Identification of a 37 kDa plant protein that interacts with the turnip mosaic potyvirus capsid protein using anti-idiotypic-antibodies

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Abstract

Experimental data are provided for the presence of a plant protein that interacts with the capsid protein (CP) of turnip mosaic potyvirus (TuMV). The receptor-like protein was identified by exploiting the molecular mimicry potential of anti-idiotypic antibodies. A single-chain Fv molecule derived from the monoclonal antibody 7A (Mab-7A), which recognizes the CP of TuMV, was produced in *Escherichia coli* and the recombinant protein was used to raise rabbit antibodies. The immune serum reacted with Mab-7A but not with a monoclonal antibody of the same isotype, indicating that anti-idiotypic antibodies were produced. These anti-idiotypic antibodies recognized a 37 kDa protein from *Lactuca sativa*. Complex formation between the anti-idiotypic antibodies and the plant protein was inhibited by the CP of TuMV which indicates that the plant protein interacts with the viral protein. The 37 kDa protein was localized in chloroplasts and was detected in other plant species.

Introduction

The infection of susceptible plant hosts by RNA viruses depends on the association of host and viral factors for the replication of the viral genome, the expression of viral genes and the movement of virus throughout the plant [44]. Consequently, plant and viral protein-protein interactions are presumed to play a pivotal role in disease development. One viral protein believed to interact with host components is the capsid protein (CP). The CP has a structural role but is also necessary for virion translocation in the plant and aphid transmissibility [2, 9, 25, 37, 41]. Furthermore, in the case of alfalfa mosaic virus (AIMV), the CP participates in viral RNA replication [30]. In addition, the CP has been reported to be the interface in the defense response in *Nicotiana sylvestris* carrying the *N'* resistance gene against tobacco mosaic virus (TMV) [6] and in *Solanum tuberosum* carrying the *Rx* resist-

ance gene against potato virus X [16, 33]. The plant proteins involved in the interaction with the CP are thought to be early components of a signal transduction pathway similar to that of the TMV resistance *N* gene of *Nicotiana tabacum* [42]. However, the identity of the plant components interacting with CPs has not been established. Protein-protein interactions, such as receptor-ligand complexes, have been studied using the idiotypic-anti-idiotypic network approach [23]. This approach involves the preparation of an anti-ligand monoclonal antibody (Mab) which is subsequently used as immunogen to obtain anti-anti-ligand or anti-idiotypic antibodies (anti-ids). If the anti-ligand antibody and the receptor protein interact with the same region of the ligand, a sub-population of the anti-ids could potentially mimic the ligand epitope and bind to the receptor protein. Anti-ids have been used to identify cell surface receptors for hormones and animal viruses [16, 31] and in vaccination [27]. Likewise, Akimitsu *et al.* [1] used anti-ids for the detection of the plant cell surface receptor for victorin. The anti-idiotypic

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approach was used in this study to identify plant proteins interacting with the CP of turnip mosaic potyvirus (TuMV). The CP of potyviruses is a three-domain protein with the N- and C-terminal ends exposed on the surface of the virion and the central domain forming the core subunit structure [39]. The highly conserved central region is needed for encapsidation of the viral RNA while the highly variable, surface-exposed, N-terminal and C-terminal regions are involved in host-vector-virus interactions and/or transport functions [2, 9, 25]. Monoclonal antibodies (Mab) have previously been raised against the 33 kDa CP of TuMV [21]. Here, an antibody derivative of Mab TuMV-7A was synthesized. This derivative, known as a single-chain Fv (sc-Fv) molecule [24], consisted of the heavy- and light-chain variable domains of the parent immunoglobulin joined together by a flexible peptide linker and was expressed in *Escherichia coli*. A polyclonal serum was raised against the recombinant protein which also reacted with a 37 kDa protein found in chloroplasts of *Lactuca sativa*. The interaction between the anti-scFv-7A serum and the chloroplast protein was inhibited by the presence of TuMV CP. These characteristics suggest that the chloroplast protein may interact with the CP of TuMV.

Materials and methods

Bacterial strains and plasmids

E. coli strains XL1-Blue (Stratagene) and BL21(DE3) (Novagen) were used as bacterial hosts. The expression vector pET22b (Novagen) was used for recombinant protein expression in *E. coli*.

