Proteome Analysis of Plant-Virus Interactome

COMPREHENSIVE DATA FOR VIRUS MULTIPLICATION INSIDE THEIR HOSTS*

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Known host-parasite molecular interactions are widespread among parasite families, but these interactions have to be particularly large considering that viruses generally encode few proteins. Although some particular virus-host interactions are well described, no global study has yet shown multiple and simultaneous interactions in a host-parasite biological system. To prove that these multiple interactions occur in biological conditions, the complexes formed by a plant virus (rice yellow mottle virus) and the proteins of its natural host (rice) were extracted and purified from infected tissue sample. Remarkably mass spectrometry permitted the identification of a large number of proteins from the complexes that are involved in different functions not encoded by the virus but probably essential for its biological life cycle. This recruiting of proteins was strongly confirmed by the repetition of experiments using different pairs of virus-host and the use of high salt concentration to extract the complexes. We mainly identified proteins involved in plant defense, metabolism, translation, and protein synthesis and some proteins involved in transport. This study demonstrates that viruses are able to recruit many proteins from their hosts to ensure their development. Among different pairs of virus-host, similar protein functions were identified suggesting a particular importance of these proteins for viruses. The identification of particular paralog proteins among multigenic families suggests the high specificity of the recruiting for some protein functions. ***Molecular & Cellular Proteomics 5:2279–2297, 2006.***

Although a new virus was discovered recently, the mimivirus that encodes about 1,200 genes (1), viruses such as some phages, poxviruses, phycodnaviruses, iridoviruses, and asfarviruses encode only about 500 genes, and plant viruses belonging to the Geminiviridae and Reoviridae encode only 4–12 genes (2).

To cope with the virus life cycle complexity (decapsidation-encapsidation, translation, replication, and transport) and host defenses, viruses probably recruit host proteins to carry out needed functions that are not encoded by the viral genome. To confirm this virus recruiting, we have used the rice yellow mottle virus (RYMV)[1] as virus model and rice (*Oryza sativa*), which is fully sequenced and widely used for genomics studies, as plant model (3). RYMV causes substantial economic losses in rice production in Africa (4). Its distribution and biology are well known (5). This virus is a monopartite positive RNA strand virus about 4,450 nucleotides, and its genome organization is quite simple as it is composed of four ORFs that encode at least five proteins (5). Three isoforms (compact, transitional, and swollen) were identified according to the presence of divalent ions and pH and were localized in different cell compartments such as cytoplasm, nucleolus, vacuole, vesicle, and cell wall (6, 7). Viral particles were also suspected to be localized in chloroplast (6).

Here we report for the first time a range of analyses from the isolation of virus-host protein complexes to the identification of host proteins by mass spectrometry, allowing us to demonstrate the specific recruiting of numerous host proteins by the virus. A purification method of virus-host protein complexes was developed from infected material using gel exclusion chromatography, separation on SDS-PAGE, analysis by nano-LC-MS/MS after tryptic digestion, and protein identification (8). This new molecular method could also be applied to human and animal viruses for the purpose of finding new therapeutic targets.

EXPERIMENTAL PROCEDURES

Plant and Virus Materials—We used two rice cultivars showing a contrasted response to RYMV infection: a highly susceptible one, IR64 (*O. sativa indica*), and a partially resistant and tolerant one, Azucena (*O. sativa japonica*). IR64 is a high yielding cultivar developed at the International Rice Research Institute, and Azucena is a traditional upland cultivar from the Philippines. For both cultivars, seeds were sown separately, and plants were grown in a confined greenhouse in controlled conditions: 12-h light at 28 °C and 12-h dark at 24 °C. Two weeks after seedling, plants were mechanically inoculated with purified RYMV particles from a virulent isolate of Burkina Faso (BF1) at a concentration of 100 μg/ml in inoculation buffer (20 mM phosphate, pH 7) as described previously (9). This experiment was repeated twice. Leaves of non-inoculated and RYMV-inoculated

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[1] The abbreviations used are: RYMV, rice yellow mottle virus; CP, coat protein; wpi, weeks postinfection; wps, weeks postseedling; SCPMV, southern cow pea mosaic virus; FHV, flock house virus; TAPS, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; nano-LC-MS/MS, nanoscale capillary LC-MS/MS; TIGR, The Institute for Genomic Research.

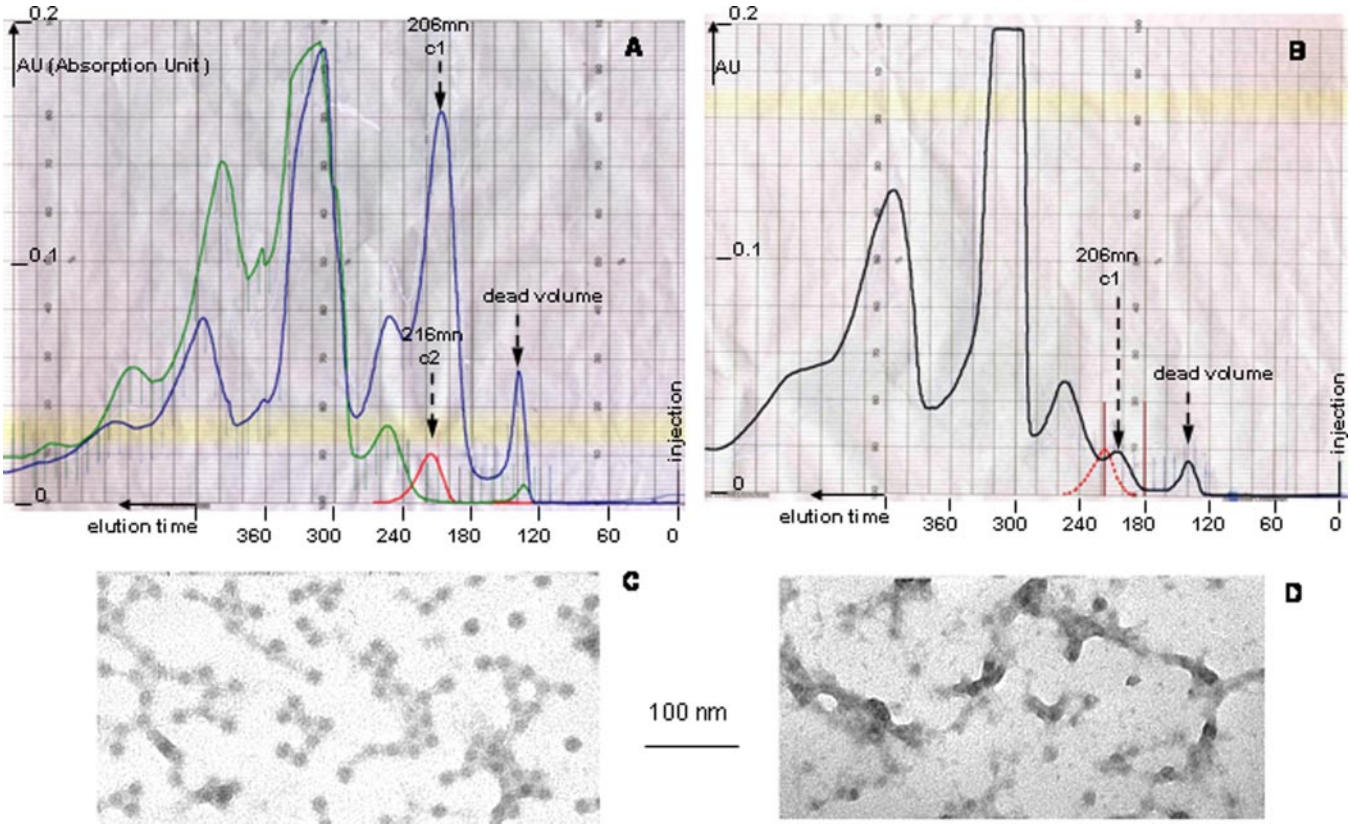

FIG. 1. **Elution profiles of IR64 protein extraction solutions from *in vivo* and *in vitro* binding experiments.** 10 ml of concentrated protein extract solution are injected with an automated injection syringe (ATKTA Prime system), and elution was carried out at 1.5 ml/min with elution buffer (20 mM TAPS, pH 7.6, 100 mM NaCl). *Dead volume* represents the fraction of membrane fragments and/or protein aggregates that are still present in solution. Collected fractions containing RYMV-host protein complexes correspond to the first ⅔ part of peaks labeled *c1*. *A*, size exclusion chromatography elution profiles of purified RYMV (*red*) and of soluble proteins extracted from IR64 non-infected leaves (control experiment in *green*) and IR64 infected leaves (*blue*). *B*, size exclusion chromatography of *in vitro* binding experiment with soluble proteins extracted from IR64 healthy plants added with purified RYMV. *C*, negative contrast electron micrograph of collected fraction stained with uranyl acetate containing purified RYMV particle from fraction corresponding to the peak *c1* eluted at 216 min. *D*, RYMV particle associated with host materials in fraction corresponding to the peak *c2* eluted at 206 min. The *bar* represents 100 nm.

plants were harvested at 1, 2, and 3 weeks postinoculation (wpi), frozen in nitrogen, and conserved at −80 °C.

To compare the specificity of the recruited host proteins we used *Phaseolus vulgaris* (non-host for RYMV and host for southern cowpea mosaic virus (SCPMV)) and *Nicotiana tabacum* (non-host for RYMV). Infected and/or non-infected plants were cultivated and harvested in the same conditions as rice cultivars.

Different viruses were used in our experiments. RYMV isolate BF1 (Swiss-Prot accession number Q9DGX1) was used to infect rice cultivars, for *in vitro* binding experiments with rice host plant, and with *N. tabacum* as non-host plant. SCPMV belongs to the same genus (*Sobemovirus*) but has a different genomic organization and a limited dicotyledonous host range (10). Flock house virus (FHV) is a member of the insect and animal virus family Nodaviridae.

RYMV Extraction—Rice leaves were ground in liquid nitrogen and homogenized in 0.1 M phosphate buffer, pH 5.0. Virus particles were precipitated with 6% polyethylene glycol 8000 and resuspended in phosphate buffer. Further purification was performed by centrifugation through a 10–40% sucrose gradient. Virus concentration was estimated by spectrophotometry using an extinction coefficient of 6.5.

Plant Protein Extraction—About 10 g of frozen leaves were crushed in a liquid nitrogen-cooled mortar, and 50 ml of extraction buffer, pH 7.5 (50 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 25 mM glucose, 5 mM EGTA, 5 mM DTT, 5% glycerol, 0.1% Triton X-100, protease inhibitor mixture tablets (Roche Applied Science)) were added to the resulting powder. The suspension was centrifuged at 22,000 × *g* for 30 min at 3 °C, and the supernatant was filtered through a 0.45-μm syringe filter.

Extraction of Virus-Protein Complexes—Frozen infected leaves (1, 2, and 3 wpi) were treated with the protocol used for plant protein extraction (see below) and then concentrated to 10 ml by ultrafiltration with Centricon Plus 20 (Millipore) at 3 °C.

