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-encapsulation, 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 divergent 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 seeding, 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

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-aminoopropanesulfonic acid; nano-LC-MS/MS, nanoscale capillary LC-MS/MS; TIGR, The Institute for Genomic Research.
Plants were harvested at 1, 2, and 3 weeks post-inoculation (wpi), frozen in nitrogen, and conserved at 
/1100280 °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 AKTAPrime system (Amersham
Biosciences) was used for injection, detection, and collection of the
proteins. Fractions corresponding to the first ½ peak of virus-protein
complexes were collected and lyophilized (Fig. 1, c1).

**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 (AKTAPrime 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 ½ 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.
cysteine residues were reduced with dithiothreitol at 57 °C and alkylated.

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% NH4HCO3 (25 mM), 50% ACN. The cysteine residues were reduced with diithiothreitol 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 NH4HCO3 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 C18, 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 .pkf 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. 1A) or from in vitro binding experiments with soluble proteins extracted from non-infected leaves supplemented with purified virus (Fig. 1B).

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. 1A, green) and overlapped slightly with the peak at 216 min corresponding to the experiment with the virus purified alone (Fig. 1A, 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. 1A, c1, blue). The same additional peak at 206 min was found in experiments using the extraction buffer added with 0.5 or 1 u 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. 1B, c1). After the purification/elution cycle, electron microscopy revealed the presence of intact virus particles in the collected fraction corresponding to peak c2 (Fig. 1C). In collected fractions corresponding to peak c1, virus particles were associated with electron dense materials (Fig. 1D). 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. 2B) 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 | IR64 in vivo | IR64 in vitro |
|---|---|---|---|---|---|
| 5-methyl-2-thioacetamidopyridine-5-nitromethane 5-methyltransferase | 11696 | m12092 | 8549 | 10 | 10 | 14 |
| 5-methyl-2-thioacetamidopyridine-5-nitromethane 5-methyltransferase | 11696 | m12093 | 8456 | 15 | 29 | 4 |
| CoA-lyase, putative | 1197 | m1318 | 6795 | 5 | 15 | 10 |
| Fructose-bisphosphate aldolase class I | 11696 | m13527 | 5679 | 14 | 18 | 19 |
| Fructose-bisphosphate aldolase class I | 11696 | m13528 | 3870 | 5 | 5 | 4 |
| Fructose-bisphosphate aldolase class I | 11696 | m13529 | 3870 | 5 | 5 | 4 |
| Fructose-bisphosphate aldolase class I | 11696 | m13530 | 3870 | 5 | 5 | 4 |
| tRNA-dependent glutamate synthase (fragments) | 11673 | m13550 | 17863 | 33 | 7 | 7 |
| nucleoside diphosphate kinase III, chloroplast/membrane protein | 11692 | m13559 | 25392 | 3 | 4 | 4 |
| Rubisco bisphosphate carboxylase, small subunit putative | 11696 | m13585 | 14205 | 11 | 9 | 12 |
| Oxygen evolving enhancer protein 3 (PEP) | 11673 | m13546 | 22567 | 9 | 10 | 10 |
| mitochondrial chaperone 60 | 11676 | m13561 | 60850 | 3 | 4 | 4 |
| Porin class | 11697 | m13558 | 32710 | 6 | 7 | 8 |
| Subtilisin family protease | 11697 | m13560 | 78181 | 6 | 8 | 7 |
| reversibly glycosylated polypeptide | 11698 | m13564 | 41322 | 11 | 9 | 6 |
| probable DNA-binding protein GDP10 - rice | 11696 | m13570 | 43170 | 8 | 9 | 12 |
| glyceraldehyde-3-phosphate dehydrogenase, C-terminal domain, putative | 11670 | m13580 | 42828 | 9 | 6 | 6 |
| glyceraldehyde-3-phosphate dehydrogenase, type I | 11670 | m13592 | 36722 | 9 | 5 | 7 |
| glyceraldehyde-3-phosphate dehydrogenase, type II | 11670 | m13598 | 36722 | 9 | 5 | 7 |
| Saposin-like nucleosome modulator, putative | 11696 | m13553 | 106182 | 6 | 8 | 8 |
| expressed protein | 11696 | m13561 | 41082 | 4 | 8 | 6 |
| class F molecular chaperone hsp70 - rice (fragment) | 11697 | m13557 | 71985 | 2 | 12 | 6 |
| Protease inhibitor 1,4 and proline-rich protein | 11697 | m13565 | 12282 | 2 | 3 | 2 |
| Protease inhibitor 1,4 and proline-rich protein | 11696 | m13567 | 11283 | 2 | 3 | 2 |
| 3-hydroxyisocitrate dehydrogenase, C-terminal domain, putative | 11696 | m13543 | 70840 | 4 | 8 | 4 |
| beta-lactamase | 11699 | m13566 | 59300 | 9 | 7 | 14 |
| Proteins A-type and B-type, putative | 11696 | m13530 | 20527 | 9 | 11 | 9 |
| Proteins A-type and B-type, putative | 11696 | m13541 | 20527 | 9 | 11 | 9 |
| Protease A-type and B-type, putative | 11698 | m13525 | 20527 | 9 | 11 | 9 |
