Identification and Characterization of Host Proteins Interacting with NSm, the *Tomato Spotted Wilt Virus* Movement Protein

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Abstract

To search for host proteins involved in systemic spreading of *Tomato spotted wilt virus* (TSWV), the virus-encoded NSm movement protein has been utilized as a bait in yeast two-hybrid interaction trap assays. J-domain chaperones from different host species and a protein denominated At-4/1 from *Arabidopsis thaliana* showing homologies to myosins and kinesins were identified as NSm-interacting partners. In this communication we illustrate that following TSWV infection, J-domain proteins accumulated in systemically infected leaves of *A. thaliana*, whereas At-4/1 was constitutively detected in leaves of *A. thaliana* and *Nicotiana rustica*.

Keywords: *Tomato spotted wilt virus* (TSWV); *Arabidopsis thaliana; Nicotiana rustica*; movement protein; J-domain protein; yeast two-hybrid system

INTRODUCTION

Protection from viruses depends on measures of precaution, i.e. controlling virus disease occurs in an indirect way, e.g. by breeding of resistant crop plants or by repelling or reducing virus-transmitting pests. Achieving substantial insight into the molecular mechanisms during virus replication and movement in infected plant tissues should allow new strategies of direct virus control in the near future.

The host range of *Tomato spotted wilt virus* (TSWV) currently includes more than 800 plant species, and losses to world agriculture were estimated to be about USD one billion per year (PRINS & GOLDBACH 1998). Because of its tripartite RNA genome along with negative and ambisense coding strategies, TSWV is technically not well suited for conventional reverse genetics, hence, host factors required for TSWV replication and systemic spreading are still terra incognita. Therefore, alternative approaches occurred to be the method of choice to eludidate on the role of virus – as well as host proteins during infection. Using the yeast two-hybrid system and far-western analysis, J-domain chaperones and a protein of unknown function aligning to myosins and kinesins, denominated At-4/1, have been identified as interacting partners of the TSWV encoded NSm movement protein (VON BARGEN *et al.* 2001; SOELLICK *et al.* 2000).

In this short report we present determinations of J-domain and At-4/1 protein levels using polyclonal antisera following TSWV infection of *A. thaliana* and *N. rustica*.

MATERIALS AND METHODS

Arabidopsis thaliana (L.) Heynh. (ecotype Col) plants were grown for 6 to 8 weeks under short day conditions (8 h light/16 h darkness) in a Percival 35LL growth chamber. Three rosette leaves were mechanically inoculated with freshly prepared extracts derived from TSWV (L3, DSMZ No. PV 0182) systemically infected *Nicotiana rustica* L. leaves using Carborun-

dum (silicium carbide). After 28 days, systemically infected leaves were subjected to protein analysis. For heat-shock experiments, the ambient temperature in the growth cabinet was shifted from 21°C to 37°C for 4 hours (16°C shift, according to NOVER 1991) and immediately, leaves were harvested and frozen. *Nicotiana rustica* L. plants were grown on Vermiculite and fertilized with Hoagland solution under long day conditions. Two leaves of plants developed to a 6 to 8 leaf stage were mechanically inoculated with TSWV as described for *A. thaliana*. After 14 days, systemically infected leaves were subjected to protein analysis.

For western blot analyses, equal volumes of protein extracts were separated on SDS/PAGE gels and subsequently blotted onto PVDF membranes. The presence of J-domain proteins in soluble protein fractions of infected *A. thaliana* leaves (obtained from supernatants after grinding in 100mM Tris/Cl pH 8.0, 10mM EDTA, 5mM DTT and centrifugation at 4°C, 30 000 × g for 30 min) was determined in an indirect immunoassay using the polyclonal antiserum Pab21-A39 in a 1:3000 dilution and anti-rabbit IgGs conjugated with alkaline phosphatase as secondary antibodies by imbibing filters in NBT/BCIP solution. For inspections of the At-4/1 protein in *A. thaliana* and *N. rustica*, a polyclonal antiserum raised against two synthetic peptides deduced from the C-terminus of the At-4/1 amino acid sequence (in 1:5000 dilutions) was utilized and western blot analysis was performed as described for the J-domain protein detections. TSWV infection of systemic leaves was confirmed by western blots or ELISAs utilizing nucleocapsid-specific antibodies (data not shown).

RESULTS

The antiserum used in the experiments examinating J-domain protein levels was raised against the heterologously expressed protein from a full-length cDNA clone derived from the *A. thaliana* atDjB60 gene. The atDjB60 encoded J-domain protein (formerly denominated At-A39) was shown to specifically interact with NSm (SOELLICK *et al.* 2000). Preliminary experiments using deletion mutants of At-A39 revealed that the antibodies were able to detect solely the J-domain region of the polypeptide (not shown). Hence, we expected that using this antiserum, evidently a number of different, individual J-domain proteins would be determined in immunoblots using crude *A. thaliana* protein extracts. Actually, sequencing of the complete *A. thaliana* genome revealed that 89 individual