In Vitro Virus-Protein Binding Assay—3.5 mg of purified virus were added to 100 mg of proteins extracted with buffer as described above and then concentrated to 10 ml by ultrafiltration with Centricon Plus 20 at 3 °C. Contact between virus and proteins was ~90 min until injection on the chromatographic column. All purification steps were done in a cold room at 4 °C.

Purification of Virus-Protein Complexes—The concentrated mixtures were injected on a 1,000-mm long, 26-mm-diameter column filled with Sephacryl S500 (Amersham Biosciences), and TAPS buffer, pH 7.6, 100 mM NaCl as eluent. The ÁKTAprime system (Amersham Biosciences) was used for injection, detection, and collection of the proteins. Fractions corresponding to the first ⅔ peak of virus-protein complexes were collected and lyophilized (Fig. 1, *c1*).

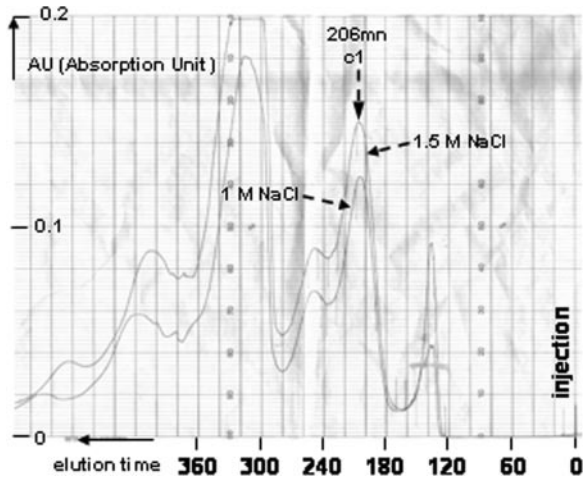

FIG. 2. **Elution profiles of IR64 protein extraction solutions from *in vivo* experiments using high salt concentration.**

SDS-PAGE of Virus-Protein Complexes—The lyophilized pellets were resuspended with deionized water (Milli-Q), and the resulting solution was desalted using PD10 columns (Amersham Biosciences). The amount of protein was estimated using the BCA protein assay kit (Pierce), and about 40 μg of proteins were denatured for 5 min at 100 °C after adding 2× SDS loading buffer (100 mM Tris-HCl, pH6.8, 200 mM DTT, 4% SDS, 0.2% bromphenol blue, 20% glycerol) and loaded on a precast 4–12% acrylamide gel (Invitrogen). Gels were stained with colloidal Coomassie Blue, and sample bands were obtained from gels under sterile conditions to avoid keratin contamination (Fig. 2).

Mass Spectrometry and Protein Identification—In-gel digestion was performed with an automated protein digestion system, MassPREP Station (Waters, Milford, MA). The gel plugs were washed three times with a mixture of 50% NH_4HCO_3 (25 mM), 50% ACN. The cysteine residues were reduced with dithiothreitol at 57 °C and alkylated with iodoacetamide. After dehydration with acetonitrile, the proteins were digested in-gel with 30 μl of 12.5 ng/μl modified porcine trypsin (Promega, Madison, WI) in 25 mM NH_4HCO_3 overnight and at room temperature. Then a double extraction was performed: the first was with 60% acetonitrile in 5% formic acid, and the second was with 100% acetonitrile. The resulting peptide extracts were directly injected for nanoscale capillary LC-MS/MS (nano-LC-MS/MS) analysis.

Nano-LC-MS/MS analysis of the digested proteins was performed using a CapLC capillary LC system (Waters) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF II, Waters). Chromatographic separations were conducted on a reverse-phase capillary column (PepMap C_{18}, 75-μm inner diameter, 15-cm length; LC Packings) with a 200 nl/min flow rate.

Mass data acquisitions were piloted by MassLynx software (Waters) using automatic switching between MS and MS/MS modes as described previously (11, 12). To improve the quality of MS/MS spectra during nano-LC-MS/MS analysis, we empirically derived energy curves depending on the *m/z* value of the selected precursor ion: for each *m/z* value, three different collision energies were applied. Fragmentation was performed using argon as the collision gas.

The mass data recorded during nano-LC-MS/MS analysis were processed and converted into MassLynx .pkl peak list format prior to searching with the search engine Mascot (Matrix Science, London, UK). The searches were performed on a local Mascot server running on a 3-GHz Pentium IV processor with a tolerance on mass measurements of 70 ppm in MS mode and 0.25 Da in MS/MS mode. One missed cleavage per peptide was allowed, and some variable modifications were taken into account such as carbamidomethylation for cysteine, oxidation for methionine, and *N*-acetylation of the protein. Searches were performed without constraining protein molecular weight or isoelectric point. The rice (Nippon Bare) pseudomolecule database (release 3) accessible from The Institute for Genomic Research (TIGR) (www.tigr.org/tdb/e2k1/osa1/) website was used for the identification of rice proteins, and a compilation database gathering Swiss-Prot, TrEMBL, and TrEMBLnew protein databases was used for the identifications of the other host plant proteins (*P. vulgaris* and *N. tabacum*). A total of 531 proteins were annotated for *P. vulgaris*, and 1,988 proteins were annotated for *N. tabacum* in this compilation database (July 2005). Our rice protein identification method using the complete pseudomolecule database allowed us, in some cases, to discriminate paralog genes among multigenic families (8).

Due to the strategy developed here, the virus coat protein was present in all gel bands and in very high concentration independently of the spot position on the gel. To circumvent the superabundance of the viral coat protein, we used an informatics exclusion program to prevent the selection of the coat protein peptides during the nano-LC-MS/MS analysis. This procedure allowed the identification of lower abundance proteins.

A Python language script was used to extract data from files generated by Mascot software, and Access database software (Microsoft) was used to gather and compare datasets according to the different conditions of the experiments. In this study, we validated an identification when the protein was identified by at least two peptides, both having an MS/MS ion score higher than 39.

RESULTS

Methodology Used to Isolate RYMV Host Protein Complexes in Vivo and in Vitro—RYMV-host protein complexes were isolated from infected plants (Fig. 1*A*) or from *in vitro* binding experiments with soluble proteins extracted from non-infected leaves supplemented with purified virus (Fig. 1*B*).

According to our protocol (see "Experimental Procedures"), after injection of purified virus (6.5 mg) we detected an elution peak at 216 min. The first elution peak of soluble proteins extracted from non-infected *O. sativa* (IR64) leaves, which was the control without virus (negative control), was eluted at 255 min (Fig. 1*A*, *green*) and overlapped slightly with the peak at 216 min corresponding to the experiment with the virus purified alone (Fig. 1*A*, *c2*, *red*). The eluted profile of soluble proteins extracted from infected leaves showed an additional peak at 206 min corresponding to virus-protein complexes (Fig. 1*A*, *c1*, *blue*). The same additional peak at 206 min was found in experiments using the extraction buffer added with 0.5 or 1 M NaCl (Fig. 2). In experiments with *in vitro* virus-host protein complexes, the same additional peak at 206 min was detected at a lower concentration in accordance with the amount of added purified virus (3.5 mg) (Fig. 1*B*, *c1*). After the purification/elution cycle, electron microscopy revealed the presence of intact virus particles in the collected fraction corresponding to peak c2 (Fig. 1*C*). In collected fraction corresponding to peak c1, virus particles were associated with electron dense materials (Fig. 1*D*). This method allowed us to isolate *in vivo* and *in vitro* virus-host protein complexes and

TABLE I

Proteins identified from in vivo and in vitro complexes

Non-redundant proteins identified in the 137 gel bands (Fig. 2*B*) and from the different *in vivo* and *in vitro* experiments are shown. In the first part, proteins were classified according to their similarity and number of identifications among the Azucena and IR64 cultivar *in vivo*; in the second part proteins were classified according to their differences and number of identifications between Azucena and IR64 cultivar *in vivo*. Columns annotated with 1, 2, and 3 wpi correspond to experiments realized with rice plants 1, 2, and 3 weeks postinoculation with the virus. Other columns refer to the experiments realized with different combinations of virus and host. The number inside dark rectangles indicates the number of peptides used to identify the protein, and crosses indicate that a similar function was identified in Swiss-Prot, TrEMBL, or TrEMBLnew protein databases. Some of the multigenic families are represented by two or more paralogs that were identified by a discriminating peptide. rubisco, ribulose-bisphosphate carboxylase/oxygenase.