| Protease A-type and B-type, putative | 11696 | m13525 | 20527 | 9 | 11 | 9 |
| protease subunit alpha type 3 (ec 3.4.25.1) | 11669 | m13525 | 28802 | 9 | 9 | 9 |
| protease subunit alpha type 3 (ec 3.4.25.1) | 11669 | m13525 | 28802 | 9 | 9 | 9 |
| protease subunit beta type 1 (ec 3.4.25.1) | 11661 | m13502 | 24369 | 10 | 6 | 6 |
| protease subunit beta type 1 (ec 3.4.25.1) | 11661 | m13502 | 24369 | 10 | 6 | 6 |
| 205 protease subunit 4 subunit | 11668 | m13510 | 23493 | 9 | 10 | 11 |
| catalytic subunit serine protease | 11668 | m13510 | 23493 | 9 | 10 | 11 |
| Carbonic anhydrase, putative | 11697 | m13533 | 29117 | 12 | 5 | 10 |
| trypsin-like protease | 11688 | m13547 | 90218 | 17 | 13 | 21 |
| phosphoglycerate kinase | 11692 | m13511 | 50452 | 5 | 3 | 2 |
| aminotransferase, class V | 11674 | m13532 | 44567 | 6 | 2 | 3 |
| reversibly glycosylated protein | 11673 | m13544 | 41553 | 6 | 5 | 6 |
| chaperone protein DnaK | 11696 | m13513 | 73905 | 6 | 8 | 6 |
| ribosomal protein S15, putative | 11695 | m13522 | 58904 | 6 | 8 | 6 |
| Protease inhibitor 1,4, putative | 11698 | m13542 | 11499 | 3 | 2 | 2 |
| Protease inhibitor 1,4, putative | 11698 | m13543 | 11499 | 3 | 2 | 2 |
| Protein Name | Accession | pI | MW (kDa) | LogP | Molar Extinction | Stock Concentration | Stock Volume | Stock Protein | Stock Date |
|--------------|-----------|----|----------|------|------------------|--------------------|--------------|--------------|-----------|
| 35S ribosomal protein subunit 1 (I) | 11686000100 | 7.1 | 100 | 0.5 | 12000 | 1000 | 1000 | 100 | 100 |
| 35S ribosomal protein subunit 2 (I) | 11686000100 | 7.1 | 100 | 0.5 | 12000 | 1000 | 1000 | 100 | 100 |
| 35S ribosomal protein subunit 3 (I) | 11686000100 | 7.1 | 100 | 0.5 | 12000 | 1000 | 1000 | 100 | 100 |
| 35S ribosomal protein subunit 4 (I) | 11686000100 | 7.1 | 100 | 0.5 | 12000 | 1000 | 1000 | 100 | 100 |

For the full dataset, please refer to the original publication.
### TABLE I—continued

| Name of the common proteins to Azucena in vivo and IR64 in vivo | accession number | MW | Azucena in vivo | IR64 in vivo in vitro | IR64 in vitro |
|---------------------------------------------------------------|------------------|----|-----------------|-----------------------|--------------|
| phosphoenolpyruvate carboxykinase, putative                  | 11864.m01485     | 73158 | 6                | 3                     | X            |
| Copper/zinc superoxide dismutase, putative                   | 11874.m04529     | 21301 | 2                | 2                     | 4            |
| Peroxidase, putative                                         | 11892.m00333     | 37613 | 3                | 2                     | 10           |
| expressed protein                                            | 11897.m01727     | 42739 | 4                |                       |              |

| Name of the specific proteins to Azucena in vivo and to IR64 in vivo in vitro |
|-----------------------------------------------------------------------------|
| glycine dehydrogenase                                                      | 11867.m05023     | 11356 | 8                | 11                     | 8            |
| fructose-1,6-bisphosphatase                                                 | 11867.m04002     | 111401| 6                | 10                     | 8            |
| putative glyoxysomal malate dehydrogenase                                  | 11869.m05674     | 36699 | 4                | 4                      | 6            |
| peptidy/peptidyl isomerase (EC 5.2.1.8) Cypase - rice                     | 11868.m00200      | 18349 | 4                | 2                      | 3            |
| probable chitinase (EC 3.2.1.14) L1b - rice                              | 11870.m09999      | 25151 | 8                | 4                      | 4            |
| alpha-mannosidase                                                          | 11871.m02967      | 114157| 7                | 8                      | 3            |
| chsK protein                                                               | 11871.m08805      | 83849 | 7                | 10                     | 10           |
| proteasome subunit beta type 3 (ec 3.4.25.1) (20s proteasome alpha subunit c) | 11861.m00801     | 22789 | 6                | 6                      | 8            |
| putative aldehyde oxidase                                                  | 11869.m05935      | 145284| 23               | 7                      | 6            |
| phenylalanine ammonia-lyase                                                | 11870.m09345      | 75901 | 7                | 15                     | 9            |
| FMN-dependent dehydrogenase                                               | 11873.m00499      | 40219 | 2                | 10                     |              |
