc, TIP2;1::c-Myc, TIP3;1::c-Myc and CMV 1a::HA expression plasmids (pGBK7T/TIP1;2, pGBK7T/TIP2;1, pGBK7T/TIP3;1 and pGADT7/CMV 1a, respectively) were used as templates for in vitro translation with an amino acid mixture (TNT T7 Coupled ReticuloCyte Lysate System; Promega). As TIPs are membrane proteins, after 1 h incubation at 30°C and centrifugation at 10 000 g, the pellet fraction harbouring the membrane proteins was dissolved in PBS buffer (2–7 mM KCl, 4–3 mM Na2HPO4, 1–8 mM KH2PO4, 137 mM NaCl, pH 7–2) containing 1% Triton X-100 and cleared via centrifugation (Kleizen et al., 2005). Ten microlitres of CMV 1a::HA in vitro translation products were mixed with 10 μl in vitro translation products harbouring TIP1;2::c-Myc, TIP2;1::c-Myc or TIP3;1::c-Myc. These mixtures were precipitated with anti-HA polyclonal antibody and then the protein complexes were bound with Protein A beads. The bead-bound proteins were then eluted and resolved via SDS-PAGE, and transferred to nitrocellulose membranes. The in vitro interaction of TIPs and CMV 1a was visualized via immunoblotting, in which development was achieved with anti-HA polyclonal antibody (Fig. 2b, lanes 5–7). This result suggested that TIP1;2 and TIP 2;1, specifically interacted with CMV 1a in vitro. All reactions were performed at least twice with consistent results.

TIP isoforms have been used as marker proteins for different types of vacuoles (Jaul et al., 1998, 1999; Jiang et al., 2000; Moriyasu et al., 2003). To verify that TIP1;2 and TIP2;1 reside on the tonoplast, the entire TIP1;2 or TIP2;1 cDNA
Fig. 1. Interaction analysis between TIPs and CMV 1a (a) or CMV 2a (b) using an SRS. *cdc25H* yeast strains harbouring plasmids designed to express the indicated proteins constitutively were diluted in sterile water to an OD$_{600}$ of 1.0, 0.5, 0.05 or 0.005. Each of the dilutions (5 μl) was spotted onto SD-Leu-Ura plates and cultured at 25 °C for 52 h (left) or 37 °C for 66 h (right). pMyr MAFB and pSos MAFB were used as positive control (PC) and pMyr Lamin C and pSos MAFB were used as negative control (NC).
without the termination sequences encoding the codon was fused to the sequences encoding the C-terminal region of green fluorescent protein (GFP) in the smGFP gene and the resulting constructs were expressed transiently in onion cells via biolistic gene bombardment and labelled with FM4-64 (Molecular Probes). The vital membrane-staining dye FM4-64, a known endocytic tracer, can be used to visualize vacuolar membranes in yeast (Vida & Emr, 1995) and plant cells (Ueda et al., 2001). Onion cells that transiently overproduced TIP1;2 or TIP2;1 (12 h incubation after bombardment) were incubated with 30 μM FM4-64 for 5 min in water, washed with fresh water and incubated for 10 h. FM4-64 and GFP fluorescence in the cells were analysed using an Axiovert fluorescence microscope.

As is shown in Fig. 3(a), the GFP fluorescence of TIP1;2- or TIP2;1-tagged constructs in onion cells was observed in the outer vacuolar membrane and ring structures. Actually, the intensity of the fluorescence being emitted from these ring structures appeared to be far greater than that of the outer vacuolar membrane. These staining patterns were similar to those observed for TIP1;1 (At2g36830; c-TIP1), and the ring structures were referred to as 'bulbs' (Saito et al., 2002). Approximately 10 h after labelling, the fluorescence of FM4-64, transported via endocytosis to the tonoplasts, overlapped perfectly with the GFP fluorescence in the cells expressing TIP1;2::GFP and TIP2;1::GFP. These observations showed that TIP1;2::GFP and TIP2;1::GFP were both primarily localized to the tonoplasts.

In vivo TIP1;2::GFP and TIP2;1::GFP targeting was used to investigate the possibility that CMV 1a is co-localized subcellularly with TIP1;2 or TIP2;1 in protoplasts of Arabidopsis (Fig. 3b). Also, a red fluorescent protein (RFP)-tagged CMV 1a (CMV 1a::RFP) as well as TIP1;2::GFP and TIP2;1::GFP were introduced into the Arabidopsis protoplasts prepared from whole seedlings by the polyethylene-glycol-mediated transformation procedure (Jin et al., 2001). When only CMV 1a::RFP was introduced into the Arabidopsis protoplasts, CMV 1a was detected on the tonoplast, similarly to the bulb structures after 72 h incubation (Fig. 3c). When the plasmids encoding CMV 1a::RFP and TIP1;2::GFP, or CMV 1a::RFP and TIP2;1::GFP were co-transformed into the Arabidopsis protoplasts, CMV 1a was shown to overlap completely with TIP1;2 or TIP2;1 in the tonoplasts (Fig. 3d). Thus, it appears probable that CMV 1a forms a complex with TIP1;2 and/or TIP2;1 in the tonoplasts of plant cells.

