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ANALYSIS OF THE INTERACTION BETWEEN *TOMATO TORRADO VIRUS* PROTEINS USING THE YEAST TWO-HYBRID SYSTEM

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Abstract: Ten years ago for the first time the new picorna-like virus species – *Tomato torrado virus* (ToTV) – was found and described on tomato plants. The isolates of this pathogen were reported in Europe, America, and Oceania including Australia. Because of its unique biological and molecular features, ToTV was classified to the new genus *Torradovirus*, in the Secoviridae family. In Poland, three isolates: Wal'03, Kra, and Ros ToTV were identified on greenhouse tomato cultivars. At present, the biology and the genome structure of this virus are characterised. But there is no data extending beyond the bioinformatics analyses about the function of viral proteins, polyproteins, and non-coding sequences, as well as possible interactions between viral, host and vector factors that may be important for the infection process, encapsidation, transport in plants, and transmission. In this study, we have undertaken a search for the possible protein-protein interaction of ToTV encoded proteins using the yeast two-hybrid (Y2H) system. The viral genome fragments covering full sequences for nine known proteins of ToTV were amplified using specific primers with characteristic recombination sites. This process enabled the construction of basic entry clones for each protein that further facilitated manipulations with prepared constructs using Gateway technology. Two-hybrid assays were performed in the yeast strain and tested interactions of ToTV proteins were analysed in several combinations using auxotrophy markers. Our analyses did not reveal the presence of interactions between ToTV domains. Surprisingly, no interactions were found in the case of various CP subunits as well as between CP subunits and 3A protein, that in some virus families are known to play a role in viral life cycle. This role includes virion assembly or cell-to-cell transport. The lack of interactions may be a result of the limitation of this experimental system, or suggest that these proteins may interact indirectly, or require the presence of genomic RNAs or some host factors.

Key words: gateway strategy, *Tomato torrado virus*, viral proteins interaction, yeast two-hybrid system

INTRODUCTION

Tomato torrado virus (ToTV) has been reported in several countries of the world such as France (Verdin *et al.* 2009), Hungary (Alfaro-Fernández *et al.* 2009), Poland (Pospieszny *et al.* 2007a), Spain (Alfaro-Fernández *et al.* 2007; Verbeek *et al.* 2007; Alfaro-Fernandez *et al.* 2010), Italy (Davino *et al.* 2010), Australia (Gambley *et al.* 2010), Colombia (Verbeek and Dullemans 2012), and Panama (Herrera-Vasquez *et al.* 2009). In 2009, the ICTV working group on plant Picornavirales, classified this new pathogen to the newly established genus *Torradovirus*, in the Secoviridae family (Sanfacon *et al.* 2009). The virus is transmitted by the whitefly species *Trialeurodes vaporariorum* and *Bemisia tabaci* (Amari *et al.* 2008; Pospieszny *et al.* 2007b). Preliminary results showed that this virus could also be spread with tomato seeds (Gambley *et al.* 2010; Pospieszny *et al.* 2012). The natural host of ToTV is *Solanum lycopersicum* L., but the virus may also infect weeds (Alfaro-Fernández *et al.* 2008). Since 2003, three isolates of this virus have been found in Poland, on the greenhouse tomatoes: Wal'03, Kra and Ros (Budziszewska *et al.* 2008; Pospieszny *et al.* 2008). The symptoms of pathogenesis caused by the above mentioned isolates vary. This variation may result from a diversity in the genome sequence, especially in the 3`untranslated region (unpublished data), that may in consequence influence virus accumulation which has been shown previously (Budziszewska *et al.* 2011). *Tomato torrado virus* causes local necrotic spots on the leaves, fruits, and causes systemic necrosis of whole plants. The resulting permanent withering leads to significant crop reduction. In 2009, the virus was included in the EPPO (European and Mediterranean Plant Protection Organization) alert list (EPPO 2009; Alfaro-Fernández *et al.* 2010). The ToTV genome consists of two (+)ssRNAs which contain three open reading frames (ORFs): RNA1 encodes ORF1 for the polyprotein with the characteristic functional motifs of protease cofactor (Pro-co), protease (Pro), helicase (Hel) and RNA-dependent RNA polymerase (RdRp), the RNA2 encodes for ORF1 of protein with no specified function found, and ORF2 for a polyprotein that carries information of three CP (coat protein) subunits Vp23, Vp26, Vp35; and movement protein 3A, which are cleaved from the polyprotein precursor by proteolytic cleavage (Verbeek *et al.* 2007; Budziszewska *et al.* 2008) (Fig. 1). Both RNA strands contain 5`and 3`UTR regions and polyA tract at 3`