| fructose-1,6-bisphosphatase                                                | 11867.m06181      | 37012 | 2                | 3                      |              |
| glyceraldehyde dehydrogenase                                               | 11870.m05219      | 43968 | 3                | 8                      |              |
| glycine dehydrogenase                                                     | 11849.m05785      | 40364 | 2                | 9                      | X            |
| Glycolysyl hydrodats family 17                                              | 11867.m07148      | 35582 | 9                | 3                      |              |
| nucleoside diphosphate kinase i (ec 2.7.4.6) (ndk i)(ndk kinase i)(ndk i) | 11873.m02349      | 16331 | 3                | 2                      | X            |
| pyruvate kinase                                                            | 11871.m00409      | 57254 | 2                | 4                      | 2            |
| triphosphatase isomerase                                                   | 11871.m02939      | 22373 | 2                | 10                     |              |
| putative glutathione S-transferase                                         | 11871.m00515      | 24758 | 5                | 2                      |              |
| expressed protein                                                          | 11870.m00879      | 42909 | 2                | 3                      |              |
| Peptidase family M1, putative                                              | 11868.m01161      | 58301 | 4                | 3                      |              |
| sucrose-UDP glucosyltransferase 2                                         | 11867.m09240      | 52949 | 6                | 6                      | 8            |
| AL161582 amino peptidase-like protein                                      | 11874.m03033      | 91231 | 2                | 4                      |              |
| translation elongation factor eEF-1 beta - rice                           | 11873.m01415      | 24847 | 3                | 4                      |              |
| isoleucyl-tRNA synthetase, putative                                        | 11868.m00459      | 155097| 9                | 5                      | 14            |
| Oxido reducerase NAD-binding domain, putative                             | 11868.m00008      | 40638 | 5                | 2                      | 2            |
| Subtilase family, putative          | 11668.m04269 | 145477 |
|-----------------------------------|--------------|--------|
| glutamate dehydrogenase           | 11668.m05875 | 44534  |
| ferredoxin-N-trik reductase        | 11668.m05196 | 73400  |
| Peptidase family M1, putative     | 11674.m04536 | 99461  |
| phosphoenolpyruvate carboxylase   | 11674.m03727 | 10566  |
| phosphoglycerate/pyridoxal phosphate phosphatase family, putative | 11670.m03689 | 40194 |
| ribose-5-phosphate isomerase A    | 11673.m00734 | 29179  |
| Calreticulin family, putative     | 11673.m01387 | 59127  |
| TCP-1p60 chaperonin family        | 11669.m00441 | 61045  |
| peroxiredoxin Q                   | 11680.m00923 | 23722  |
| putative valyl tRNA synthetase    | 11680.m04802 | 118588 |
| D-isomer specific 2-hydroxyacid dehydrogenase, NAD binding domain, putative | 11668.m00117 | 44789 |
| Cuprin, putative                  | 11674.m03549 | 21861  |
| 3-hydroxyacyl-CoA dehydrogenase, NAD binding domain, putative | 11667.m02406 | 79292 |
| Ribulose bisphosphate carboxylase large chain, N-terminal domain | 11662.m03356 | 18430 |
| glutaminyl-RNA synthetase, putative | 11682.m00871 | 99249  |
| AlpC/MTS family, putative        | 11668.m00916 | 23179  |
| Remorin, C-terminal region, putative | 11670.m04374 | 22415  |
| Similar to serine carboxypeptidase II-like protein | 11668.m04044 | 39574  |
| hypothetical protein              | 11668.m04855 | 20724  |
| LyfM domain, putative             | 11681.m03358 | 38861  |
| rubisco subunit binding-protein alpha subunit, chloroplast precursor/60 kda chl a | 11666.m01770 | 61003 |
| co-chaperone GrpE, putative       | 11668.m03631 | 35355  |
| proteasome subunit alpha type 7 (ec 3.4.25.1) (20s proteasomealpha c subunit | 11661.m03206 | 27197  |
| oxidoreductase, aldo/keto reductase family | 11668.m05657 | 38427  |
| CAA300T15.1 protein               | 11670.m05668 | 35205  |
| HMN (high mobility group) box, putative | 11660.m05109 | 14124  |
| putative 14-3-3 protein            | 11669.m06565 | 29160  |
| 6-phosphogluconate dehydrogenase, decarboxylating | 11660.m00122 | 52721  |
| adenosine kinase                   | 11668.m03867 | 39893  |
| lactoyl/lactate lyase, putative    | 11674.m03666 | 32553  |
| legumin-like protein               | 11662.m00174 | 38195  |
| UDP-glucose-1-phosphate uridylyltransferase | 11661.m03401 | 54886  |