Construction and expression of the scFv-7a gene

The scFv derivative of Mab-7A was prepared essentially as described [5] with modifications: first-strand cDNA was prepared from 10 µg of total RNA using murine leukemia virus reverse transcriptase (BRL) and the constant heavy-chain-1 primer MOCG12FOR (5'-CTCAATTTTCTTGTCACCTTGGTGC-3') and constant κ chain primer CKFOR (5'-CTCATTCCTGTGAAGCTCTTGAC-3'). *Thermus aquaticus* DNA polymerase (Bio/Can Scientific) was used for amplification and reaction conditions were as recommended by the manufacturer. Polymerase chain reaction (PCR) amplifications of the V_L V_H coding regions were carried out independently. The cDNAs were amplified

for 30 cycles and each cycle consisted of a 1 min denaturation (94 °C), 1 min annealing (60 °C) and a 2 min extension (72 °C). Primers VH1FOR-2 (5'-TGAGGAGCAGGTGACCGTGGTCCCTTGGCCCC-3') and VH1BACK (5'-AGGTSMARCTGCAGSAGTCWGG-3'; where S = C or G; M = A or G; W = A or T) were used to amplify the V_H region. Primers VK2BACK (5'-GACATTGAGCTCACCCAGTCTCCA-3') and VK4FOR (consisting of an equimolar mix of MJK1FONX, 5'-CCGTTTGATTTCAGCTTGGTGCC-3'; MJK2FONX, 5'-CCGTTTATTTCCAGCTTGGTCCC-3'; MJK4FONX, 5'-CCGTTTATTTCCAACCTTTGTC-CC-3'; MJK5FONX, 5'-CCGTTTCAGCTCCAGCTTGGTCCC-3') were used to amplify the V_K region. The linker DNA was similarly amplified from pscFvD1.3 using primers MO-LINK-BACK and MO-LINK-FOR (complementary to VH1FOR-2 and VK2BACK, respectively). Gel-purified V_H and V_K amplified fragments (100 ng each) were mixed with 20 ng of the linker DNA fragment encoding the peptide (Gly4Ser)₃ in a 50 µl reaction mixture without primers and amplified for seven cycles (94 °C for 1 min, 72 °C for 2.5 min) with Vent DNA polymerase (New England Biolabs) to randomly join the fragments, then amplified for 23 cycles (94 °C for 1 min, 60 °C for 2 min, and 72 °C for 2 min) using 25 pmol each of VH1BACK and VK4FOR, which contained *Nco*I and *Not*I restriction sites respectively. The *Nco*I/*Not*I scFv gene fragment was subcloned into identically restricted pET22b to create pETscFv-7A.

Recombinant protein expression, serum production and immunodetection

E. coli BL21(DE3), harboring pETscFv-7A, was grown in Luria Broth with ampicillin (100 µg/ml) at 37 °C. At $A_{600} = 0.5$, 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added and the cells were incubated for an additional 2 h at 22 °C after which they were harvested by centrifugation at $4000 \times g$. The cells were resuspended in 50 mM Tris-HCl pH 8.0, 1.0 mM EDTA, 100 mM NaCl and lysed with 2 mg/ml lysozyme and several freeze-thawing cycles. Inclusion bodies were purified according to Sambrook *et al.* [38].

Recombinant scFv-7A was purified by SDS-PAGE in a 12% acrylamide gel. After staining the gel with Coomassie brilliant blue R-250 in water, the band was excised and passed through a 21G hypodermic needle. Rabbits were immunized as described in

Robbins *et al.* [35]. Western blot analyses were performed as described [38] using polyvinylidene difluoride (PVDF) or nitrocellulose membranes.

ELISAs were performed at room temperature except where noted. Plates were washed six times with phosphate buffered saline (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.0) containing 0.1% Tween-20 (PBS-T) between each incubation. Proteins suspended in coating/extraction buffer (15 mM Na₂CO₃, 35 mM NaHCO₃ pH 9.6, 2% polyvinylpyrrolidone (PVP)) were bound to microtiter plates (Falcon) for 4 h and wells were then blocked in 3% BSA-PBS-T for 4 h. Plates were subsequently incubated 1 h with the rabbit serum and developed using anti-rabbit IgG conjugated with alkaline phosphatase or horse-radish peroxidase. For competitive ELISA, two-fold serial dilutions of *E. coli* lysate in conjugate buffer (PBS-T, 2% PVP, 0.2% BSA) were added and incubated overnight at 4 °C.