Name of the common proteins to Azucena in vivo and IR64 *in vivo*	accession number	MW	Azucena *in vivo* 1 wpi	Azucena *in vivo* 2 wpi	Azucena *in vivo* 3 wpi	IR64 *in vivo* 1 wpi	IR64 *in vivo* 2 wpi	IR64 *in vivo* 3 wpi	IR64 *in vitro* 1 wpi	IR64 *in vitro* 2 wpi	IR64 *in vitro* 3 wpi	IR64 - FHV *in vitro*	*N. Tabacum* - RYMV *in vivo*	*P. Vulgaris* - RYMV *in vivo*	*P. Vulgaris* - SCPMV *in vivo*
5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase	11686.m04282	84584	10	17	28	25	20	13	16	14	19		X		
5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase	11686.m04283	84666	10	15	33	26	19	14	16	14	20				
CoA-ligase, putative	11667.m01918	67855	5	13	21	19	11	14	2	13	15	2			
Fructose-bisphosphate aldolase class-I	11667.m06837	38799	14	15	18	17	15	19	10	13	13		X		X
Fructose-bisphosphate aldolase class-I	11674.m00179	38370	5	5	6	4	5	5			4				
Fructose-bisphosphate aldolase class-I	11680.m03968	37731	15	13	15	17	14	14	4	11	10				
Fructose-bisphosphate aldolase class-I	11682.m03129	38839	15	10	16	16	16	19	8	12	10				
Fructose-bisphosphate aldolase class-I	11687.m00638	41980	4	3	20	5	9	15	15	6	12	7			
ferredoxin-dependent glutamate synthase (fragments)	11673.m04580	176883	33	54	74	49	47	53	7	18	42		X		
nucleoside diphosphate kinase iii, chloroplast/mitochondrialprecursor (ec 2.7.4.	11682.m04999	25921	3	3	4	2	5	3	4		4				
Ribulose bisphosphate carboxylase, small subunit, putative	11686.m01931	14940	11	10	8	12	12	7	15	9	12	12	X		
Oxygen evolving enhancer protein 3 (PsbQ)	11673.m03487	22967	9	10	10	13	13	16		11	15				
mitochondrial chaperonin-60	11676.m02851	60850	3	14	11	9	14	10	10	17	25	3	X		
Peroxidase, putative	11667.m02169	37210	6	6	7	6	3	6	4	3	4	4	X		
Subtilase family, putative	11667.m06510	78811	5	6	8	5	4	5		2					
reversibly glycosylated polypeptide	11669.m03987	41322	11	9	16	10	11	13	20	19	25		X	X	
probable DNA-binding protein GBP16 - rice	11682.m02647	43170	8	7	8	4	14	12				11			
glyceraldehyde-3-phosphate dehydrogenase, C terminal domain, putative	11670.m03659	42689	8	9	4	9	6		11	9	14	9	X	X	X
glyceraldehyde-3-phosphate dehydrogenase, type I	11670.m03922	36673	9	6	4	5	5		7	6	7				
glyceraldehyde-3-phosphate dehydrogenase, type I	11674.m00238	36390	10	8	11	9	9		11	7	7				
Staphylococcal nuclease homologue, putative	11668.m03043	108182	6	26	5	20	7			8	27				
expressed protein	11669.m06021	41020	4	8	8	2	6								
dnaK-type molecular chaperone hsp70 - rice (fragment)	11687.m04489	71185	2	12	6		4	5	10		4	12	X	X	X
Protease inhibitor/seed storage/LTP family, putative	11667.m06303	12282	2	3		4	2	3							
Protease inhibitor/seed storage/LTP family, putative	11686.m00141	11338	2	5		4	3	5			2				
3-hydroxyacyl-CoA dehydrogenase, C-terminal domain, putative	11668.m01643	78460		11	7	7	9	14	4		8				
beta-glucosidase	11669.m04987	56836		9	7	15	18	22	10						
Proteasome A-type and B-type, putative	11668.m05203	26257		9	11	5	7	9	6	5	10				
Proteasome A-type and B-type, putative	11680.m00541	29766		10	9	4	7	9	6	5	9				
Proteasome A-type and B-type, putative	11681.m03080	65875		13	5	7	5	5		9	9				
proteasome subunit alpha type 3 (ec 3.4.25.1) (20s proteasome alphasubunit g	11667.m05921	28132		8	9	2	2	8	2		6			X	
proteasome subunit alpha type 5 (ec 3.4.25.1) (20s proteasome alphasubunit e	11687.m03741	25977		6	7	2	3	3	3		9			X	
proteasome subunit beta type 1 (ec 3.4.25.1) (20s proteasome alphasubunit f)	11681.m03002	24266		10	6	3	6	8	3	6	9		X		
proteasome subunit beta type 3 (ec 3.4.25.1) (20s proteasome alpha subunit c	11680.m04278	35345		4	6	4	10	9	6	4	10		X	X	
20S proteasome beta 4 subunit	11669.m04911	23463		9	10	8	7	11	7	7	10		X	X	
putative exoglucanase precursor	11669.m05450	67735		10	5	3	4	3			8				
Carbonic anhydrase, putative	11667.m04353	29117	12	12	4	10			14				X	X	
transketolase	11680.m00347	80028	17	17	5	13			21		8		X		
phosphoglycerate kinase	11682.m03991	50492	5	3	2	2			17		5	10	X	X	X
aminotransferase, class V	11674.m03932	44397	6	2	2		4		9				X		X
reversibly glycosylated polypeptide	11673.m04044	41253	6	5	10		4		10	6	16				
chaperone protein DnaK	11682.m02183	73505	2	3	4			6	8		4	6			
ribosomal protein S15, putative	11686.m01022	58994	6	8		8	2			10	12	17			
Protease inhibitor/seed storage/LTP family, putative	11686.m00142	11499	3	2		2	2								
Protease inhibitor/seed storage/LTP family, putative	11686.m00143	11506	2	2		2	2								

retrotransposon protein, putative, unclassified	11667.m05749	100020	8	10		11	3			12	10	10			
thioredoxin peroxidase	11668.m03158	28097	4		7	4		3	3					X	
probable proteasome endopeptidase complex (EC 3.4.25.1) alpha chain - rice	11674.m04395	35352		2	6	4	8		22	14	19				
Proteasome A-type and B-type, putative	11680.m00661	27026		7	6	3		6	3	3	5				
Proteasome A-type and B-type, putative	11682.m00922	28979		5	5	3		4	4		3				
proteasome subunit alpha type 6 (ec 3.4.25.1) (20s proteasomealpha subunit a	11669.m00798	27556		10	11	5		2	5	3	6		X		
ferredoxin--nadp reductase, leaf isozyme, chloroplast precursor(ec 1.18.1.2) (fr	11680.m00092	39982		2	5		7	3	3		4		X		
pyruvate decarboxylase	11669.m01843	64677		8	3		3	3			2				
Eukaryotic aspartyl protease	11673.m04582	46276		3	8		5	3		2					
phenylalanine ammonia-lyase	11668.m03971	75498		16	31		15	22	19		23	3			
prolyl-tRNA synthetase	11686.m02474	57950		6		3	2	4							
4-alpha-glucanotransferase, putative	11673.m04620	105744	7	4		3			10	4	11	9			
2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltrans	11670.m03088	48283	3	4		3					6	5			
Similar to ribulose-bisphosphate carboxylase large subunit	11687.m03018	9711	2	3		2				3	3	3	X	X	
RNA binding protein, putative	11686.m02218	44701	2	5		10			5	9	13	7			
putative basic protein	11669.m05052	13461	2	3			3		2	2	3				
phosphoglucomutase	11669.m05086	62910	2	2				3	4				X		
translationally controlled tumor protein homolog (tctp).	11687.m04135	18934	3	2				2	4						
putative basic blue copper protein	11669.m06498	12821		2	3	3									
proteasome subunit alpha type 1 (ec 3.4.25.1) (20s proteasome alphasubunit f	11668.m00341	29612		4	5	2			4	5	5				
DREPP2 protein	11668.m01751	24034		3	2		3		2						
proteasome subunit alpha type 3 (ec 3.4.25.1) (20s proteasome alphasubunit g	11682.m03938	27203		9	7			4			6				
Malic enzyme, N-terminal domain, putative	11667.m05146	71456		10	12			9	4		9		X	X	
Peroxidase, putative	11670.m05864	38181		3	10			2							
Elongation factor TS, putative	11686.m03492	121014		17		4	7		19	13	19	22			
TCP-1/cpn60 chaperonin family	11680.m00146	64085		7		4	12			7	19	8			
proteasome subunit alpha type 2 (ec 3.4.25.1) (20s proteasome alphasubunit b	11668.m04045	25828		10			2	7	3	6	8		X		
methyl-binding domain protein MBD106, putative	11686.m04243	31491		5			7	5							
ATP synthase F1, beta subunit	11676.m01781	60260	3			5			10		4	9			
translation elongation factor EF-1, subunit alpha	11669.m00767	49262	2			2			6		7	3	X	X	
expressed protein	11680.m01526	16656	2			3						3			
Elongation factor G, domain IV, putative	11668.m03009	93961	4				11		5						
dihydrolipoamide S-acetyltransferase	11674.m03308	52541		4		2				2		2			
citrate (Si)-synthase, eukaryotic	11668.m00931	52229		5		3									
glyceraldehyde-3-phosphate dehydrogenase, type I, putative	11669.m00306	47081		3		5			6	4		7			
glutaminyl-tRNA synthetase, putative	11667.m00855	89847		7		4									
glutamyl-tRNA synthetase	11676.m01897	80836		7		3					6	5			
heat shock protein 82	11674.m03916	70703		10		2				3	17	5			
Elongation factor 1 gamma, conserved domain, putative	11680.m03639	47390		4			6			4	14				
elongation factor 1-gamma (ef-1-gamma) (eef-1b gamma).	11668.m01176	112373		2			11			5	18				
adenosylhomocysteinase	11687.m02417	49299		4				3	6		7				
thiazole biosynthesis enzyme, putative	11673.m03325	37201		6				2							
nucellin-like aspartic protease	11687.m00750	44732			2			3							
fructose-1,6-bisphosphatase, putative	11670.m01491	42245			9			6	8						
Fructose-bisphosphate aldolase class-I, putative	11667.m00198	178342			2			2							
GDSL-like Lipase/Acylhydrolase	11682.m01126	38959			10		9	7							
Glutamine synthetase, catalytic domain, putative	11670.m05561	49428			2		2		6	4		4	X		
expressed protein	11669.m05809	50657			2		2								
malate dehydrogenase, NAD-dependent	11682.m04796	35414			4			3	2						
cytoplasmic malate dehydrogenase	11676.m02982	35546			9			6	2				X		X

TABLE I—*continued*

Name of the common proteins to Azucena in vivo and IR64 *in vivo*	accession number	MW	Azucena *in vivo* 1 wpi	Azucena *in vivo* 2 wpi	Azucena *in vivo* 3 wpi	IR64 *in vivo* 1 wpi	IR64 *in vivo* 2 wpi	IR64 *in vivo* 3 wpi	IR64 *in vitro* 1 wpi	IR64 *in vitro* 2 wpi	IR64 *in vitro* 3 wpi	IR64 - FHV *in vitro*	*N. Tabacum* - RYMV *in vivo*	*P. Vulgaris* - RYMV *in vitro*	*P. Vulgaris* - SCPMV *in vivo*
phosphoenolpyruvate carboxykinase, putative	11669.m01495	73158			6			3							
Copper/zinc superoxide dismutase, putative	11674.m04529	21301			2			2	4				X		
Peroxidase, putative	11682.m00373	37613			3			2							
expressed protein	11667.m07127	43759			4			10							
Name of the specific proteins to Azucena *in vivo* and to IR64 *in vivo/in vitro*															
glycine dehydrogenase	11667.m05023	111356	8	11	8				16	4	6		X		
glycine dehydrogenase	11680.m04002	111401	6	10	8				20	4	6				
putative glyoxysomal malate dehydrogenase	11669.m05674	36699	4	4					6						
peptidylprolyl isomerase (EC 5.2.1.8) Cyp2 - rice	11668.m00205	18349	4	2					3						
probable chitinase (EC 3.2.1.14) IIb - rice	11670.m03999	25151	8	4					4		4				
alpha-mannosidase	11687.m02967	114157		7	8				3						
dnaK protein	11667.m00805	93049		7	10						10				
proteasome subunit beta type 3 (ec 3.4.25.1) (20s proteasome alpha subunit c)	11668.m00821	22769		5	6						8				
putative aldehyde oxidase	11669.m05835	145064		23	7				5		5				
phenylalanine ammonia-lyase	11670.m04234	75991		7	15						9				
FMN-dependent dehydrogenase	11673.m00499	40219	2						10						
fructose-1,6-bisphosphatase	11667.m06481	37012	2						3						
glycine cleavage system T protein	11670.m05219	43968	3						8						
glycolate oxidase	11669.m05782	40384	2						9				X		
Glycosyl hydrolases family 17	11667.m07188	35682	9						3						
nucleoside diphosphate kinase i (ec 2.7.4.6) (ndk i) (ndp kinase i)(ndpk i)	11673.m02949	16851	3						2				X		
pyruvate kinase	11687.m00429	57284	2						4		2				
triosephosphate isomerase	11681.m03239	32373	2						10						
putative glutathione S-transferase	11676.m03515	24758	5						2						
expressed protein	11673.m00079	42609	2						3						
Peptidase family M1, putative	11668.m01161	98391		4					3						
sucrose-UDP glucosyltransferase 2	11669.m02894	92849		6							6	8			
AL161582 aminopeptidase-like protein	11674.m03033	91231		2					4						
translation elongation factor eEF-1 beta - rice	11673.m04142	24847		3						4					
isoleucyl-tRNA synthetase, putative	11680.m04299	155097		9						5	14	2			
Oxidoreductase NAD-binding domain, putative	11668.m00036	40638		5							2	2			