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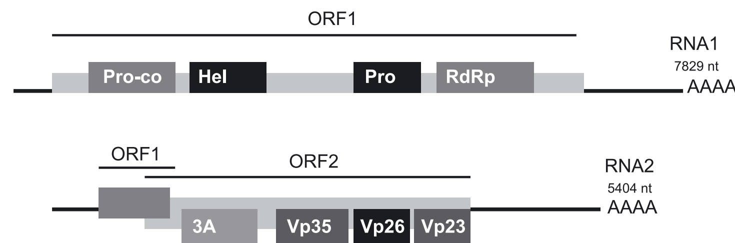

Fig. 1. The scheme of ToTV genome organization. The particular motifs of protein domains are indicated within the ORF1 encoding polyprotein on RNA1 strand, as well as in ORF1 and within ORF2 encoding polyprotein. Both are present on RNA2 strand

ends. At present, the data on functions of ToTV encoded proteins were obtained using *in silico* analysis-based predictions, with the exception of viral CP subunits that were additionally analyzed by mass spectrometry (Verbeek *et al.* 2007). Based on this analysis, several possible aminoacid regions of cleavage sites between Vp35 and Vp26 as well as between Vp26 and Vp23 were suggested, although *in silico* screening of these sites indicated no homologies with known protease recognition sites. Because of the high diversity of the protease recognition sites among plant picornavirales, the exact positions of these sites should be, however, determined experimentally (Verbeek *et al.* 2007). Overall, the current function of ToTV proteins in pathogenesis, including their role in initial phases of the infection, virus-host-vector interactions, virus replication, capsid assembly process, as well as in the structure of ToTV proteins, still remains unknown. Previous studies were focused primarily on the characteristics of nucleotide sequences of known ToTV isolates, their phylogeny or diagnostics (Wieczorek and Obrępalska-Stęplowska 2013).

The protein-protein interaction network plays an important regulatory role in many known biological processes. In the viral infection cycle such interactions are investigated, for example, for replication, the encapsidation process, and the local and systemic transport of viral particles within host tissue. There are a few reports that discuss the interaction between CP during capsid assembly, its stability, and dynamics. The virus capsid is necessary for genome protection from degradation, hence, to establish successful infection. Up to now three strategies for capsid assembly have been recognized. The first one depends on direct CP interactions, identified, for example, in bromoviruses, comoviruses, tobamoviruses or picornaviruses (Hull 2001; Mateu 2013). The second one requires additionally scaffolding proteins and is characterized among animal dsDNA also dsRNA viruses and in DNA phages. The last strategy depends on simultaneous CP – viral nucleic acid interaction in a condensation process and is exemplified by cucumoviruses, alfamoviruses where the protein-RNA interaction predominates (De Graaff *et al*. 1995; Bol 1999; Hull 2001; Mateu 2013).

In the present study, we have tried to find out the interactions between predicted ToTV protein domains. We have mainly tried to test ToTV capsid proteins and the movement protein domain that are multifunctional viral factors in many virus species. The analyses were performed using the yeast two-hybrid (Y2H) system. For finding possible interactions between complete proteins as well as for detecting interacting protein domains, the Y2H system is considered the method of choice (Causier and Davies 2002; Auerbach and Stagljar 2005).