Cell fractionation

Pellet (P-100) and soluble (S-100) fractions were obtained by grinding 7 g of fresh lettuce leaves in 35 ml of ice-cold 50 mM Tris-HCl pH 8.0, 5.0 mM EDTA, 0.1% 2-mercaptoethanol for one minute in a blender. The extract was centrifuged at $20\,000 \times g$ for 15 min at 4 °C, and the resulting supernatant was further centrifuged at $100\,000 \times g$ for 45 min at 4 °C. The pellet (P-100) was resuspended in 1.0 ml 50 mM Tris-HCl pH 8.0, 5 mM EDTA. Chloroplasts were isolated from 50 g of young lettuce leaves according to the method of Palmer *et al.* [32]. The chloroplast suspension was adjusted to 60 mM Na₂CO₃ pH 10.0, 60 mM DTT and 2% SDS and insoluble material was removed by centrifugation at $14\,400 \times g$ for 30 min at 4 °C. Mitochondria were purified using the extraction protocol outlined by Hanson *et al.* [19]: two mitochondrial fractions were collected, the 1.6 M/2.0 M sucrose interface (M1) and the 1.2 M/1.6 M interface (M2).

Results

Production of antibodies against scFv-7A

In this study, a scFv molecule derived from the Mab-7A cell line [21] was engineered in order to obtain antibodies directed against the paratope of the monoclonal antibody. This strategy was chosen to minimize production of anti-isotypic and anti-allotypic antibodies.

The TuMV 7A monoclonal cell line was produced using purified TuMV nucleoprotein; for TuMV, the virion is composed of ca. 2000 copies of the CP, surrounding the RNA to which is attached one copy of the virus-encoded VPg protein [21]. The cDNA encoding the scFv molecule was obtained via RT-PCR using RNA extracted from the mouse hybridoma cell line; the amplified fragments coded for the variable domain of both the light and heavy chains. scFv molecules built using the RT-PCR approach used here have been shown to bind their respective antigen similar to that of their parent immunoglobulin molecules [22]. The assembled gene was sequenced and characterized as an IgG 2a-subclass immunoglobulin (data not shown). The Mab-7A had previously been identified as an IgG 2a by immunoanalysis using isotypic-specific antibodies (data not shown). The chimeric gene was cloned into expression vector pET22b for high-level expression in *E. coli*. A fusion protein of the predicted size (31 kDa) was found to accumulate in inclusion bodies as indicated by SDS-PAGE (Figure 1A). An anti-mouse Fab serum (ICN) reacted with the recombinant protein, confirming that an immunoglobulin-derived molecule had been synthesized (Figure 1B). The recombinant protein was purified and injected into rabbits to raise antibodies. As expected, the resulting serum recognized the scFv-7A protein (Figure 1C). Because the scFv-7A molecule is made up exclusively of the variable domains of the parent immunoglobulin, it was expected that anti-ids were predominantly produced. An ELISA was performed to test for their presence in the serum. The wells were coated either with Mab-7A or the control Mab-7-10A of unrelated specificity but of the same isotype (IgG 2a) [7] and incubated with various dilutions of the anti-scFv-7A serum and a non-immune serum. The immune serum reacted preferentially with Mab-7A (Figure 2, filled squares), which indicates that scFv-7A was indeed a derivative of Mab-7A and that anti-ids were mainly produced since the rabbit serum did not react with the isotypic control antibody.

Recognition of a plant protein by the anti-scFv-7A serum

Since anti-ids were produced against Mab-7A, it was hypothesized that a portion of them would mimic the CP epitope that was recognized by Mab-7A. If this CP epitope interacts with a plant protein, the anti-ids representing the internal image of the CP should also interact with the plant protein. This was verified by

Figure 1. Analyses of scFv-7A expressed in *E. coli* BL21(DE3). After SDS-PAGE in a 10% polyacrylamide gel, proteins were either stained with Coomassie blue (A, top) or transferred to nitrocellulose and incubated with an anti-Fab serum (B, middle) or the anti-scFv-7A serum (C, bottom). Lane 1, total protein from *E. coli* not harboring pET22b; lane 2, total protein from *E. coli* harboring pETscFv-7A before induction; lane 3, total protein from *E. coli* harboring pETscFv-7A after a 2 h induction; lane 4, inclusion bodies from *E. coli* harboring pETscFv-7A after a 2 h induction.