| chitinase (EC 3.2.1.14) III C10701 - rice | 11667.m04722 | 32909  |
| Multicopper oxidase, putative     | 11673.m03122 | 61913  |
| Peroxidase, putative              | 11667.m07348 | 39638  |
| putative pollen allergen           | 11667.m03610 | 25282  |
| Protease inhibitor-seed storage/LTP family, putative | 11667.m06648 | 13923  |
| C2 domain, putative               | 11673.m03026 | 18605  |
| Phosphatase-induced protein 1 conserved region | 11668.m06695 | 34152  |
| Phosphatase-induced protein 1 conserved region | 11668.m01130 | 33015  |
| Phosphophenylalanine/Urdeine kinase family, putative | 11668.m04554 | 44387  |
| proteasome subunit alpha type 2 (ec 3.4.25.1) (20s proteasome alpha subunit b | 11669.m02741 | 25644  |
| reversibly glycosylated polypeptide | 11670.m05570 | 49005  |
| ATP synthase F1, beta subunit     | 11667.m04784 | 59493  |
| Name of the common proteins to Azucena in vivo and IR64 in vivo | accession number | MW | Azucena in vivo | IR64 in vivo | IR64 in vitro |
|---------------------------------------------------------------|-----------------|----|----------------|-------------|--------------|
| ATP synthase F1, alpha subunit                               | 11681.m00716    | 55235 | 6 | 2 | 2 |
| Similar to ferredoxin-nitrite reductase (EC 1.7.7.1) precursor - rice | 11687.m03487    | 55745 | 3 | 8 | 2 |
| flavodoxin, putative                                          | 11667.m05707    | 21705 | 3 | 3 | 2 |
| glucose-6-phosphate isomerase, putative                       | 11674.m03720    | 67229 | 2 | 3 | 6 |
| glutamate dehydrogenase 2 (EC 1.4.1.3) (gdh 2), maize-ear cross | 11670.m04472    | 45594 | 2 | 2 | 2 |
| glutamate-1-semialdehyde-2,1-aminomutase                     | 11674.m04228    | 50237 | 3 | 6 | 6 |
| isocitate dehydrogenase, NADP-dependent                       | 11667.m04430    | 45013 | 3 | 10 | 10 |
| NAD binding domain of 6-phosphogluconate dehydrogenase, putative | 11668.m03371    | 30496 | 3 | 3 | 10 |
| probable transaminase (EC 2.6.1.1) T161.1.170 [similarity] - Arabidopsis thaliana | 11666.m01908    | 49657 | 3 | 5 | 5 |
| ribulose-5-phosphate-3-epimerase                             | 11686.m00690    | 20034 | 2 | 2 | 2 |
| Thiamine pyrophosphate enzyme, central domain, putative      | 11667.m03019    | 48657 | 2 | 2 | 2 |
| triosephosphate isomerase                                     | 11667.m00476    | 23122 | 3 | 6 | 6 |
| ubiquitin-activating enzyme ε 1                               | 11667.m00732    | 117047 | 4 | 4 | 4 |
| dnaK protein                                                  | 11682.m03345    | 74227 | 3 | 3 | 3 |
| protein disulfide isomerase 2 precursor                      | 11668.m00870    | 58198 | 5 | 5 | 5 |
| translation elongation factor Tu                             | 11666.m03555    | 50392 | 5 | 13 | 13 |
| putative aldehyde oxidase                                     | 11666.m05834    | 145336 | 5 | 5 | 5 |
| Hsp90 protein, putative                                       | 11674.m03730    | 130665 | 2 | 2 | 2 |
| Lipoxigenase, putative                                        | 11674.m00935    | 194494 | 2 | 2 | 2 |
| cytochrome oxidase, zinc-binding dehydrogenase family, putative | 11674.m02863    | 335868 | 2 | 2 | 2 |
| PstP                                                          | 11673.m02084    | 26389 | 3 | 3 | 3 |
| putative trypanothione-dependent peroxidase                   | 11696.m02804    | 63049 | 3 | 3 | 3 |
| putative salt-induced protein                                 | 11696.m02877    | 19971 | 3 | 3 | 3 |
| Similar to salt-stress root protein ns1                      | 11667.m01289    | 21801 | 4 | 4 | 4 |
| Atg19170/MV11.8                                              | 11666.m01316    | 121131 | 2 | 2 | 2 |
| NADP-ribosylation factor family                              | 11667.m01605    | 25005 | 3 | 3 | 3 |
| retrotransposon protein, putative, unclassified              | 11668.m05463    | 245827 | 3 | 3 | 3 |
| pyruvate dehydrogenase E1 beta subunit isoform 3             | 11667.m03092    | 40225 | 2 | 2 | 2 |
| Similar to 26S proteasome subunits                           | 11670.m03566    | 47567 | 5 | 5 | 5 |
| 6,7-dimethyl-8-benzylazaine synthase, putative               | 11670.m04040    | 22471 | 5 | 5 | 5 |
| ATP synthase F1, alpha subunit                               | 11670.m14577    | 55660 | 6 | 13 | 13 |
| citrate synthase I                                           | 11698.m01280    | 56490 | 4 | 4 | 4 |
| similar to glucosidase II alpha subunit                      | 11699.m01126    | 72311 | 4 | 4 | 4 |
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. 3A). 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. 3A) 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. 3A). 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. 3A).