Subtilase family, putative	11668.m04289	145477		17								12				
glutamate dehydrogenase	11669.m05875	44341			2					2						
ferredoxin--nitrite reductase	11668.m05166	72403			3					6						
Peptidase family M1, putative	11674.m04538	99461			3					10						
phosphoenolpyruvate carboxylase	11674.m02727	10986			8					2						
phosphoglycolate/pyridoxal phosphate phosphatase family, putative	11670.m03968	40194			3					3						
ribose 5-phosphate isomerase A	11673.m00734	29179			3					6						
Calreticulin family, putative	11673.m01387	50127			2					3						
TCP-1/cpn60 chaperonin family	11669.m00441	61045				7	9	7		2		16				
peroxiredoxin Q	11680.m00923	23722				3	9	4								
putative valyl tRNA synthetase	11669.m04902	119588				9	2									
D-isomer specific 2-hydroxyacid dehydrogenase, NAD binding domain, putative	11668.m00017	44786					18	2								
Cupin, putative	11674.m03549	21861					2	3								
3-hydroxyacyl-CoA dehydrogenase, NAD binding domain, putative	11667.m02406	79392				4						2				
Ribulose bisphosphate carboxylase large chain, N-terminal domain	11682.m03338	16430				2						3				
glutaminyl-tRNA synthetase, putative	11682.m00871	90249				2										
AhpC/TSA family, putative	11668.m00918	23179				2				8						
Remorin, C-terminal region, putative	11670.m04374	22415				3						2				
Similar to serine carboxypeptidase II-like protein	11668.m04044	39574				3										
hypothetical protein	11668.m04855	20724				5										
LysM domain, putative	11681.m03358	38661				4										
rubisco subunit binding-protein alpha subunit, chloroplast precursor(60 kda cha	11686.m01770	61093					9				16	15	5	X		
co-chaperone GrpE, putative	11668.m03831	36535					2					4				
proteasome subunit alpha type 7 (ec 3.4.25.1) (20s proteasomealpha subunit d	11681.m03266	27197					4			3				X		
oxidoreductase, aldo/keto reductase family	11668.m05657	36427					2									
CAA303715.1 protein	11670.m05868	35205					4									
HMG (high mobility group) box, putative	11680.m05109	14124					2						2			
putative 14-3-3 protein	11669.m05065	29160					2			2	2	3				
6-phosphogluconate dehydrogenase, decarboxylating	11680.m00122	52721						3		2						
adenosine kinase	11668.m03967	36993						3		2						
lactoylglutathione lyase, putative	11674.m00866	32553						4								
legumin-like protein	11682.m00174	38195						4								
UTP--glucose-1-phosphate uridylyltransferase	11681.m03401	54886						4		4						
chitinase (EC 3.2.1.14) III C10701 - rice	11687.m04472	32996						3								
Multicopper oxidase, putative	11673.m03122	61913						4		2						
Peroxidase, putative	11667.m07348	39638						5								
putative pollen allergen	11676.m03619	29282						2								
Protease inhibitor/seed storage/LTP family, putative	11667.m06048	13923						2								
C2 domain, putative	11673.m03026	18905						5								
Phosphate-induced protein 1 conserved region	11668.m05085	34162						2								
Phosphate-induced protein 1 conserved region	11680.m01136	33015						2								
Phosphoribulokinase / Uridine kinase family, putative	11668.m04554	44387								3	6			X	X	X
proteasome subunit alpha type 2 (ec 3.4.25.1) (20s proteasome alphasubunit b	11669.m02741	25844									6	6				
reversibly glycosylated polypeptide	11670.m05573	46005									3	8				
ATP synthase F1, beta subunit	11667.m04784	59463								4		9	5			

TABLE I—*continued*

Name of the common proteins to Azucena in vivo and IR64 *in vivo*	accession number	MW	Azucena *in vivo* 1 wpi	Azucena *in vivo* 2 wpi	Azucena *in vivo* 3 wpi	IR64 *in vivo* 1 wpi	IR64 *in vivo* 2 wpi	IR64 *in vivo* 3 wpi	IR64 *in vitro* 1 wpi	IR64 *in vitro* 2 wpi	IR64 *in vitro* 3 wpi	IR64 - FHV *in vitro*	*N. Tabacum* - RYMV *in vivo*	*P. Vulgaris* - RYMV *in vitro*	*P. Vulgaris* - SCPMV *in vivo*
ATP synthase F1, alpha subunit	11681.m00716	55325							2						
Similar to ferredoxin-nitrite reductase (EC 1.7.7.1) precursor - rice	11667.m02487	55745							8						
flavodoxin, putative	11667.m05707	21705							3						
glucose-6-phosphate isomerase, putative	11674.m03720	67229							2						
glutamate dehydrogenase 2 (ec 1.4.1.3) (gdh 2). [mouse-ear cress	11670.m04472	45594							2						
glutamate-1-semialdehyde-2,1-aminomutase	11674.m04228	50237							6						
isocitrate dehydrogenase, NADP-dependent	11667.m04493	46013							10						
NAD binding domain of 6-phosphogluconate dehydrogenase, putative	11668.m03371	30496							3						
probable transaminase (EC 2.6.1.-) T16L1.170 [similarity] - Arabidopsis thalian	11669.m01908	49857							5						
ribulose-5-phosphate-3-epimerase	11669.m00693	29034							2						
Thiamine pyrophosphate enzyme, central domain, putative	11667.m03019	49557							2						
triosephosphate isomerase	11667.m00476	26152							6						
ubiquitin-activating enzyme e1 2	11687.m00052	117047							4						
dnaK protein	11682.m03345	74267							3						
protein disulfide isomerase 2 precursor	11687.m00870	56819							5						
translation elongation factor Tu	11668.m03655	50382							2			13			
putative aldehyde oxidase	11669.m05834	145336							5						
Hsp90 protein, putative	11674.m03795	130865							2						
Lipoxygenase, putative	11674.m03993	104494							2						
oxidoreductase, zinc-binding dehydrogenase family, putative	11674.m02863	39558							2						
PsbP	11673.m00394	26939							3						
putative trypanothione-dependent peroxidase	11669.m02934	63249							3						
putative salt-induced protein	11669.m02877	19971							3						
Similar to salt-stress root protein rs1	11667.m01299	21801							4						
At3g19170/MVI11_8	11668.m05126	121131							2						
ADP-ribosylation factor family	11667.m01605	25003							3						
retrotransposon protein, putative, unclassified	11668.m05463	205627							2						
pyruvate dehydrogenase E1 beta subunit isoform 3	11681.m03028	40226								2					
Similar to 26S proteasome subunits	11670.m03506	47567								2		6			
6,7-dimethyl-8-ribityllumazine synthase, putative	11670.m04040	22471									5		X		
ATP synthase F1, alpha subunit	11670.m01497	55665									8	13	X		
citrate synthase I	11668.m01280	56640									4	4			
similar to glucosidase II alpha subunit	11669.m01126	72311									4				

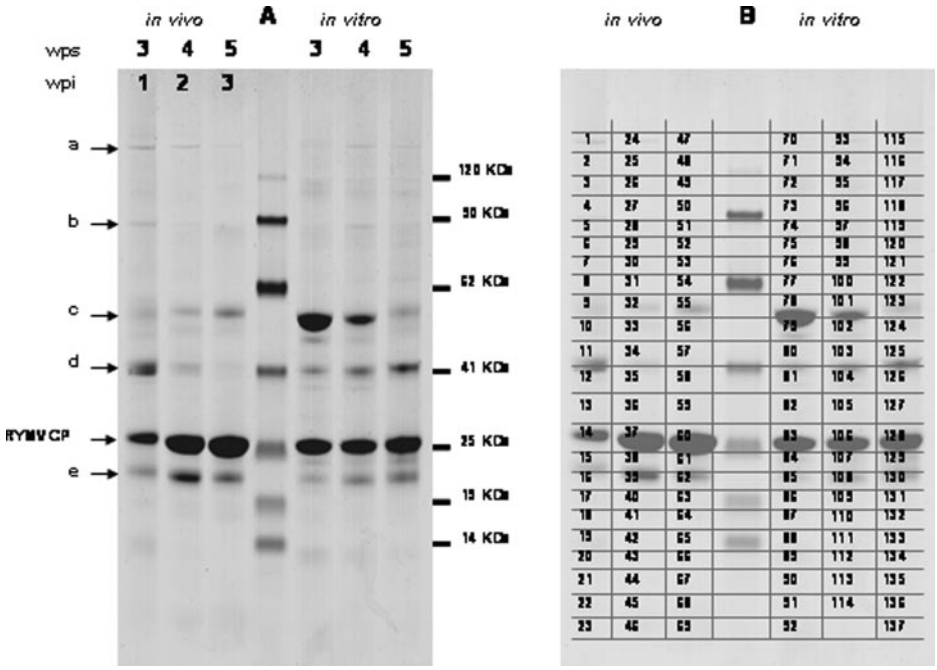

FIG. 3. **Gels of denatured RYMV-IR64 protein complexes.** *A*, SDS-PAGE of RYMV-IR64 protein complexes collected after size exclusion chromatography from *in vitro* IR64 infected leaves and from *in vitro* binding experiments. Plants at 1, 2, and 3 wpi correspond to plants at 3, 4, and 5 wps. *B*, same gel showing the numbering and localization of samples analyzed by LC-MS-MS. Each gel lane was cut and analyzed after tryptic digestion.

was further used to identify virus-host protein partners by nano-LC-MS/MS.

SDS-PAGE Separation—At different times of plant development (3, 4, and 5 weeks postseedling (wps)) and during the time course of infection (1, 2, and 3 wpi) virus-host protein complexes were isolated from *in vivo* and *in vitro* experiments, denatured, and separated by SDS-PAGE gel (Fig. 3*A*). Gel protein profiles were reproducible when virus, plant varieties, and stage of development were identical (data not shown). In contrast the protein profiles for *O. sativa* IR64 variety (Fig. 3*A*) differed according to the stage of development and the time course of infection for *in vivo* experiments or stage of development for *in vitro* experiments. When comparing the same stage of development, we observed specific profiles for *in vivo* and *in vitro* experiments. This observation supports the idea that complexes isolated from infected plants were already present in the *in vivo* tissues and were not formed during the protein extraction process. Thus, we observed for visible *bands a*, *b*, *c*, *d*, and *e* different accumulations between *in vivo* and *in vitro* experiments at different stages of development (Fig. 3*A*). To identify these proteins, systematic cutting and nano-LC-MS/MS analysis of these gel lanes were performed.