Figure 2. Presence of anti-idiotypic antibodies determined by ELISA. Wells were first coated with either Mab-7A (filled symbols) or Mab-7-10A (open symbols) and subsequently incubated with dilutions of the anti-scFv-7A serum (squares) or a non-immune serum (circles). Complex formation was revealed by incubation with a goat anti-rabbit serum conjugated with peroxidase. Abbreviations: Anti-7A, anti-scFv-7A serum; NRS, non-immune serum; 7A, Mab-7A; control, Mab-7-10A.

western blot analysis. Proteins from *Lactuca sativa* cv. Calmar, which is susceptible to infection by TuMV, were separated by SDS-PAGE, transferred to PVDF membrane and incubated (without renaturation) with the anti-scFv-7A serum. A protein of 37 kDa, which was not observed after incubation with the pre-immune serum (Figure 3, lane 1), reacted with the immune serum (Figure 3, lane 2). Incubation of the membrane with the anti-scFv-7A serum was also done in the presence of scFv-7A proteins in order to compete with the membrane-bound 37 kDa protein. That competition reduced the intensity of the band observed in the 37 kDa region of the gel (Figure 3, lane 3), indicating that the signal was the result of specific interaction of anti-scFv-7A antibodies with the 37 kDa plant protein. This set of experiments indicated that anti-scFv-7A antibodies specifically recognized a 37-kDa protein from *L. sativa*.

Competition between CP of TuMV and 37 kDa protein for binding anti-scFv-7A serum

Since the plant protein was recognized by an anti-anti-CP serum, the 37 kDa plant protein was thus speculated to interact with the TuMV CP. If this is the case, the viral protein (CP) must be able to compete with the anti-scFv-7A antibodies for the binding of the 37 kDa plant protein. The competition was first per-

Figure 3. Immunoblotting analysis of plant proteins with the anti-scFv-7A serum. *Lactuca sativa* leaves were homogenized and proteins were separated by SDS-PAGE in a 12% polyacrylamide gel and transferred onto PVDF membrane. The membrane was cut into strips which were incubated with: lane 1, the pre-immune serum; lane 2, the anti-scFv-7A serum; lane 3, the anti-scFv-7A serum containing an *E. coli* lysate expressing scFv-7A.

formed by immunoblotting: total plant proteins were separated by SDS-PAGE, transferred onto PVDF membrane and incubated with the anti-scFv-7A serum in the absence or presence of CP. As control, recombinant scFv-7A was also transferred. Although the signal for the scFv-7A-anti-scFv-7A complex decreased only slightly when CP was present during the incubation, the signal for the 37 kDa protein was only 40% of the original signal (Figure 4). To confirm the displacement seen by immunoblotting, competition experiments in ELISA plates were performed in which wells were coated with plant proteins and incubated with the anti-scFv-7A serum and with increasing quantities of *E. coli* lysate containing CP [35]. The CP lysate competed with anti-scFv-7A antibodies for binding the 37 kDa protein, showing an average 35% decrease in signal strength with the addition of lysate containing 1.5 ng of CP, compared to that generated with the negative control lysate containing an equal amount of protein. The displacement was also observed using CP purified from dissociated virus particles (results not shown). The lack of complete displacement could be explained by the higher affinity an immunoglobulin has for its ligand (anti-7a/37 kDa protein) than that found in a protein-protein (CP/37 kDa protein) complex, the complementary determining regions of the immunoglobulin playing an important role in the increased affinity [14]. These experiments suggest that the CP of TuMV is able to interact with the plant protein recognized by anti-ids directed against Mab-7a.

Figure 4. Competitive immunoblot analysis between CP and anti-scFv-7A serum. Total proteins from *Lactuca sativa* were separated by SDS-PAGE in a 12% polyacrylamide and transferred to PVDF membrane. The membrane was incubated with the anti-scFv-7A serum only (A) or with the anti-scFv-7A serum in the presence of an *E. coli* lysate expressing the CP of TuMV (B); lane 1, inclusion bodies from *E. coli* harboring pETscFv-7A after a 2 h induction; lane 2, *L. sativa* proteins. The signals were quantified from the digitized image using GPTools software (Biophotonics Corp.). Data represent percentage decrease of signal in the presence of CP (C, bottom).

Cellular localisation and prevalence of the 37 kDa protein

The cellular localization of the plant protein was determined. Leaves from *L. sativa* were homogenized and the cellular content was fractionated by centrifugation into soluble (S-100), membrane (P-100), mitochondria and chloroplast components. The proteins from each fraction were separated by SDS-PAGE and analyzed by immunoblotting. The anti-scFv-7A serum recognized the 37 kDa protein in the chloroplast fraction (Figure 5, lane 3).