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. 3B, 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 *in vivo* and *in 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...
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 experiments 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 glycolaldehyde-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 glycolaldehyde-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 experimentations (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 NaCl 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. 6A). 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. 5B), 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).
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."
### TABLE II—continued

| Name of the proteins | accession number | MW | 0.1 M NaCl | 0.3 M NaCl | 1 M NaCl | 0.1 M NaCl | 0.3 M NaCl | 1 M NaCl |
|----------------------|------------------|----|------------|------------|----------|------------|------------|----------|
| glycine dehydrogenase | 11687.m05023     | 111258 | 15 | 6 | 4 | 9 |
| phosphophotolipase carboxylase | 11674.m07127 | 10986 | 36 | 27 | 13 |
| phosphonolipase K3 | 11682.m03911 | 50492 | 10 | 10 | 6 |
| proteasome subunit alpha type 3 (ec 3.4.25.1) | 11682.m03938 | 27200 | 11 | 6 | 3 |
| putative 90 ribosomal protein | 11689.m05035 | 24227 | 2 | 4 | 3 |
| ribosomal protein L7A12, putative | 11687.m04571 | 18579 | 5 | 5 | 3 |
| Similar to acidic ribosomal protein P2A-2 | 11687.m09016 | 11800 | 2 | 2 | 2 |
| Similar to ribulose 1,5-bisphosphate carboxylase | 11676.m01782 | 36459 | 4 | 3 | 6 |
| thioredoxin peroxidase | 11686.m03158 | 26097 | 11 | 7 | 6 |
| dnaK protein | 11689.m01895 | 71056 | 9 | 5 | |
| 2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase | 11676.m03086 | 46283 | 3 | 11 | |
| alpha-mannosidase | 11687.m02687 | 114157 | 16 | 2 | |
| arabinofuranosyltransferase I [EC:2.4.2.7] | 11687.m02288 | 73421 | 2 | 2 | |
| ATP synthase F1, alpha subunit | 11670.m01497 | 55095 | 3 | 3 | |
| expressed protein | 11689.m02084 | 72905 | 8 | 4 | |
| expressed protein | 11689.m03961 | 77842 | 2 | 6 | |
| glutamate dehydrogenase | 11689.m05755 | 44431 | 10 | 3 | |
| Glutamine synthetase, catalytic domain | 11686.m04942 | 38176 | 12 | 7 | |
| Glutamine synthetase, catalytic domain, putative | 11670.m05561 | 49428 | 7 | 14 | |
| glutaminyl-tRNA synthetase | 11676.m01897 | 80386 | 7 | 3 | |
| glyceraldehyde-3-phosphate dehydrogenase, type I, putative | 11689.m03036 | 47081 | 3 | 4 | 4 |
| glycolate oxidase | 11689.m05782 | 40834 | 7 | 4 | |
| hAT family dimerisation domain, putative | 11682.m02184 | 80786 | 2 | 6 | |
| heat shock protein 62 | 11687.m03316 | 70720 | 32 | 26 | 3 |
| histone deacetylase HD2, putative | 11682.m05012 | 21171 | 2 | 3 | |
| HsP00 protein, putative | 11681.m02792 | 91604 | 15 | 10 | |
| Hsp90 protein, putative | 11681.m02845 | 80150 | 37 | 21 | |
| isoacceptor-tRNA synthetase, putative | 11680.m02496 | 155097 | 4 | 5 | 5 |
| Lipoyltransferase | 11686.m03561 | 104621 | 7 | 11 | |
| LysA enzyme, N-terminal domain, putative | 11687.m03146 | 71456 | 20 | 8 | |
| phosphophotolipase carboxylase | 11681.m01380 | 106863 | 16 | 10 | |
| Proteasome A-type and B-type, putative | 11682.m00922 | 26979 | 4 | 2 | |
| PifA ssDNA and RNA-binding protein | 11687.m01560 | 33200 | 4 | 3 | |
| putative glycine hydroxymethyltransferase | 11686.m03432 | 56380 | 11 | 2 | |
| Similar to 28S proteasome subunits | 11670.m03506 | 47507 | 2 | 3 | 2 |
| Transferase family, putative | 11687.m07012 | 110473 | 4 | 4 | |
| translation elongation factor Tu | 11668.m03655 | 53820 | 5 | 5 | |
| 60s ribosomal protein 1A | 11674.m02392 | 20285 | 4 | 6 | |
| acidic ribosomal protein P3a - maize | 11680.m04433 | 11887 | 2 | 2 | 2 |
| ferredoxin--naad reductase, leaf isozyme, chloroplast precursor (ec 1.18.11) | 11680.m00922 | 39982 | 7 | 8 | 3 |
| histone-like protein | 11689.m05256 | 25612 | 4 | 2 | |
| Ositoedroductase NAD-binding domain, putative | 11688.m00306 | 40638 | 7 | 4 | 4 |
| Paroxysm, putative | 11689.m02189 | 37210 | 3 | 4 | 3 | 2 |
| proteasome subunit beta type 3 (ec 3.4.25.1) | 11668.m00821 | 22769 | 5 | 4 | |
| reversibly glycosylated polypeptide | 11670.m03573 | 46605 | 8 | 3 | 9 |
| 14-3-3 protein | 11674.m03731 | 28982 | 3 | | |
| 5-methyltetrahydrodipetroxyglutamate--homocysteine S-methyltransferase | 11686.m02482 | 84584 | 20 | 11 | 14 |
| AAA family ATPase, CDC48 subfamily | 11676.m02643 | 90857 | 7 | | |
| aconitate hydratase 1 | 11689.m00378 | 106235 | 2 | | |
| adenylate homologues | 11687.m00117 | 49929 | 6 | | |
| Acyl-CoA synthase 1 | 11689.m01970 | 103830 | 7 | | |
| ATP synthase F1, beta subunit | 11687.m04784 | 58463 | 2 | | |
| Catabolism family, putative | 11673.m01387 | 50127 | 4 | | |
| Catalase, putative | 11689.m00325 | 56728 | 9 | | |