MATERIALS AND METHODS

RNA isolation and PCR reactions

The nucleotide sequences of analyzed ToTV proteins in Polish isolates share a 98–100% identity therefore the protein interaction study was based on the sequences of Wal'03 ToTV isolate. Viral particles were isolated from virus-infected plants of *Nicotiana benthamiana* maintained in greenhouse conditions at 18/26°C, as described previously (Pospieszny *et al.* 2010). RNA was phenol/SDS-extracted and ethanol precipitated (Sambrook *et al*. 2001). In the next step, 0.5 μg of RNA was used for cDNA synthesis that was performed with random primers using Revert Aid First Strand cDNA Synthesis Kit (Fermentas). Afterwards, the series of PCR reactions were performed in order to obtain the amplified gene sequences corresponding to known ToTV domains. The amplification reactions were carried out in a 50 μl volume of the reaction mixture using 2.5 U of PfuUltra II Fusion HS DNA Polymerase (Invitrogen), 0.2 μM sense and antisense primers flanked with specific adapter sequences (Table 1), designed on the basis of the nucleotide sequence of RNA1 (EU563948) and RNA2 (EU563947), 1x reaction buffer, 250 μM of each dNTP, and 1 μl of cDNA obtained from 50–500 ng of RNA template. The amplification conditions were: initial denaturation at 95°C for 2 min, followed by 30–35 cycles of: 20 sec at 95°C, 20 sec at the annealing temperature optimal for particular primer pairs (Table 1) and 30 sec/1 kb at 72°C. The PCR products were analyzed electrophoretically, and then eluted using Wizard SV Gel and the PCR clean-up system (Promega), according to the manufacturer's instructions.

Construction of the entry clones

In this work, Gateway™ technology (Invitrogen) was used. Therefore, all cloning steps were based on the site-specific recombination, which requires the addition of *att* sites in PCR primers. Obtained *att*B-flanked PCR products were subsequently cloned into pDONR221 vector (Invitrogen) that contains *att*P-sites. To generate entry clones for each ToTV protein, BP recombination reactions were performed by using the BP clonase (Invitrogen), and

Table 1. The sequences of primers used in PCR amplifications of ToTV protein domains. The optimal annealing temperatures as well as the length of the predicted products are shown. The *att*B1 (in forward primers) and *att*B2 sites (in reverse primers) were underlined

Primer	Amplified protein motifs	Ta [°C]	Sequence 5'-3'
B1Pro	protease	64°C	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCGTTTTAATGTGATGATGTCC
B2Pro		302 bp	GGGGACCACTTTGTACAAGAAAGCTGGGTCTACCATTGCTACTTTAACCGG
B1Hel	helicase	65°C	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGACAAGATATATGGGGAG
B2Hel		503 bp	GGGGACCACTTTGTACAAGAAAGCTGGGTCAGAAGTGGGAGCATCAGTG
B1Pro Co	Protease cofactor	65°C	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGTTGCAAAGATTG
B2Pro Co		698 bp	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTGCTCCCTTAGATG
B1RdRp	RdRp	65°C	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCAAAGATGAGCGC
B2RdRp		755 bp	GGGGACCACTTTGTACAAGAAAGCTGGGTCAAAACCTCTGCTTAG
B1Vp23	Vp23	60°C	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTTTTCATATGGGGCTGT
B2Vp23		650 bp	GGGGACCACTTTGTACAAGAAAGCTGGGTCATTTTCAAAACTCCTTA
B1Vp26	Vp26	64°C	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCACAATTTGGTATGA
B2Vp26		710 bp	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTTCCTTTCTCATCAA
B1Vp35	Vp35	62.2°C	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGTGGCCCAAACTAGTGT
B2Vp35		737 bp	GGGGACCACTTTGTACAAGAAAGCTGGGTCACGAGGAGGCTGCATGAT
B1_3A	3A	62.2°C	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAAAAACCTGCTTCGCAA
B2_3A		653 bp	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGCTTCGCTTGGCACAT
B1Orf1	Orf1	60°C	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCATTTATTTCTCG
B2Orf1		563 bp	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACTTGGGCCCCT

then 1 µl of the mixture was used to transform *E. coli* One Shot OmniMAX™ 2-T1R Chemically Competent Cells (Invitrogen), according to the supplier's protocols. Then, recombined plasmids were isolated from the positive transformants, using Nucleo Plasmid isolation Kit (Macherey-Nagel). Sequencing followed, to confirm the presence of inserts, the correctness of analyzed sequences, as well as the in-frame position. The following study on viral protein interactions was performed using ProQuest Two Hybrid with Gateway™ Technology (Invitrogen) with two destination vectors (pDEST™32 which contains GAL4 DNA binding domain, and pDEST™22 with GAL4 activation domain). Both vectors may be fused with each of the ToTV proteins, and tested as a 'bait', and a 'prey', respectively. The application of this system assumes that the two interacting proteins, each fused with one of two basic GAL4 domains (binding and activating), will reconstitute an active GAL4 transcription factor resulting in the expression of the reporter genes. The interaction between the two viral proteins tested is, thereby confirmed. The pDEST™32 and pDEST™22 vector include *TRP1* and *LEU2* genes, respectively, as a selection markers for tryptophan and leucine auxotrophy.