Identification of Virus Host Protein Partners by Mass Spectrometry—As expected, we identified the RYMV coat protein (CP) (Swiss-Prot accession number Q9DGX1) around 26 kDa in accordance with the results obtained by Western blot using monoclonal antibody against the virus (data not shown). For the same total amount (20 μg) of proteins loaded on each lane, the amount of CP increased during the time course of infection, whereas equal amounts were observed for complexes purified from *in vitro* experiments (Fig. 3*A*).

The virus coat protein was present at such a high concentration that it was identified in all gel bands independently from location in the gel. As the nano-LC-MS/MS automatic acquisition program selected the most prevalent peptides for fragmentation, the high abundance of the coat protein prevented the identification of less abundant proteins. To circumvent this problem, we have established an exclusion program to informatically exclude the masses of the coat protein tryptic peptides, thereby preventing their selection and allowing the selection of peptides belonging to other proteins (8). As shown in Fig. 3*B*, 137 gel bands were analyzed by nano-LC-MS/MS for the experiments with the IR64 cultivar with RYMV, and 2,017 proteins were identified in the rice pseudomolecules release 3 database (data not shown). Nevertheless most of the proteins were identified several times, and we have presented here only the 223 non-redundant identified proteins (Table I).

Functional Distribution of Recruited Host Proteins—For the 223 identified proteins using the IR64 cultivar, the following distribution was found. 19% were identified only *in vivo*, 41% were identified both in *vivo* and *vitro*, and 40% were present only *in vitro*. The major functional category corresponded to proteins involved in metabolism functions (Fig. 4, *Me*), mainly glycolysis, photosynthesis, amino acid, lipid, and cell wall metabolism. The second category corresponded to functions involved in translation and protein synthesis (*T*) including translation factors, elongation factors, tRNA synthetases, protein-disulfide isomerase, chaperone proteins, and proteasome. The third category was related to defense (*D*) with protein chaperones (*i.e.* 70, 82, and 90 kDa); proteins involved in defense pathways such as superoxide dismutase, phenylalanine-ammonia lyase, homocysteine *S*-methyltransferase, and lipoxygenase; proteins related to oxidative stress with

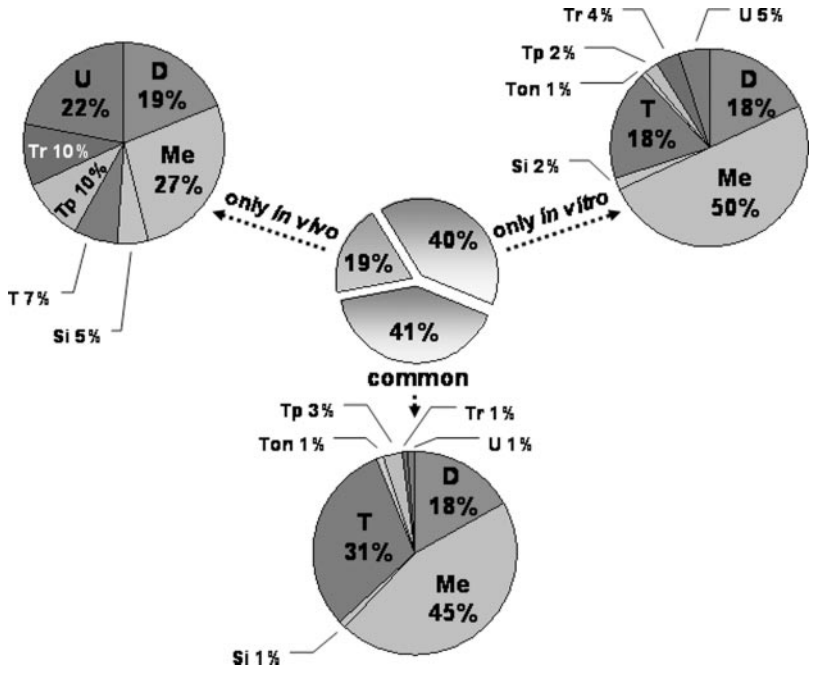

FIG. 4. **Distribution of proteins identified from RYMV-IR64 experiments in Table I.** The *central circle* is divided into proteins detected only in *in vivo* experimentations, only in *in vitro* experimentations, and *in vivo* as well *in vitro* experimentations (*common*). For each group, functional distribution into metabolism (*Me*), defense-stress (*D*), transcription (*Tr*), translation/protein synthesis (*T*), transport (*Tp*), signal transduction (*Si*), unknown (*U*), and transposon (*To*) is shown.

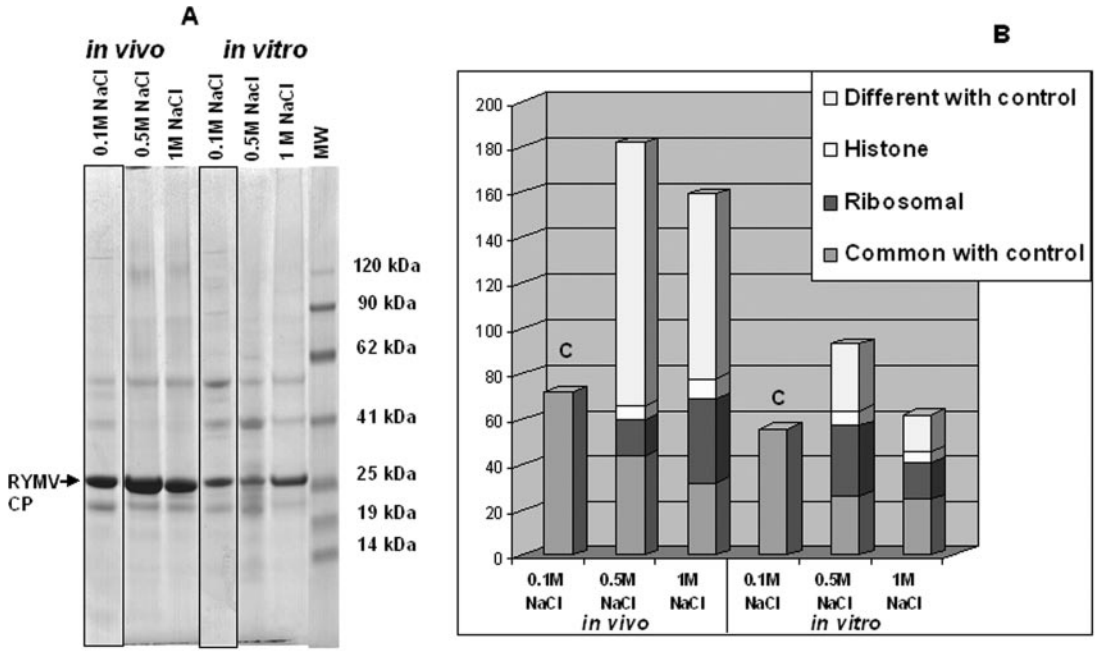

FIG. 5. **Effects of increased NaCl concentration during extraction-purification of complexes.** *A*, SDS-PAGE of RYMV-host protein complexes extracted and purified by size exclusion chromatography from 2 wpi *in vivo* IR64 infected leaves or from *in vitro* binding experiments at different salt concentrations. Extractions and exclusion size chromatography were realized with buffers added with 0.1 M NaCl, 0.5 M NaCl, and then 1 M NaCl. *B*, histogram of non-redundant proteins identified by LC-MS-MS (see Table II) in comparison with a control, *C* (IR64 2 wpi 0.1 M NaCl *in vivo* and IR64 4 wps 0.1 M NaCl *in vitro*). Ribosomal proteins and histone proteins (except ribosomal protein S15, putative 11686.m01022) were identified only at 0.5 and 1 M NaCl.

thioredoxin, peroxiredoxin, and oxidoreductase NAD binding; and glutathione *S*-transferase as well as pathogenesis-related proteins including peroxidase and chitinases. Other categories, less represented, were related to unknown functions (*U*), transport (*Tp*), and transcription (*Tr*).

Proteins identified only in *in vivo* experimentations (19%) might be explained by the specificity of a recruiting made *in planta* and by the inability for these already coated virus particles to recruit more proteins during the extraction process. Proteins identified only *in vitro* experimentations (40%)

might be explained by a recruiting due to the solubilization of proteins that were not accessible for the virus *in planta*. Some of these proteins were identified in *in vivo* experimentations with Azucena cultivar (*i.e.* putative aldehyde oxidase 11669.m05835) and might be involved in a specific recruiting *in planta*, but for the others we could not exclude an artifact recruiting. For the common proteins, it was highly probable that they could be recruited *in planta*, and they were recruited *in vitro* because they are extracted in *in vitro* experimentations.

Paralog Identification—In some cases, we were able to discriminate which paralog gene among a multigenic family was specifically recruited and present in the virus-host protein complex (see "Experimental Procedures"). This was the case for the paralog 11668.m03971 for phenylalanine-ammonia lyase (defense category) specifically identified among 10 paralogs (TIGR database) from this multigenic family. In addition, some specific paralogs were recruited only *in vivo* such as peroxidase, putative 11682.m00373; others were recruited only *in vitro* such as reversibly glycosylated polypeptide 11670.m05573. Additionally we observed the same paralogs identified *in vivo* and *in vitro* (peroxidase, putative 11667.m02169, for example). Of interest was the example of glyceraldehyde-3-phosphate dehydrogenase (in the metabolism category) where the number of recruited paralogs varied with the experimental conditions: four paralogs (Table I) were recruited *in vivo* and *in vitro* by the virus at 1 week postinfection, three paralogs were still recruited *in vivo* at 2 wpi instead of four *in vitro*, and finally no paralog from glyceraldehyde-3-phosphate dehydrogenase was recruited *in vivo* at 3 wpi, whereas three paralogs were identified *in vitro* at 3 wpi. These results suggested that some specific paralogs among multigenic families were recruited at specific points during the time course of virus infection.

Specificity of Host Protein Recruiting—To investigate the specificity of host protein recruiting by RYMV, we used the following strategy. (i) We used different NaCl concentrations to extract and isolate complexes from *in vivo* or *in vitro* experiments to confirm that some of the protein interactions occurred at low ionic strength and may have been unspecific. (ii) We then investigated whether the recruited proteins were host- and virus-dependent by testing different virus-host pairs.

Varying NaCl Concentration—Protein profiles were quite different according to the salt concentration used in *in vivo* and *in vitro* extractions (Fig. 5*A*). As expected, the amount of CP from *in vitro* experiments at different salt concentrations was similar. On the contrary, in *in vivo* experiments we observed a higher amount of CP at 0.5 and 1 M NaCl, suggesting that a higher amount of complexes was extracted with a high ionic strength buffer probably due to the better extraction of some subcellular compartments. The higher number of proteins identified at 0.5 and 1 M NaCl confirmed this result, specifically the ribosomal proteins, which were localized in nucleolus, mitochondria, and chloroplasts, and also histone proteins, which were localized in nucleus (*in vivo* and *in vitro*, Fig. 5*B*). Furthermore proteins from proteasome, which localized to cytoplasm, are well identified at low as well as high ionic strength (because the cytoplasmic compartment is easier to extract) (Table II). We confirmed that the amount of proteins extracted from Azucena cultivar infected leaves showed an 18% increase when using buffer added with 0.5 and 1 M NaCl compared with 0.1 M NaCl (data not shown). We noticed a decrease in the number of proteins that were common with proteins identified at 0.1 M NaCl due to the possible abolition of low ionic strength interactions. We observed an expected decrease of total proteins at 1 M NaCl (151 *versus* 163 at 0.5 M for *in vivo* and 62 *versus* 93 at 0.5 M for *in vitro*). The common proteins that were still identified at 1 M NaCl suggest that the binding was specific and that they strongly interacted with virus particles (Table II).