| Name of the proteins | accession number | MW   | 0.1 M NaCl | 0.5 M NaCl | 1 M NaCl | 0.1 M NaCl | 0.5 M NaCl | 1 M NaCl |
|----------------------|------------------|------|------------|------------|----------|------------|------------|----------|
| CoA-ligase, putative | 11687.m01918     | 6785 | 11         | 4          | 13        | 11         | 4          | 13       |
| co-chaperone GrpE, putative | 11687.m03896 | 31491 | 8          | 2          |          | 8          | 2          |          |
| co-chaperone GrpE, putative | 11687.m02441 | 39868 | 2          | 1           | 2         | 2          | 1          | 2        |
| Copper/zinc superoxide dismutase, putative | 11687.m04329 | 21301 | 2          | 1          | 2         | 2          | 1          | 2        |
| CPSF A subunit region, putative | 11682.m04969 | 121803 | 2          | 1           | 2         | 2          | 1          | 2        |
| cystathionine beta-lyase | 11680.m00746 | 41884 | 3          | 2          |          | 3          | 2          |          |
| cytoplasmic malate dehydrogenase | 11674.m02962 | 35540 | 2          | 1           | 2         | 2          | 1          | 2        |
| dnaK protein | 11688.m00151 | 73345 | 5          | 3          |          | 5          | 3          |          |
| dnaK protein, putative | 11688.m04698 | 98309 | 3          | 2          |          | 3          | 2          |          |
| Elongation factor 1 gamma, conserved domain, putative | 11688.m03639 | 47930 | 6          | 6          | 4         | 6          | 6          | 4        |
| Elongation factor G, domain IV, putative | 11688.m03009 | 93961 | 11         | 8          |          | 11         | 8          |          |
| expressed protein | 11687.m03764 | 34816 | 2          | 1           | 2         | 2          | 1          | 2        |
| expressed protein | 11688.m02220 | 25774 | 2          | 1           | 2         | 2          | 1          | 2        |
| expressed protein | 11688.m05132 | 16008 | 2          | 1           | 2         | 2          | 1          | 2        |
| FF domain, putative | 11687.m03297 | 106808 | 4          | 2          | 4         | 2          | 2          | 4        |
| FMN-dependent dehydrogenase | 11673.m00499 | 40219 | 4          | 2          | 4         | 2          | 2          | 4        |
| fructose-1,6-bisphosphatase, putative | 11670.m01491 | 42245 | 6          | 4          |          | 6          | 4          |          |
| Fructose-bisphosphatase class-I | 11674.m05837 | 38799 | 15         | 5          | 13        | 15         | 5          | 13       |
| Glutamine dehydrogenase 2 (ec 1.4.1.3) (gdh 2), mouse-ear cress | 11680.m04472 | 45594 | 13         |          |          | 13         |          |          |
| glutathione-disulfide reductase | 11689.m08534 | 62215 | 11         |          |          | 11         |          |          |
| glycine-RNA synthetase beta subunit, putative | 11680.m00041 | 116990 | 3          |          |          | 3          |          |          |
| hydroxylase, carbon-nitrogen family, putative | 11688.m03118 | 33442 | 4          |          |          | 4          |          |          |
| legumin-like protein | 11682.m00174 | 38195 | 2          |          |          | 2          |          |          |
| LIM domain, putative | 11688.m01279 | 140397 | 2          |          |          | 2          |          |          |
| Lipoygenase, putative | 11674.m03993 | 104494 | 5          |          |          | 5          |          |          |
| malate dehydrogenase, NAD-dependent | 11682.m04796 | 35414 | 5          |          |          | 5          |          |          |
| methyl-binding domain protein MBD106, putative | 11688.m04243 | 31491 | 7          | 9          | 7         | 9          | 7          | 9        |
| Nucleoside diphosphate kinase | 11686.m03549 | 16663 | 6          |          |          | 6          |          |          |
| nucleoside diphosphate kinase I (ec 2.7.4.6) (ndk I) (ndk kinase I) | 11673.m02949 | 16851 | 4          |            | 4         |          |            |          |
| nucleoside diphosphate kinase II, chloroplast/mitochondrial precursor (ec 11682.m04999 | 25921 | 5          | 3          |          | 5         | 3          |          |          |
| Peptidase family M1, putative | 11688.m01161 | 98391 | 2          |          |          | 2          |          |          |
| Peroxidase, putative | 11687.m02168 | 33279 | 5          |          |          | 5          |          |          |
| phosphoenolpyruvate carboxylase, putative | 11689.m01495 | 73158 | 3          |          |          | 3          |          |          |
| phosphoenolpyruvate carboxylase | 11681.m01307 | 110405 | 20         |            | 20        |            | 20        |          |
| phosphoribosylaminomimidazole carboxylase, ATPase subunit, putative | 11667.m00991 | 68388 | 13         |            | 13        |            | 13        |          |