Construction of the destination vectors

The destination 'bait' and 'prey' vectors were generated through the LR recombination reactions between previously obtained entry clones, containing the particular genes of interest flanked by *att*L-sites, and the pDEST™32 and pDEST™22 vectors that contain specific *att*R-sites, according to the producer's instructions. Subsequently, 1 µl of LR reaction mixture was used for the transformation of One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen). Bacterial cells transformed with pDEST™32 or pDEST™22 vectors grew up at 37°C on LB plates with 10 µg/ml gentamicin, or 100 µg/ml ampicillin, respectively. We obtained expression clones for pDEST™32 and pDEST™22 vectors fused with the target sequence of proteins encoded by both RNA strands. Then, the plasmid DNA samples were isolated using Nucleo Spin Plasmid Kit (Macherey-Nagel) and verified using appropriate restriction enzymes to confirm the presence of the insert. The generated plasmids carrying the protease cofactor, helicase, protease domain were digested with *Eco*RI; RdRp domain with *Sac*I; ORF1-RNA2 with *Xba*I; Vp35 with *Nco*I, Vp26 with *Xba*I and *Eco*RI; Vp23 with *Kpn*I; and 3A domain with *Xho*I.

Tests of the Specificity of Two-Hybrid Interaction

All yeast experiments were performed with *S. cerevisiae* strain MaV203 which carries reporter genes such as *lacZ* and auxotrophic markers like histidine (*HIS3*) and uracil (*URA3*). Competent yeast cells were performed using the *S.c.* Easy Comp Transformation Kit (Invitrogen), according to the producer's instructions. Prepared cells (50 μl) were co-transformed with 0,5 μg generated 'bait' (DB-X) and 'prey' (AD-Y) destination vectors to test ToTV protein interactions in several combinations, that are shown in table 2, as well as with control vectors (Table 3) supplied by producer. The transformation reactions were incubated for 1 h at 30°C, and mixed every 15 min. After centrifugation, the cell pellet was resuspended in TE buffer and then plated onto yeast dropout media without tryptophan and leucine (SC-Leu-Trp, Sigma–Aldrich). This was done to check the transformation efficiency and to select colonies that were obtained after 72 h of growth at 30°C and then maintained on new plates with the aforementioned medium. Then, the auto-activation tests were performed using the procedures described by the producer. The yeast colonies chosen to identify possible ToTV protein interactions, were assayed based on their ability to activate the transcription of the above mentioned reporter genes. Then, the obtained yeast phenotypes were analyzed according to the producer's protocol. Additionally, the X-gal assay was performed to evaluate *lacZ* gene expression in analyzed yeast cells. Selected colonies grew up on the nitrocellulose filter on the plates with YAPD medium. For this purpose, the membrane was frozen in liquid nitrogen. Incubation followed at 37°C on plates with Whatman papers saturated with X-gal solution (1 mg/ml) in Z buffer and 50 mM β-mercaptoethanol for blue/white screening for 24 h (Sambrook *et al*. 2001).

Table 2. The scheme of the tested interactions between ToTV protein domains. Tested combinations of protein partners are dotted, self-interactions were not tested and are shaded. The inversions of analyzed interaction were not tested and are indicated by dash

Destination 'bait' vector / Destination 'prey' vector	RNA1				RNA2				
	pDEST™32/ Hel	pDEST™32/ RdRp	pDEST™32/ Pro-co	pDEST™32/ Pro	pDEST™32/ ORF1-RNA2	pDEST™32/ 3A	pDEST™32/ Vp35	pDEST™32/ Vp26	pDEST™32/ Vp23
pDEST™22/Hel		–	–	–	–	–	–	–	–
pDEST™22/RdRp	•		–	–	–	–	–	–	–
pDEST™22/Pro-co	•	•		–	–	–	–	–	–
pDEST™22/Pro	•	•	•		–	–	–	–	–
pDEST™22/ORF1-RNA2	•	•	•	•		–	–	–	–
pDEST™22/3A	•	•	•	•	•		–	–	–
pDEST™22/Vp35	•	•	•	•	•	•		–	–
pDEST™22/Vp26	•	•	•	•	•	•	•		–
pDEST™22/Vp23	•	•	•	•	•	•	•	•	