Our results suggested two effects of salt concentration. First an increase of salt concentration to 0.5 M increased the number of proteins identified in complexes, resulting from a better extraction of complexes due to the ability of high salt concentration to extract proteins highly bounded to the membranes (13, 14) and to break membrane compartments (osmotic pressure) but not solubilize the membranes as a detergent could do. On the contrary, the increase of salt concentration to 1 M reduced the number of common proteins that were also identified at 0.1 M NaCl, suggesting that proteins still identified at high salt concentration were strongly specific to the complexes.

Different Pairs of Virus-Host Plant—To see further whether this affinity was host- and virus-dependent, we studied complexes extracted and purified from various virus-hosts combinations (Fig. 6).

First we compared at 1, 2, and 3 wpi the *in vivo* interaction of RYMV with two subspecies of *O. sativa*: the susceptible *O. sativa* ssp. *indica* (IR64) and the tolerant *O. sativa* ssp. *japonica* (Azucena) (Fig. 6*A*). More proteins were identified for Azucena than for IR64 (171 *versus* 135 proteins identified when we cumulated 1, 2, and 3 wpi identifications). Among these proteins, a large number (100) were common for both subspecies (Table I), confirming that the recruiting among subspecies was quite similar. It was likely that the proteins identified differentially in Azucena and IR64 take part in the tolerance or susceptibility of these subspecies (Table I).

We further investigated the recruiting between different viruses and different host plants: (i) a compatible interaction between another *Sobemovirus* (SCPMV) and its host plant *P. vulgaris* and (ii) three incompatible interactions, one between insect virus FHV and IR64 and two other pairs, RYMV-*N. tabacum* and RYMV-*P. vulgaris.* We identified some identical protein functions among all the different complexes (Fig. 5*B*), but we could not make precise conclusions about the percentage of similarity of these recruiting activities due to the lack of protein databases concerning these two others plants (Table I).

TABLE II

Proteins identified from in vivo and in vitro complexes at different salt concentration

Non-redundant proteins identified with the IR64 cultivar from the different *in vivo* and *in vitro* experiments using an extraction buffer added with 0.1, 0.5, and 1 M NaCl. Proteins were classified according to their identification in all experiments compared with proteins identified only with 1 M NaCl *in vivo*. rubisco, ribulose-bisphosphate carboxylase/oxygenase.

Name of the proteins	accession number	MW	*in vivo* 0,1 M NaCl	*in vivo* 0,5 M NaCl	*in vivo* 1 M NaCl	*in vitro* 0,1 M NaCl	*in vitro* 0,5 M NaCl	*in vitro* 1 M NaCl
Fructose-bisphosphate aldolase class-I	11687.m00638	41980	9	10	8	6	10	10
mitochondrial chaperonin-60	11676.m02851	60850	14	27	19	17	28	16
20S proteasome beta 4 subunit	11669.m04911	23463	7	9	7	7	9	8
probable proteasome endopeptidase complex (EC 3.4.25.1) alpha chain	11674.m04395	35352	8	8	5	14	7	2
Proteasome A-type and B-type, putative	11668.m05203	26257	7	12	11	5	11	9
Proteasome A-type and B-type, putative	11680.m00541	29766	7	12	7	5	7	6
Proteasome A-type and B-type, putative	11681.m03080	65875	5	11	8	9	10	6
proteasome subunit alpha type 2 (ec 3.4.25.1) (20s proteasome alphasu	11668.m04045	25828	2	9	6	6	9	8
proteasome subunit beta type 1 (ec 3.4.25.1) (20s proteasome alphasub	11681.m03002	24266	6	10	11	6	9	6
proteasome subunit beta type 3 (ec 3.4.25.1) (20s proteasome alpha sul	11680.m04278	35345	10	6	4	4	5	5
retrotransposon protein, putative, unclassified	11667.m05749	100020	3	9	12	12	11	8
reversibly glycosylated polypeptide	11669.m03987	41322	11	20	21	10	22	21
reversibly glycosylated polypeptide	11673.m04044	41253	4	9	12	6	18	14
ribosomal protein S15, putative	11686.m01022	58994	2	7	10	10	8	7
Ribulose bisphosphate carboxylase, small subunit, putative	11686.m01931	14940	12	9	11	9	9	7
rubisco subunit binding-protein alpha subunit, chloroplast precursor(60 k	11686.m01770	61093	9	9	15	16	11	6
proteasome subunit alpha type 3 (ec 3.4.25.1) (20s proteasome alphasu	11667.m05921	28132	2	12	5		4	2
proteasome subunit alpha type 5 (ec 3.4.25.1) (20s proteasome alphasu	11687.m03741	25977	3	12	6		6	4
TCP-1/cpn60 chaperonin family	11669.m00441	61045	9	18	13		21	12
TCP-1/cpn60 chaperonin family	11680.m00146	64085	12	13	13	7	15	8
elongation factor 1-gamma (ef-1-gamma) (eef-1b gamma).	11668.m01176	112373	11	11	12	5		5
ferredoxin-dependent glutamate synthase (fragments)	11673.m04580	176883	47	42	13	18	6	
dnaK-type molecular chaperone hsp70 - rice (fragment)	11687.m04489	71185	4	9	8		3	
phenylalanine ammonia-lyase	11668.m03971	75498	15	32	15		20	
proteasome subunit alpha type 7 (ec 3.4.25.1) (20s proteasomealpha su	11681.m03266	27197	4	7	4		6	
5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferas	11686.m04283	84666	19	12	4	14		
Elongation factor TS, putative	11686.m03492	121014	7	12	27	13		
glyceraldehyde-3-phosphate dehydrogenase, C terminal domain, putativ	11670.m03659	42689	6	4	7	9		
glyceraldehyde-3-phosphate dehydrogenase, type I	11674.m00238	36390	9	2	3	7		
aminotransferase, class V	11674.m03932	44397	4	3	4			
co-chaperone GrpE, putative	11668.m03831	36535	2	9	4			
6,7-dimethyl-8-ribityllumazine synthase, putative	11670.m04040	22471		4	3		2	2
EF-1 guanine nucleotide exchange domain, putative	11673.m04615	23800		7	9		4	2
histone H2B	11667.m00490	16520		2	2		2	2
histone H3 - maize	11667.m06477	15259		3	3		6	2
histone h4	11667.m06180	11402		5	4		5	5
L1P family of ribosomal proteins	11667.m06421	27084		3	3		5	4
L1P family of ribosomal proteins	11674.m04492	24467		3	3		5	4
Proteasome A-type and B-type, putative	11680.m00661	27026		5	3	3	4	2
proteasome subunit alpha type 1 (ec 3.4.25.1) (20s proteasome alphasu	11668.m00341	29612		8	2	5	9	3
proteasome subunit alpha type 2 (ec 3.4.25.1) (20s proteasome alphasu	11669.m02741	25844			6	6	9	8
proteasome subunit alpha type 6 (ec 3.4.25.1) (20s proteasomealpha su	11669.m00798	27556		13	9	3	6	5
ribosomal protein L1, putative	11682.m03006	38756		2	10		9	6
Ribosomal protein L11, RNA binding domain, putative	11668.m04566	17706		4	4		4	4
ribosomal protein L21, putative	11668.m01493	23338		3	5		2	6
Ribosomal protein S8e	11668.m02727	26870		4	4		5	5
60s ribosomal protein l30	11667.m01697	12356			4		3	3
Similar to ribulose-bisphosphate carboxylase large subunit	11687.m03018	9711		2	3	3	3	3
translation elongation factor eEF-1 beta - rice	11673.m04142	24847		6	6	4	3	4
60s Acidic ribosomal protein	11674.m00142	11076		2	4			3
60s acidic ribosomal protein p2b	11682.m03504	11592		6	7			2

TABLE II—*continued*

Name of the proteins	accession number	MW	in vivo 0,1 M NaCl	in vivo 0,5 M NaCl	in vivo 1 M NaCl	in vitro 0,1 M NaCl	in vitro 0,5 M NaCl	in vitro 1 M NaCl
glycine dehydrogenase	11667.m05023	111356		15	6	4	9	
phosphoenolpyruvate carboxylase	11674.m02727	10986		36	27		13	
phosphoglycerate kinase	11682.m03991	50492		10	10			6
proteasome subunit alpha type 3 (ec 3.4.25.1) (20s proteasome alphasu	11682.m03938	27203		11	6		3	
putative 9S ribosomal protein	11669.m05635	24227		2	4			3
ribosomal protein L7/L12, putative	11667.m04571	18579		5	5		3	
Similar to acidic ribosomal protein P2a-2	11667.m00908	11600		2	2			2
Similar to ribulose 1,5-bisphosphate carboxylase	11676.m01782	38459		4	3		6	
thioredoxin peroxidase	11668.m03158	28097		11	7			6
dnaK protein	11669.m01695	71056		9	5			
2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succin	11670.m03088	48283		3	11			
alpha-mannosidase	11687.m02967	114157		16	2			
arabinoxylan arabinofuranohydrolase isoenzyme AXAH-II	11687.m00288	73421		2	2			
ATP synthase F1, alpha subunit	11670.m01497	55665		3	3			
expressed protein	11669.m02084	73965		8	4			
expressed protein	11669.m03961	77842		2	6			
glutamate dehydrogenase	11669.m05875	44341		10	3			
Glutamine synthetase, catalytic domain	11668.m04892	39176		12	7			
Glutamine synthetase, catalytic domain, putative	11670.m05561	49428		7	14			
glutamyl-tRNA synthetase	11676.m01897	80836		7	3			
glyceraldehyde-3-phosphate dehydrogenase, type I, putative	11669.m00306	47081		3	4	4		
glycolate oxidase	11669.m05782	40384		7	4			
hAT family dimerisation domain, putative	11682.m01384	80786		2	6			
heat shock protein 82	11674.m03916	70703		33	23	3		
histone deacetylase HD2, putative	11682.m05012	21171		2	3			
Hsp90 protein, putative	11680.m05012	95365		8	11			
Hsp90 protein, putative	11681.m02782	89164		15	10			
Hsp90 protein, putative	11681.m02845	80150		37	21			
isoleucyl-tRNA synthetase, putative	11680.m04299	155097		4	5	5		
Lipoxygenase	11686.m03661	104621		7	11			
Malic enzyme, N-terminal domain, putative	11667.m05146	71456		20	8			
phosphoenolpyruvate carboxylase	11668.m01380	109863		16	10			
Proteasome A-type and B-type, putative	11682.m00922	28979		4	2			
PurA ssDNA and RNA-binding protein	11667.m01560	33300		4	3			
putative glycine hydroxymethyltransferase	11669.m05342	56380		11	2			
Similar to 26S proteasome subunits	11670.m03506	47567		2	3	2		
Transferase family, putative	11667.m07012	110473		4	4			
translation elongation factor Tu	11668.m03655	50382		5	5			
60s ribosomal protein l7a	11674.m02302	29285		4			8	
acidic ribosomal protein P3a - maize	11680.m04833	11887		2			2	2
ferredoxin--nadp reductase, leaf isozyme, chloroplast precursor(ec 1.18.	11680.m00092	39982	7	8			3	
histone-like protein	11669.m05928	29612		4			2	
Oxidoreductase NAD-binding domain, putative	11668.m00036	40638		7			4	4
Peroxidase, putative	11667.m02169	37210	3	4		3		2
proteasome subunit beta type 3 (ec 3.4.25.1) (20s proteasome alpha sul	11668.m00821	22769		5			4	
reversibly glycosylated polypeptide	11670.m05573	46005		8		3	9	
14-3-3 protein	11674.m03731	28982		3				
5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferas	11686.m04282	84584	20	11		14		
AAA family ATPase, CDC48 subfamily	11676.m02643	90857		7				
aconitate hydratase 1	11669.m00378	106235		2				
adenosylhomocysteinase	11687.m02417	49299		6				
At2g47390/T8I13.23	11669.m01970	103830		7				
ATP synthase F1, beta subunit	11667.m04784	59463		2				
Calreticulin family, putative	11673.m01387	50127		4				
catalase, putative	11669.m00325	56728		9				