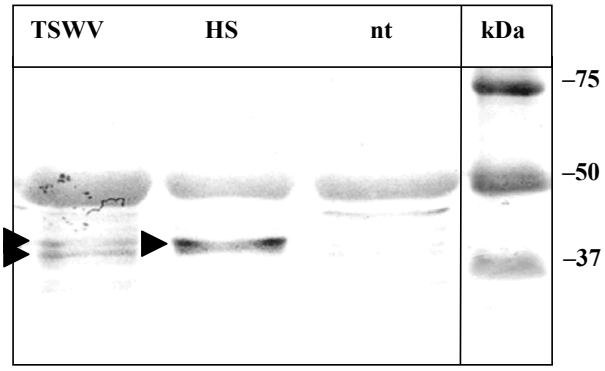

Figure 1. Western blot of soluble protein fractions derived from *A. thaliana* leaves utilizing polyclonal antiserum for the detection of J-domain chaperones in TSWV infected or heat-shocked (HS) as wells as non-treated (nt) tissues. Arrowheads denote proteins revealing molecular masses of about 40 kDa, solely appearing after heat-shock and in TSWV systemically infected leaves

open reading frames do exist encoding polypeptides comprising a J-domain (MIERNYK 2001). As shown in Figure 1, a protein with a molecular mass of about 50 kDa was detectable in TSWV, heat- and non-treated leaves of *A. thaliana*. In addition, two proteins with molecular masses of about 40 kDa appeared in TSWV infected leaves, and after heat-shock, one single band emerged corresponding to the TSWV induced proteins, however, it cannot be ruled out that here both proteins were also present but not well separeted due to electrophoresis artifacts.

The antiserum raised against synthetic peptides of the At-4/1 protein detected one single polypeptide exhibiting a molecular mass of about 26 kDa, which

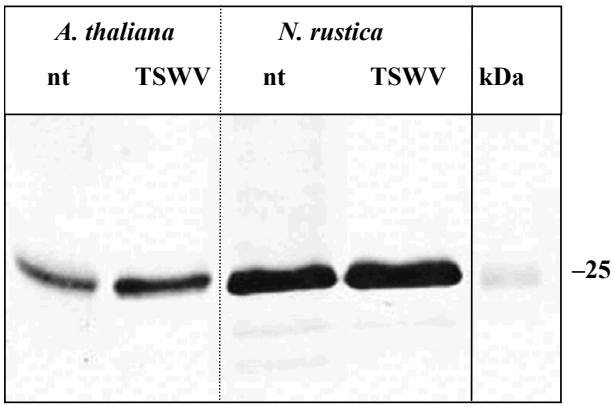

Figure 2. Western blot of crude protein extracts from *A. thaliana* and *N. rustica* prepared from non-treated (nt) and TSWV systemically infected leaves utilizing polyclonal antiserum for the detection of the At-4/1 polypeptide

was present in TSWV – as well as non-treated leaves from *A. thaliana* and *N. rustica* (Figure 2).

DISCUSSION

It is generally accepted that virus-encoded movement proteins interfere with endogenous mechansisms for intra- and intercellular transport of macromolecules to transfer viral genomes throughout the plant (HAYWOOD *et al.* 2002). Not unlikely, molecular chaperones are involved in these processes, as has been suggested from (i) examinations on the function of particular transcription factors (KN1; KRAGLER *et al.* 1998) and (ii) the discovery of a homolog to Hsp70 chaperones encoded by closteroviruses, which was shown to be indispensable for movement of beet yellows virus (ALZHANOVA *et al.* 2001). From this point of view, it is reasonable to suggest that chaperones could be involved also in the systemic movement of TSWV. In our experiments, J-domain proteins with a molecular mass of about 40 kDa were detected after TSWV infection or heat-shock, in contrast to a polypeptide of 50 kDa, which appeared to be constitutively present in leaf tissues of *A. thaliana* (Figure 1). Because the antiserum used in this experiment was capable to detect single J-domain regions (stretches of less than 90 amino acids, data not shown), we assume that the 50 kDa protein represents one subfamily of J-domain chaperones not influenced by biotic or abiotic stress, whereas the 40 kDa proteins, very likely expressed from a subfamily consisting of six genes (MIERNYK 2001) including the atDjB60 gene encoding the NSm-interacting At-A39 polypeptide (calculated molecular mass: 38.2 kDa), seem to respond to environmental changes like virus attack. Each of the six genes arranged in this subgroup represents a paralog of the Hsp40 (DnaJ) like chaperone family, shown to participate in biological processes such as cell cycle control by DNA tumor viruses, clathrin uncoating or protein translocation (KELLEY 1998). Further examinations are now on their way to study a proposed biological function of the NSm-J-domain protein interference in more detail.

The second NSm interacting partner, At-4/1, appeared to be a protein of unknown function, i.e. its role, to our knowledge, has not been biochemically or genetically clarified. One outstanding feature of its amino acid sequence, as enlightened by computer analysis, are coiled-coil domains, which are typical e.g. for proteins involved in intracellular motile processes like myosins or kinesins. However, compared to archetypical myosin or kinesin, At-4/1 is only composed of 247 amino acids, hence not signified to be a protein unequivocally linked to cellular trafficking along microtubuli or microfilaments. As shown in Figure 2, At-4/1 is present in TSWV – as well as non-treated leaf tissues, suggesting that, determined on our measurements of steady-state protein levels, expression of the corresponding gene is not in uenced in *A. thaliana* and *N. rustica* in the presence of TSWV. Nevertheless, not only in account of the current controversial discussion on the putative role of microtubuli or actin filaments during the movement of tobacco mosaic virus (BOYKO *et al.* 2002; GILLESPIE *et al.* 2002) we are encouraged to further explore the role of At-4/1 in infected as well as healthy plant tissues.

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