Table 3. The scheme of performed transformation reactions

Destination 'bait' vector	Destination 'prey' vector	Purpose
pEXP™32/Krev1*	pEXP™22/RalGDS-wt*	strong positive interaction control
pEXP™32/Krev1*	pEXP™22/RalGDS-m1*	weak positive interaction, control
pEXP™32/Krev1*	pEXP™22/RalGDS-m2*	negative interaction control
pDEST™32**	pDEST™22**	negative self-activation control
pDEST™32/tested 'bait' protein***	pDEST™22**	test of self-activation
pDEST™32/tested 'bait' protein***	pDEST™22/tested 'prey' protein***	tested interaction

*Invitrogen control vector; **Invitrogen destination vector, without the sequence of tested protein; ***Invitrogen destination vector, with the sequence of tested as 'bait' or 'prey' protein, respectively

RESULTS

This study aimed to identify ToTV protein domain interactions applying the Y2H system, based on Gateway™ technology. This strategy constitutes a powerful tool that provides a rapid and highly efficient way to introduce and subclone DNA sequences into multiple destination vector systems for various functional analyses and for protein expression (Invitrogen).

In the first step, the donor vectors with DNA encoding all protein domains of ToTV, were constructed. The analyses of the obtained sequences of the entry clones for ToTV-encoded proteins confirmed their correctness permitting subsequent recombination reactions leading to the construction of the destination 'bait' and 'prey' vectors used in the two-hybrid assays. The auto-activation tests indicated that all of ToTV proteins used as 'baits' did not characterize any self-activation ability of the GAL4 activation domain, hence they could be used in subsequent selection assays. The tests of selected yeast colonies transformed with destination vectors fused with viral protein domains used as a 'bait' or 'prey' (Table 2) gave different phenotypes. The use of different selection media (Fig. 2) was the reason for the different phenotypes. We observed that all the tested yeast colonies indicated the phenotypes that were typical for commercial negative interaction controls, supplied by the producer (Invitrogen) (Fig. 2). The performed phenotype analyses of obtained transformants on plates lacking auxotrophic markers, suggested the absence of positive interactions between the analysed ToTV proteins. The parallel performed colorimetric assays for β-galactosidase activity confirmed these results. In a few cases, though, they gave faint blue-colored colonies, that might suggest some weak positive interactions among the analysed viral proteins. Since the interactions were so slight, they were not considered as positive interactions.

DISCUSSION

The development of the two-hybrid system and its modifications provide for a greater possibility of testing and detecting protein-protein interactions *in vivo* in bacterial, yeast, and mammalian host cells. Nowadays, there are a lot of commercial systems based on different vectors that are destined for this type of analyses.

In this work, the performed Y2H assay indicates the absence of interactions between tested protein partners. This fact is quite surprising, especially in the case of three different CP subunits as well as 3A protein containing movement motif that, as we hypothesised, should interact with each other. The Vp35, Vp26 and Vp23 proteins of ToTV constitute components of the viral capsid, that characterises T = 3 icosahedral symmetry reported to be similar among all plant picornavirales (Sanfacon *et al.* 2009). Up till now, the analysis of the 3D structure of CP among the reported species of *Torradoviruses*, has not been approached. Yet, our preliminary *in silico* data suggest that this structural model may be homological to the known structure of the insect picorna-like virus *Cricket paralysis virus* (CrPV) (unpublished data). The capsid structure of CrPV is composed of four subunits VP1, VP2, VP3, and VP4 that interact with each other. The analyses of this known CP model revealed inter-subunit interaction that stabilises the capsid (Tate *et al.* 1999). The capsid assembly among several virus families has been reviewed to be dependent not only on two (or more) viral protein interactions, but also on the presence of viral RNA/DNA genomic strands, the host molecules as well as physico-chemical conditions (Mateu 2013). However, in some cases the viral protein-protein interactions are thought to be the most important factors during virion formation (Hull 2001; Mateu 2013). The CP, in addition to being a structural element of the viral protein shell, is also reported to be involved in the viral intercellular transport. At present, several different mechanisms of viral particles movement within plant tissue have been described (Schoelz *et al.* 2011). The CP-MP interactions that are required for virus movement have been well studied among virus families that contain triple gene block movement proteins (TGB), *e.g.* potexviruses, carlaviruses, pomoviruses, as well as in the case of several comoviruses (Hull 2001; Ritzenthaler 2011; Schoelz *et al.* 2011). Numerous papers investigated the "genome activation" phenomenon, in some virus families. This means that CP subunits are required for the viral replication process due to their ability to directly regulate RNA synthesis. Such interactions have been experimentally proven, for example, in ilarviruses and alfamoviruses (De Graff *et al.* 1995; Bol 1999; Van Der Heijden *et al.* 2001; Herranz *et al*. 2012).