TABLE II—*continued*

Name of the proteins	accession number	MW	in vivo 0,1 M NaCl	in vivo 0,5 M NaCl	in vivo 1 M NaCl	in vitro 0,1 M NaCl	in vitro 0,5 M NaCl	in vitro 1 M NaCl
CoA-ligase, putative	11667.m01918	67855	11	4		13		
co-chaperone GrpE, putative	11670.m03396	31491		8				
co-chaperone GrpE, putative	11674.m02441	33698		2				
Copper/zinc superoxide dismutase, putative	11674.m04529	21301		2				
CPSF A subunit region, putative	11682.m04969	121803		2				
cystathionine beta-lyase	11680.m00746	41884		3				
cytoplasmic malate dehydrogenase	11676.m02982	35546		2				
dnaK protein	11668.m00151	73345		5				
dnaK protein, putative	11668.m04669	99309		3				
Elongation factor 1 gamma, conserved domain, putative	11680.m03639	47390	6	6		4		
Elongation factor G, domain IV, putative	11668.m03009	93961	11	8				
expressed protein	11667.m03764	34816		2				
expressed protein	11668.m00220	25774		2				
expressed protein	11668.m05132	18038		2				
FF domain, putative	11667.m03297	109608		4				
FMN-dependent dehydrogenase	11673.m00499	40219		4				
fructose-1,6-bisphosphatase, putative	11670.m01491	42245		6				
Fructose-bisphosphate aldolase class-I	11667.m06837	38799	15	5		13		
Fructose-bisphosphate aldolase class-I	11680.m03968	37731	14	2		11		
glutamate dehydrogenase 2 (ec 1.4.1.3) (gdh 2). [mouse-ear cress	11670.m04472	45594		13				
glutathione-disulfide reductase	11669.m00634	60215		11				
glycyl-tRNA synthetase beta subunit, putative	11680.m00041	119090		3				
hydrolase, carbon-nitrogen family, putative	11668.m03118	33442		4				
legumin-like protein	11682.m00174	38195		2				
LIM domain, putative	11680.m01279	140367		2				
Lipoxygenase, putative	11674.m03993	104494		5				
malate dehydrogenase, NAD-dependent	11682.m04796	35414		5				
methyl-binding domain protein MBD106, putative	11686.m04243	31491	7	9				
Nucleoside diphosphate kinase	11686.m03549	16663		6				
nucleoside diphosphate kinase i (ec 2.7.4.6) (ndk i) (ndp kinase i)(ndpk i	11673.m02949	16851		4				
nucleoside diphosphate kinase iii, chloroplast/mitochondrialprecursor (ec	11682.m04999	25921	5	3				
Peptidase family M1, putative	11668.m01161	98391		2				
Peroxidase, putative	11667.m02168	33279		5				
phosphoenolpyruvate carboxykinase, putative	11669.m01495	73158		3				
phosphoenolpyruvate carboxylase	11681.m01307	110405		20				
phosphoribosylaminoimidazole carboxylase, ATPase subunit, putative	11667.m00991	68388		13				
plant acid phosphatase, putative	11667.m00911	32947		2				
prolyl-tRNA synthetase	11686.m02474	57950	2	2				
Protease inhibitor/seed storage/LTP family, putative	11686.m00139	12137		2				
putative 14-3-3 protein	11669.m05065	29160	2	4		2		
putative aldehyde oxidase	11669.m05834	145336		6				
putative cyanase	11676.m02926	18596		7				
putative eukaryotic initiation factor subunit	11676.m03873	83129		3				
Remorin, C-terminal region, putative	11670.m04374	22415		3				
retrotransposon protein, putative, unclassified	11668.m05463	205627		3				
ribosomal protein L10, putative	11669.m01776	51617		6				
Similar to 26S proteasome subunits	11670.m03506	47567		2				
Similar to arabinoxylan arabinofuranohydrolase isoenzyme AXAH-II	11687.m00283	142084		10				

TABLE II—*continued*

Name of the proteins	accession number	MW	in vivo			in vitro		
			0,1 M NaCl	0,5 M NaCl	1 M NaCl	0,1 M NaCl	0,5 M NaCl	1 M NaCl
Thiamine pyrophosphate enzyme, central domain, putative	11667.m03019	49557		3				
translation initiation factor	11682.m04971	134302		2				
60s ribosomal protein l2 (l8) (ribosomal protein tl2).	11686.m03743	28232			3		5	3
dihydrolipoamide S-acetyltransferase	11674.m03308	52541			3	2		2
histone H2A-like protein	11682.m00152	16490			2		2	2
phosphoglycerate kinase	11682.m03994	50107			11			5
putative transposases	11669.m05285	80924			7		5	
Ribosomal L37ae protein family	11667.m04734	10236			3			2
ribosomal protein L10, putative	11674.m00277	34356			6		10	
Ribosomal protein L36e	11682.m03641	12727			3		4	3
ribosomal protein L6e, putative	11668.m03614	92303			5		7	2
ribosomal protein S17, putative	11670.m04080	14937			2		3	3
ribosomal protein S17, putative	11670.m05125	17825			2			2
ribosomal protein S17, putative	11670.m05899	15966			2			4
ribosomal protein S20, putative	11667.m04725	21506			2			3
Ribosomal protein S24e, putative	11668.m01249	15739			2		2	
Ribosomal protein S8e	11670.m02661	24913			5		5	
transketolase	11680.m00347	80028			6		7	
2,3-bisphosphoglycerate-independent phosphoglycerate mutase	11667.m05987	55632			2			
4-alpha-glucanotransferase, putative	11673.m04620	105744			3	4		
60s ribosomal protein l7a	11674.m02302	29285			9			
acidic ribosomal protein P3a - maize	11680.m04833	11887			4			
ATP synthase F1, beta subunit	11682.m04581	58898			3			
ATPase, AAA family, putative	11687.m04511	51421			2			
BPG-independent PGAM N-terminus (iPGM_N)	11682.m03854	52541			2			
Carbonic anhydrase, putative	11667.m04353	28022			2			
chaperonin gamma chain, putative	11680.m03352	61219			3			
chaperonin GroEL	11668.m00030	63759			5			
CRAL/TRIO, N-terminus, putative	11682.m03352	63909			3			
Dehydrogenase E1 component, putative	11670.m00195	46115			2			
D-isomer specific 2-hydroxyacid dehydrogenase, NAD binding domain, p	11668.m00017	44786	18		2			
elongation factor 1 beta 2	11669.m02941	24639			3			
expressed protein	11667.m06687	73528			3			
glycine dehydrogenase	11680.m04002	111401			7	4		
histone deacetylase HD2, putative	11682.m05013	11747			3			
histone H3.2 protein	11669.m02779	15397			4			
Kinesin motor domain, putative	11680.m01108	137542			2			
Nuclear transport factor 2 (NTF2) domain, putative	11670.m02888	51414			2			
Proteasome/cyclosome repeat, putative	11680.m04817	98628			2			
putative 30S ribosomal protein S13	11669.m05001	19210			6			
putative TCP-1/cpn60 chaperonin family protein	11669.m04189	57193			2			
Pyridine nucleotide-disulphide oxidoreductase, putative	11667.m02297	58809			4			
pyruvate dehydrogenase complex dihydrolipoamide acetyltransferase	11680.m00066	50144			2			
Ribosomal L27e protein family, putative	11668.m01748	15569			3			
Ribosomal protein L13, putative	11667.m05375	26228			2			
ribosomal protein L4/L1 family, putative	11669.m01586	34500			5			
ribosomal protein L4/L1 family, putative	11673.m00768	44711			3			
ribosomal protein S13p/S18e	11673.m00700	17652			3			
ribosomal protein S4	11676.m03467	23408			3			
RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain), putative	11674.m00120	94958			2			
Similar to dihydrolipoamide S-acetyltransferase, putative	11686.m00772	51442			5			
Similar to myosin heavy chain	11686.m01709	71326			4			
sucrose-UDP glucosyltransferase 2	11669.m02894	92849			7			
t-complex polypeptide 1	11670.m04542	59195			4			
T-complex protein 1, epsilon subunit	11680.m03561	59094			2			
translation elongation factor EF-1, subunit alpha	11669.m00767	49262			7			

TABLE II—*continued*

Name of the proteins	accession number	MW	in vivo 0,1 M NaCl	in vivo 0,5 M NaCl	in vivo 1 M NaCl	in vitro 0,1 M NaCl	in vitro 0,5 M NaCl	in vitro 1 M NaCl
translation elongation factor G	11670.m04421	82222			5			
transposon protein, putative, CACTA, En/Spm sub-class	11680.m03562	150834			4			
Viral A-type inclusion protein repeat, putative	11670.m05138	207427			4			
Eukaryotic ribosomal protein L18	11682.m00580	21298					3	
ferredoxin--nadp reductase, leaf isozyme, chloroplast precursor (ec 1.18	11680.m00092	39982					3	
histone H2B	11667.m06216	15357					3	
protein 60S ribosomal protein L6 F2P9.8 [imported] - Arabidopsis thalian	11670.m03786	24613					5	
putative 60S ribosomal protein	11669.m05899	44461					2	
putative histone H2 protein	11669.m05383	14450					2	
Ribosomal L22e protein family	11669.m02294	14463					2	
Ribosomal protein L14, putative	11668.m03941	15378					4	
ribosomal protein L24, putative	11667.m00394	17461					2	
ribosomal protein L24, putative	11686.m00477	16920					2	
ribosomal protein L3, putative	11686.m00647	44436					7	
ribosomal protein L3, putative	11687.m00609	44463					7	
Ribosomal protein L34e	11674.m00527	13612					2	
Ribosomal protein L35Ae	11682.m04609	12653					6	
Ribosomal protein L35Ae, putative	11668.m05360	41048					4	
Ribosomal protein L7Ae, putative	11667.m05936	25792					2	
ribosomal protein S17, putative	11670.m05124	11554					3	
Ribosomal protein S24e, putative	11667.m05144	15709					2	
RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain), putative	11674.m04473	14683					2	
histone deacetylase HD2, putative	11682.m05013	11747						4
NB-ARC domain, putative	11687.m04254	124358						2

For the interaction between FHV and IR64, we identified 41 common proteins also found with the interaction RYMV-IR64 (Table I), which could explain the ability of FHV to replicate in rice (9), and suggest that a set of proteins from a host plant could interact with different viruses. The results obtained with RYMV, FHV, and SCPMV suggested that host protein recruiting occurred for different viruses and that two unrelated viruses (RYMV and FHV) could recruit the same proteins from the same plant.