The TOM2A protein has been shown to interact with itself and with TOM1, suggesting that, like TOM1, TOM2A is a constituent of the tobamovirus replication complex. TOM2A may facilitate the binding of tobamovirus replication proteins to TOM1 on the membranes, or may exert crucial effects in the formation or maintenance of the replication complex. Alternatively, TOM2A may serve to recruit other host factors required for tobamovirus replication (Tsujimoto et al., 2003). Similarly to the tobamoviruses, docking to membranes appears to be critical for the

**Fig. 2. In vitro interaction test of TIPs and CMV 1a proteins.** Equal amounts of in vitro-translated TIP3;1::c-Myc, TIP1;2::c-Myc and TIP2;1::c-Myc were incubated with in vitro-translated CMV 1a::HA, the products from each of the binding reactions were precipitated with anti-HA polyclonal antibody and then these protein complexes were precipitated with Protein A beads. The bead-bound proteins were eluted and resolved via SDS-PAGE. TIPs and CMV 1a were visualized via immunoblotting with anti-c-Myc antibody (a) or anti-HA antibody (b). SV40 large T-antigen::HA and murine p53::c-Myc were used as positive controls (PC).
formation of active CMV replication complexes. In this study, the group 1 and 2 TIPs were identified as potential interactors with CMV 1a, both in vitro and in yeast cells. TIP1;2 or TIP2;1 co-localized with CMV 1a in the tonoplast of Arabidopsis protoplasts. To check whether the interaction is specific to CMV, we also evaluated the interaction between TIPs and TMV replicase or the TMV helicase domain. However, none of the TIPs interacted in a YTHS with the helicase or replicase domains of the TMV 126 and 180 kDa proteins (see Fig. S1b, available as supplementary material in JGV Online). These results indicate that TIP1 and/or TIP2 proteins participate specifically in the formation of the CMV replication complex by anchoring the complex to the tonoplast.

When the ORFs of the TIPs were analysed, the TIP1 and TIP2 subfamilies harbour no apparent domains or sequences which would provide a clue to their interaction with CMV 1a (PSORT; Nakai & Kanehisa, 1992). However, other subfamilies of TIPs harbour additional 8–12 aa

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Fig. 3. Subcellular localization of transiently expressed TIP::GFP and CMV 1a::RFP fusion proteins in plant cells and protoplasts. (a) TIP1;2::GFP and TIP2;1::GFP were expressed in onion cells via biolistic gene bombardment, and the cells were stained with FM4-64 to visualize vacuolar structures. The fusion proteins appeared to be localized in vacuolar membranes (v, arrows) and in ring-like structures resembling the bulbs described by Saito et al. (2002) (b, arrowheads). The right panels show higher magnifications of the boxed regions. (b) GFP and TIP::GFP fusion proteins were expressed in Arabidopsis protoplasts by polyethylene glycol (PEG)-mediated transfection (Jin et al., 2001). Panels in column 1 show the bright-field image. Panels in column 2 show the GFP signal; vacuolar membranes (v) and ring-like structures (bulbs, b) are indicated. Panels in column 3 show the focus-changed image, revealing the ring-like nature of bulbs. (c) RFP and CMV 1a::RFP fusion protein were expressed in Arabidopsis protoplasts by PEG-mediated transfection. Panels in column 1 show the bright-field image. Panels in column 2 show the RFP signal. Ring-like structures (bulbs) are indicated by arrowheads. The panel in column 3 shows a higher magnification of the boxed region. (d) Co-expression of CMV 1a::RFP and TIP::GFP fusion proteins in Arabidopsis protoplasts by PEG-mediated transfection. Column 1, bright-field image; column 2, GFP signal; column 3, RFP signal; column 4, merged GFP and RFP signals. Ring-like structures (bulbs) are indicated by arrowheads.
sequences, composed of polar and charged amino acids at the C-terminal cytoplasmic region. It remains possible that these extra polar and charged amino acid residues might be involved in intrachain interactions with TIPs, which would lead to a different conformation and the consequent blockage of CMV 1a binding. However, even though CMV 1a did not show any interaction with TIP3, 4 or 5 in the SRS or in the in vitro system, there was a low level of interaction above background in a liquid YTHS (see Fig. S2, available as supplementary material in JGV Online). Thus, we cannot completely rule out the possibility that TIP3;1, TIP3;2, TIP4;1 and TIP5;1 may also interact (albeit weakly) with the CMV 1a protein.

CMV 1a and TIP1/2 fusion proteins co-localize in vacuolar structures that resemble the bulbs described by Saito et al. (2002). Co-localization of CMV 1a and TIP proteins in the regular vacuolar membrane is not clearly seen in Fig. 3, but the localization of these proteins in the tonoplast has been demonstrated by Cillo et al. (2002). Bulbs were suggested as an early form of vacuoles which can serve as a reservoir of membranes to prepare for the quick expansion of vacuoles during development, and in response to environmental change. Since the localization of CMV 1a to the membrane is an early event in CMV replication, the co-localization of CMV 1a and TIP1/2 proteins in bulbs would be significant in virus replication.

A variety of host factors have been implicated in the virus replication process (Buck, 1996), and the data presented in this study suggest that one of these host factors, namely the TIPs, might be a candidate for the membrane-associated formation of the CMV replication complex. Additionally, in the case of CMV, since the 1a protein has also been shown to affect viral movement, it is possible that TIP proteins have some role in promoting the movement of RNA from the site of synthesis on tonoplasts rather than having any direct role in replication.

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