In light of the published data, the negative results of our conducted Y2H assay may be explained by a few hypothetical assumptions. One possible suggestion is that the ToTV virion assembly process may require additional factors, such as the genomic RNA strands or some other viral or cellular factors creating the multifactorial complex. The complex is responsible for the formation, stability, and dynamics. However, as it was mentioned above, almost all of ToTV proteins (except of ORF1-RNA2 protein) originate from polyprotein precursors and are released through proteolytic cleavage. Presently, the known ToTV proteins were predicted using *in silico* analyses based on screening of the conserved, functional regions of viral sequences. The analyses were also based on the characteristic protease recognition sites deposited in available database. The actual range on amino acid sequence (especially in terminal parts) might differ when calculated bioinformatically. Therefore, the negative two-hybrid interactions between analysed domains may result from incomplete sequences of the studied protein genes that subsequently prevented the correct folding of mature proteins. In some research, it has been reported that those GAL4 domains fused to target proteins may interfere with their correct folding (Rajagopala *et al.* 2009). Other known disadvantages of the Y2H system are worth mentioning. Some experimental data based on the analyses of the interaction between bacterial motility proteins performed in several systems, differ depending on the used vectors, yeast strains, and marker genes, and were shown to produce vastly non-overlapping interactions (Rajagopala *et al*. 2009). Surprisingly, the comparative analyses have proven that commercial systems give contradictory results regarding the strength and the number