DISCUSSION

We know that the viral genome encodes a small number of genes, and the virus is thus necessarily dependent on host machinery to achieve its life cycle. The recruiting of the translational machinery from host plants is now well established for different viruses, for instance in the case of potyviruses, the interaction of eIF4E, eIFiso4E, eIF4G, and the viral protein VPg (15–18). Nevertheless the question of how the virion acts in the cellular context remains.

Indeed for each stage of the virus life cycle, it is necessary to have the required host partners in the right conditions of time, concentration, localization, and conformation. It is also necessary for the virus to have a high affinity for some host proteins. The external part of virus particles could play this crucial function in recruiting host proteins. To demonstrate this recruiting, we developed a method using RYMV, which has very stable particles (7) and replicates to a high level (4). Thus, using size exclusion chromatography, virus-protein complexes were purified from infected plants and from *in vitro* binding experiments using purified virus and soluble proteins extracted from non-infected plants. This method is reproducible and allows us to purify enough material to analyze complexes by SDS-PAGE and nano-LC-MS/MS. Host proteins from the complexes were separated and gave reproducible protein profiles for the same experimental conditions. To demonstrate the specific recruiting by the virus, we studied three critical stages for RYMV: 1 wpi (beginning of replication), 2 wpi (replication in systemically infected leaves, first symptoms), and 3 wpi (end of viral replication in susceptible variety IR64 and development of symptoms) (4). We showed that the recruiting of host proteins was different according to the infection stages. Comparing the same infection stage *in vitro* and *in vivo*, we demonstrated that the complexes isolated from infected plants were not formed during the extraction but presumably preexisted *in vivo* during the infection process. The number of identified proteins for each stage (1, 2, and 3 wpi) corresponds to a population of different complexes representative of the global situation within infected plants as the virus has the ability to infect different cell compartments. Looking at the stained SDS-PAGE gels, we clearly observed some recruited proteins showing different quantitative profiles

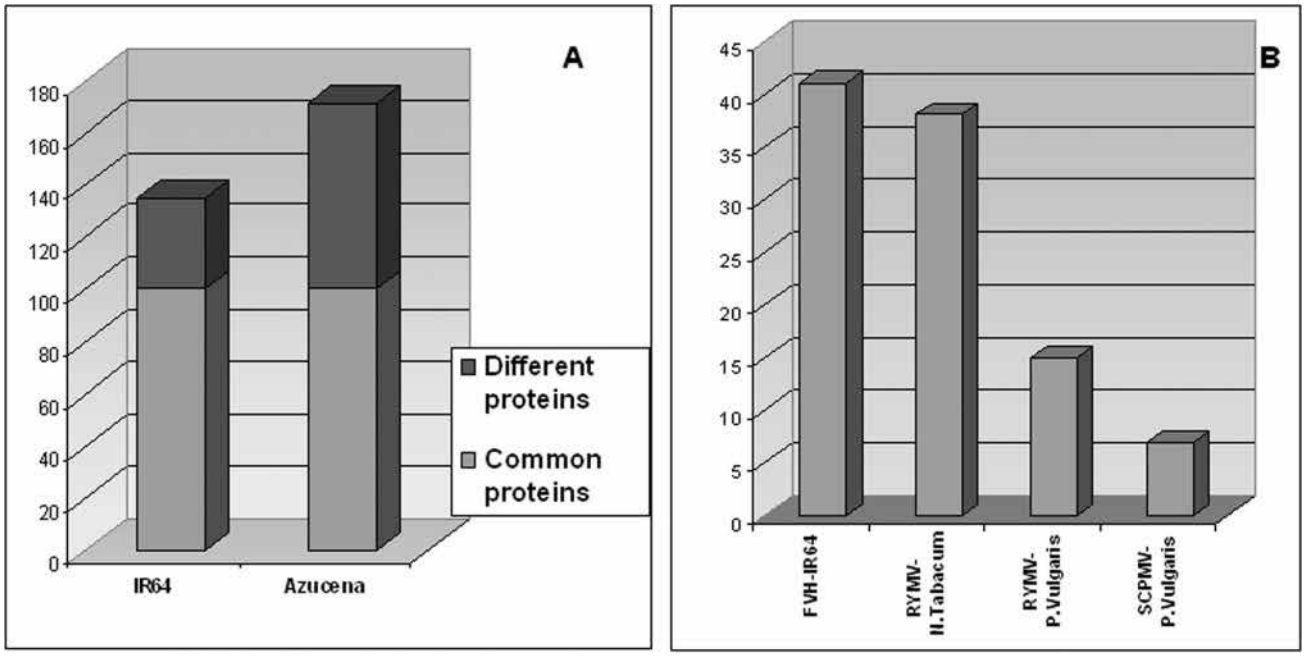

FIG. 6. **Common protein from different complexes.** *A*, histogram of cumulated number of non-redundant proteins identified by LC-MS-MS (see Table I) for IR64 and Azucena cultivars infected by RYMV at 1, 2, and 3 wpi. *B*, histogram of common proteins identified in FHV-IR64 *in vitro* interaction (see Table I) and common protein functions identified in different interactions (*in vitro* with RYMV-*N. tabacum* and RYMV-*P. vulgaris* and *in vivo* with SCPMV and *P. vulgaris*) (see Table I). Identification was made using the TIGR rice pseudomolecule for experimentations with IR64 and Azucena cultivar and using Swiss-Prot, TrEMBL, and TrEMBLnew for *P. vulgaris* and *N. tabacum.*

according to the different stages of infection (Fig. 2, *bands a*, *b*, *c*, *d*, and *e*). Because of the reproducibility of this recruiting, these results reinforce the idea that virus recruits specific host proteins during the infection process.

Among the recruited proteins, some of them have a higher affinity for RYMV. This was supported by the identification of 32 proteins that were still binding to the virus at 1 M NaCl *in vivo* (of 72 at 0.1 M NaCl for IR64 2 wpi), and among them, 25 proteins were identified *in vitro* at 1 M NaCl (Fig. 5*B*). We suppose that these proteins are most likely bound directly at the surface of the virus particle. The other proteins that were identified in lower salt concentration could have a lower affinity with the surface of the virus or could bind host proteins previously recruited by the virus. Interestingly we found a recruiting coherence through some functional categories. In the metabolism category we identified a high number of enzymes involved in glycolysis, malate, and citrate cycles presumably recruited by the virus to produce energy for virus replication. In the defense category, we identified proteins involved in reactive oxygen species (19) and detoxification (superoxide radical and hydrogen peroxide) that are presumably recruited by the virus to maintain an oxidoreduction environment compatible with viral replication. In addition, in the protein synthesis category, we identified a set of proteins involved in translation processes with ribosome, elongation factors, protein chaperones, protein-disulfide isomerase, and proteins involved in protein turnover with the 20 S proteasome. All these proteins would be recruited by the virus to optimize virus translation efficiency as soon as the virus starts decapsidation. Nevertheless we can rule out that some proteins identified belong to the plant defense system and were neutralized by sequestration.

Virus recruiting is likely to occur for other viruses as we saw a recruiting for the pair SCPMV-*P. vulgaris* (Fig. 6*B*). RYMV is able to recruit proteins from *N. tabacum* and from *P. vulgaris* in *in vitro* experimentations (Fig. 6*B*). The results obtained *in vitro* with the pair FHV-IR64 are interesting because 41 proteins were identified that where also found with the pair RYMV-IR64 (*in vivo* and/or *in vitro*), suggesting a common set of proteins recruited from rice by viruses able to replicate in this plant (it was demonstrated that the FHV is able to replicate and is systemically spread in rice plants expressing movement protein genes (20)). Inside the rice host subspecies studied, we showed that most recruited proteins are identical (Fig. 6*A*); the recruited proteins that are different between the two subspecies could be related to the susceptibility or tolerance effect observed for the IR64 and Azucena varieties. Some proteins were identified whatever the experimental conditions (*e.g.* mitochondrial chaperonin-60 11676.m02851), suggesting that they have a very strong affinity for viruses and that they play an important role in the virus biological process.

We discriminated some paralog genes belonging to a multigenic family that were specifically recruited. Some of the proteins identified in the complexes from the IR64-RYMV and Azucena-RYMV experiments were shown as deregulated in IR64 and Azucena suspension cells undergoing RYMV infection (11).

About 15 similar protein functions were found in another study using cDNA amplified fragment length polymorphism to discover genes induced or repressed during virus infection.[2]

Some of the proteins have been identified in different virus-host interactions such as Hsp60 with hepatitis B virus (21) or human immunodeficiency virus (22) and Hsp70 with plant closteroviruses (23) or human immunodeficiency virus (24) suggesting that viruses may recruit the same protein functions in different hosts. Other proteins, not identified in our experiments, were identified interacting with different viruses as pectin methylesterases (25, 26), homeodomain proteins (27), rab acceptor-related proteins (28), β-1,3-glucanase-interacting proteins (29), Fas-mediated apoptosis enhancer Daxx (30), and SUMO-1 protein (31, 32). All these results suggest that the recruiting of proteins is probably a common process for different viruses.

In this study we describe for the first time an efficient method to extract virus-host protein complexes and to identify by mass spectrometry the proteins involved in these complexes. The analysis with contrasted pathogenic isolates, in host and non-host interaction contexts, should help to identify molecular interaction mechanisms involved in viral infection. The functional relevance of these proteins remains to be evaluated using mutagenesis or silencing strategies. This method of analysis may help to identify new target proteins that may be useful to find new markers for plant selection or to develop new strategies to abort virus infection processes. It is also conceivable to use this experimental approach to isolate virus-host protein complexes from different organisms in an attempt to find new therapeutic targets in human and animal virus diseases.

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