The presence of this 37 kDa protein in chloroplasts of *Brassica perviridis*, *Nicotiana tabacum* and *Lycopersicon esculentum* was tested. Chloroplast proteins were separated by SDS-PAGE and analyzed by western blot using the anti-scFv-7A serum (Figure 6). In all plants tested, the serum reacted with a protein of

Figure 5. Cellular localisation of the 37 kDa protein by immunoblot analysis. *Lactuca sativa* leaves were homogenized and cellular content fractionated as described in Materials and methods. Proteins were separated by SDS-PAGE in a 12% polyacrylamide gel, transferred onto PVDF membrane and incubated with anti-scFv-7A serum. Lane 1, S-100 fraction; lane 2, P-100 fraction; lane 3, chloroplasts; lane 4, mitochondria 1.6 M/2.0 M sucrose interface; lane 5, mitochondria 1.2 M/1.6 M sucrose interface; lane 6, total leaf protein.

Figure 6. Distribution of the 37 kDa protein by immunoblot analysis. Chloroplast from various plant species were purified and proteins were separated by SDS-PAGE in a 12% polyacrylamide gel, transferred onto PVDF membrane and incubated with the anti-scFv-7A serum: lane 1, total leaves of *L. sativa*; lanes 2–5, chloroplasts of: lane 2, *L. sativa*; lane 3, *B. perviridis*; lane 4, *L. esculentum*; lane 5, *N. tabacum*.

37 kDa, with an additional 40 kDa species for *L. esculentum*. This indicates that the protein bearing the epitope recognized by the anti-ids is highly conserved.

Discussion

This study provides experimental data for the presence of a chloroplast protein that interacts with the CP of TuMV. This plant protein was identified by exploiting the molecular mimicry potential of anti-ids. The concept of anti-ids containing the 'internal image' of the ligand afforded a convenient alternative to its purification. Evidence for such a protein was first the observation that the anti-ids against Mab-7A recognized a 37 kDa plant protein. However, the antibodies involved

could have detected a framework feature of Mab-7A found also on the plant protein. The operational criterion supporting the identification of an interacting protein was thus the inhibition of the anti-ids-plant protein complex formation by CP, the antigen used for the production of Mab-7A. This criterion of competitive inhibition between anti-ids and antigen for binding the cellular protein has been used in other studies as evidence that the identified cellular protein is a receptor for the antigen [18, 40, 43].

The 37 kDa protein was associated with chloroplasts of *L. sativa* and polypeptides of similar molecular mass were detected in *B. perviridis*, *N. tabacum* and *L. esculentum*, with an additional form of higher molecular weight also observed in the latter. The anti-ids containing the CP internal image should bind only a small region of the 37 kDa protein. It is noteworthy that this epitope was detected on proteins of similar molecular weight in different plant species. The 37 kDa protein may thus play a role in the viral multiplication cycle. Indeed, plant viruses can replicate in most cell types and the presence of the epitope in several plant species suggests a link between the 37 kDa protein and viral infection.

Viral infections have long been associated with chloroplasts: symptoms such as mosaics, green islands, yellows or chlorosis are all largely attributable to viral interference with normal chloroplast functions [28]. Some viruses induce structural changes in that organelle; turnip yellow mosaic virus (TYMV) as well as poplar mosaic virus cause their aggregation [4, 11] and tombusviruses cause the development of multivesicular bodies within the chloroplasts [36]. TuMV caused the aggregation of chloroplasts in *Chenopodium quinoa* and electron microscopic examination revealed a layer of viral particles between the clustered chloroplasts [26]. Replication complexes in chloroplasts have also been reported for AIMV [8] and TYMV [13]. Tobacco etch potyviral RNA as well as potato virus Y CP, helper component protein and RNA have been found within infected leaf chloroplasts [12, 17]. However, there is still little evidence to support the idea that the chloroplast is the principal site of viral replication.

Furthermore, there are reports associating CP with chloroplasts. The CP of TMV was shown to accumulate in chloroplasts of infected tobacco plants [34]. Hodgson *et al.* [20] showed that the selective inhibition of photosystem II (PS II) in spinach by TMV resulted from the association of the CP with the PS II complex. Recently, the YSI/1 mutant of the common strain (U1) of TMV was found to induce a severe yellow

mosaic in *N. tabacum* instead of the light green/dark green mosaic of the parental strain. The CP accumulated several-fold in chloroplasts and the chlorosis was linked to a single amino acid substitution near the N-terminal end of the CP [3], suggesting that this region of the viral protein was responsible for more severe symptoms. However, the relationship between CP and chloroplasts is not clear.

Dramatic effects on chloroplast photosynthetic capacity *in vitro* after viral infection have been reported, but these can be alleviated with adequate nitrogen supplies to the plant; circumstances that also promote viral replication [45]. Although the purpose of the interaction between the CP of TuMV and the chloroplast protein is not known, it is possible that it is a step towards the sequestration of chloroplast components necessary to fuel viral replication [10, 29, 46].

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