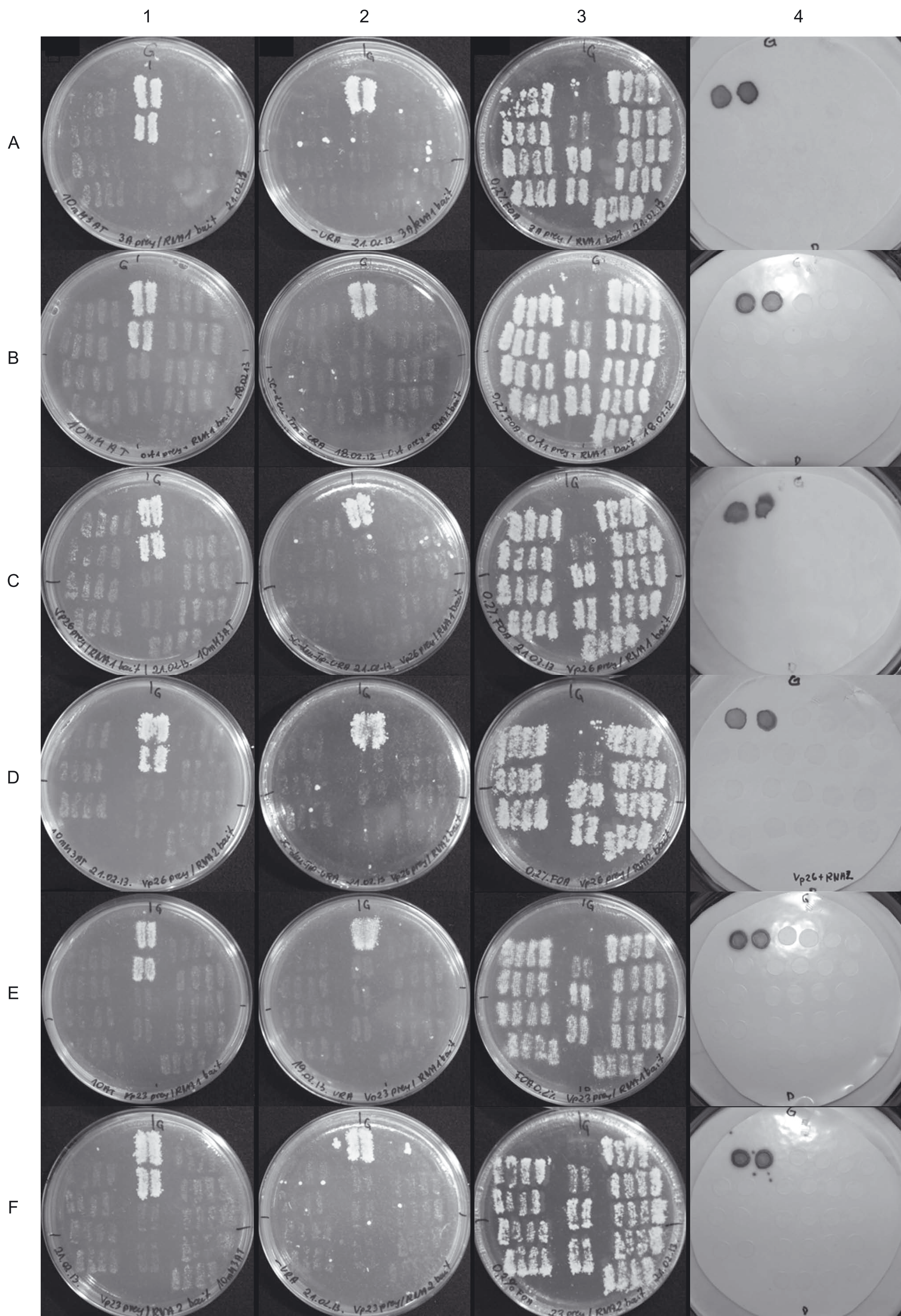

Fig. 2. The chosen results of the performed selective assays for analysed protein – protein ToTV interactions.
1–4 plates with different selective media: 1 – SC-Leu-Trp-His + 10 mM 3AT, HIS induction tested (histidine auxotrophy assay); 2 – SC-Leu-Trp-Ura, URA induction tested (uracil auxotrophy assay); 3 – SC-Leu-Trp + 0,2% 5FOA, URA induction tested (5FOA sensitivity); 4 – X-gal assay, β-galactosidase induction. On each plate the same ToTV protein used as 'prey' was tested in combination with different 'bait' ToTV proteins encoded by RNA1 or RNA2, as well as the control reactions recommended by the producer. A – 3A as a 'prey' with Pro-Co, Hel, Pro, RdRp as a 'bait'; B – protein encoded by ORF1 from RNA2 as 'prey' with Pro-Co, Hel, Pro, RdRp as 'bait'; C – Vp26 as a 'prey' with Pro-Co, Hel, Pro, RdRp as a 'bait'; D – Vp26 as a 'prey' with 3A, ORF1, Vp35 as a 'bait'; E – Vp23 as a 'prey' with Pro-Co, Hel, Pro, RdRp as a 'bait'; F – Vp23 as a 'prey' with 3A, ORF1, Vp35, Vp26 as a 'bait'

of interactions for the same tested 'bait' and 'prey' proteins (Rajagopala *et al.* 2009). Moreover, the identification of interactions between two proteins may depend on which one is used as a 'bait' and which as a 'prey'. In light of this data, our analysed potential interactions between ToTV proteins that were used as a 'prey' should now be tested in the inverse configuration (as a 'bait'). Such an analysis has not yet been carried out.

To conclude, in this study we did not find an interaction between ToTV-encoded proteins. There are two possibilities explaining our results. First of all, indeed, there are no direct interactions between analysed ToTV proteins. These proteins may interact only in the presence of other cellular or viral factors which were not considered in this work. Secondly, the Y2H assays performed on the basis of the system presented here as well as the application of protein domains predicted using bioinformatics tools, had limitations that prevented detection of the studied protein-protein interactions.

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