| plant acid phosphatase, putative | 11688.m03911 | 32947 | 2          |          |          | 2          |          |          |
| prolyl-tRNA synthetase | 11686.m04274 | 57950 | 2          | 2          | 2         | 2          | 2          | 2        |
| Protease inhibitor/seed storage LTP family, putative | 11686.m00139 | 12137 | 2          |          |          | 2          |          |          |
| putative 14-3-3 protein | 11689.m05065 | 29160 | 2          | 4          | 2         | 4          | 2          | 4        |
| putative aldehyde oxidase | 11689.m05834 | 145336 | 6          |            | 6         |            | 6          |          |
| putative cyanase | 11676.m02926 | 18599 | 7          |            | 7         |            | 7          |          |
| putative eukaryotic initiation factor subunit | 11676.m03873 | 63129 | 3          |            | 3         |            | 3          |          |
| Remorin, C-terminal region, putative | 11670.m04374 | 22415 | 3          |            | 3         |            | 3          |          |
| retrotransposon protein, putative, unclassified | 11688.m05463 | 205627 | 2          |            | 2         |            | 2          |          |
| ribosomal protein L10, putative | 11689.m01776 | 51817 | 6          |            | 6         |            | 6          |          |
| Similar to 20S proteasome subunits | 11670.m03506 | 47567 | 2          |            | 2         |            | 2          |          |
| Similar to arabinofuranosidase isoenzyme AXAII | 11687.m02883 | 140294 | 10         |            | 10        |            | 10        |          |
| Name of the proteins                                           | accession number | MW     | 0.1 M NaCl | 1 M NaCl | 1 M LiCl |
|---------------------------------------------------------------|------------------|--------|------------|----------|----------|
| Threonine pyrophosphate enzyme, central domain, putative     | 11687.m00919     | 45957  | 3          |          |          |
| translation initiation factor                                 | 11682.m04971     | 134032 | 2          |          |          |
| 60S ribosomal protein 16 (r (ribosomal protein 12).          | 11686.m07143     | 28132  | 3          | 5        | 3        |
| dihydrododecanic S-acetyltransferase                         | 11674.m03306     | 52541  | 3          | 2        | 2        |
| histone H2A-like protein                                     | 11682.m00152     | 16490  | 2          | 2        | 2        |
| phosphoglycerate kinase                                       | 11682.m03994     | 50107  | 11         |          | 5        |
| putative transposases                                        | 11666.m05268     | 63924  | 7          | 5        |          |
| Riboosomal L37ae protein family                              | 11687.m04734     | 10236  | 3          | 2        |          |
| ribosomal protein L10, putative                              | 11674.m02777     | 34356  | 6          | 10       |          |
| Riboosomal protein L68a                                      | 11682.m03541     | 12727  | 3          | 4        | 3        |
| ribosomal protein L6e, putative                              | 11688.m03614     | 93300  | 5          | 7        | 2        |
| ribosomal protein S17, putative                              | 11687.m04006     | 14937  | 2          | 3        | 3        |
| ribosomal protein S17, putative                              | 11687.m05125     | 17625  | 2          | 2        |          |
| ribosomal protein S17, putative                              | 11687.m05989     | 15966  | 2          | 4        |          |
| ribosomal protein S20, putative                              | 11687.m04725     | 21900  | 2          | 3        |          |
| Riboosomal protein S24e, putative                            | 11688.m01419     | 15739  | 2          |          | 2        |
| Riboosomal protein S8e                                       | 11670.m02661     | 24913  | 5          | 5        |          |
| transketolase                                                | 11680.m00347     | 80028  | 6          | 7        |          |
| 2,3-bisphosphoglycerate-independent phosphoglycerate mutase  | 11687.m05987     | 55632  | 2          |          |          |
| 4-alpha-gluonanotransferase, putative                        | 11673.m04620     | 105744 | 3          | 4        |          |
| 60S ribosomal protein 17a                                    | 11687.m02002     | 23865  | 9          |          |          |
| acido ribosomal protein 3a-maze                              | 11680.m04833     | 11867  | 4          |          |          |
| ATP synthase F1, beta subunit                               | 11682.m04581     | 58986  | 3          |          |          |
| ATPase, AAA family, putative                                 | 11687.m04511     | 51421  | 2          |          |          |
| BPG-independent PGAM N-terminus (PGAM_N)                    | 11682.m03854     | 52541  | 2          |          |          |
| Carboxy anhydrase, putative                                  | 11687.m04385     | 28022  | 2          |          |          |
| chaperonin gamma chain, putative                             | 11680.m03352     | 61219  | 3          |          |          |
| chaperonin GroEL                                             | 11688.m00320     | 63759  | 5          |          |          |
| CRAL/TRIO N-terminus, putative                               | 11682.m03352     | 63906  | 3          |          |          |
| Dehydrogenase E1 component, putative                        | 11670.m00195     | 46115  | 2          |          |          |
| D-isomer specific 2-hydroxyacid dehydrogenase, NAD binding domain, | 11688.m00117     | 44786  | 18         | 2        |          |
| elongation factor 1 beta 2                                   | 11689.m02941     | 24639  | 3          |          |          |
| expressed protein                                            | 11687.m06687     | 73528  | 3          |          |          |
| glycine dehydrogenase                                       | 11680.m04002     | 111404 | 4          | 7        | 4        |
| histone deacetylase HD2, putative                           | 11682.m09013     | 11747  | 3          |          |          |
| histone H3.2 protein                                         | 11689.m02779     | 15397  | 4          |          |          |
| Kinesin motor domain, putative                               | 11680.m01108     | 137542 | 2          |          |          |
| Nuclear transport factor 2 (NTF2) domain, putative           | 11670.m02868     | 51414  | 2          |          |          |
| Proteosome/Cyclosome repeat, putative                        | 11680.m04178     | 98828  | 2          |          |          |
| putative 30S ribosomal protein S13                           | 11688.m05001     | 19210  | 6          |          |          |
| putative TCP-1cpn62 chaperonin family protein                | 11689.m04189     | 57193  | 2          |          |          |
| Pyridine nucleotide-disulphide oxioreductase, putative       | 11687.m02259     | 58886  | 4          |          |          |
| pyruvate dehydrogenase complex dihydrododecanic acetyltransferase | 11680.m00666   | 50144  | 2          |          |          |
| Riboosomal L27e protein, putative                            | 11688.m01748     | 15569  | 3          |          |          |
| Riboosomal protein L13, putative                             | 11687.m03775     | 26229  | 2          |          |          |
| ribosomal protein L44.1 family, putative                     | 11689.m01586     | 34690  | 5          |          |          |
| ribosomal protein L44.1 family, putative                     | 11673.m07688     | 44711  | 3          |          |          |
| ribosomal protein S135510                                    | 11673.m07090     | 17952  | 3          |          |          |
| ribosomal protein S4                                        | 11676.m03467     | 23408  | 2          |          |          |
| RNA recognition motif, (a.k.a. RRM, RBD, or RNP domain), putative | 11674.m00120   | 94956  | 2          |          |          |
| Similar to dihydrododecanic S-acetyltransferase, putative    | 11686.m07772     | 51442  | 5          |          |          |
| Similar to myosin heavy chain                               | 11686.m01709     | 71326  | 4          |          |          |
| sucrose-UDP glucosyltransferase 2                          | 11698.m02844     | 82140  | 7          |          |          |
| t-complex polypeptide 1                                     | 11670.m04342     | 50584  | 4          |          |          |
| T-complex protein 1, epsilon subunit                        | 11680.m03661     | 59094  | 2          |          |          |
| translation elongation factor EF-1, subunit alpha           | 11689.m00767     | 40620  | 7          |          |          |
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.
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 \textit{in vivo} (of 72 at 0.1 M NaCl for IR64 2 wpi), and among them, 25 proteins were identified \textit{in vitro} at 1 M NaCl (Fig. 5B). 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 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 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. 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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. 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 cisternae (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 methyltransferases (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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