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Dissecting virus-plant interactions through proteomics approaches

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Abstract

Plant viruses exploit cellular factors, including host proteins, membranes and metabolites, for their replication in infected cells and to establish systemic infections. Besides traditional genetic, molecular, cellular and biochemical methods studying plant-virus interactions, both global and specialized proteomics methods are emerging as useful approaches for the identification of all the host proteins that play roles in virus infections. The various proteomics approaches include measuring differential protein expression in virus infected versus noninfected cells, analysis of viral and host protein components in the viral replicase or other virus-induced complexes, as well as proteome-wide screens to identify host protein - viral protein interactions using protein arrays or yeast two-hybrid assays. In this review, we will discuss the progress made in plant virology using various proteomics methods, and highlight the functions of some of the identified host proteins during viral infections. Since global proteomics approaches do not usually identify the molecular mechanism of the identified host factors during viral infections, additional experiments using genetics, biochemistry, cell biology and other approaches should also be performed to characterize the functions of host factors. Overall, the ever-improving proteomics approaches promise further understanding of plant-virus interactions that will likely result in new strategies for viral disease control in plants.

Keywords

Proteome; protein microarray; plant; virus; host factors; protein-protein interaction; RNA binding; virus replication; host-virus interaction

1. INTRODUCTION

Plant viruses usually code for only a few proteins to establish viral infection in plants. Therefore, they rely on the host by co-opting various host components during the infection cycle. The subverted host components include host proteins, subcellular membranes and metabolites. For example, the translation of the viral genomic RNA of plant RNA viruses are performed by the host ribosomes [1,2]. In addition, multiplication of the viral genome [3–9], viral RNA recombination [10–12] and transport of viral components in cells and to distant tissues in the plant are also facilitated by host proteins [13,14]. Therefore, dissecting virus-host interactions at the molecular level is important to fully understand the mechanism of virus replication and cellular responses against the infecting viruses by the host defense system. This understanding, in turn, will likely lead to the development of novel and more potent antiviral strategies to reduce the loss caused by plant viruses.

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Many methods are applied for either monitoring changes in plant cells in response to virus infections or to identify the set of host proteins that interacts with the viral proteins or the viral RNAs. Proteomics methods have several advantages over genomics approaches since they can directly measure protein levels in cells, which are not always reflected by mRNA levels due to various regulation of translation, different half-lives of proteins, post-translational modifications as well as subcellular localization of proteins in the cell. In spite of these obvious advantages, proteomics approaches have some weaknesses, including the difficulty to identify minor protein components due to limited sensitivity of the detection methods and underestimation of those proteins that only transiently interact with other components. It seems, however, that newer, more sophisticated and sensitive proteomics approaches appear every year to make these limitations less prevalent [15–18].

The application of proteomics approaches in plant virus research started two decades ago. van Loon et al. [19] used two-dimensional gel electrophoresis (2-DE) and a small antibody library of pathogenesis-related proteins (PRs) to characterize PRs from *Tobacco mosaic virus* (TMV) infected plants, to dissect virus-plant interactions. Later on, genome sequencing of different organisms became available [20,21] in combination with genetic, biochemical and bioinformatics tools, thus leading to creation of sophisticated host protein functional interaction databases [22–24]. Further advances were achieved by introducing mass-spectrometric analysis [3,25–28], and protein array approaches to screen for host proteins binding to viral components [4,6,29]. Proteomics-based studies on the host plants are likely to give an overview on how a particular viral infection affects the expression profile of the host proteome. In return, these data sets could also be useful to identify proteins involved in defensive responses as well as damage control to protect the cells. Overall, these advances using proteomics have already led to greatly improved understanding of virus-plant interactions, as described in this chapter.

2. Global protein changes in plant cells infected by plant viruses based on 2D-gel electrophoresis and mass spectrometric analysis

Due to the development of new technologies, it is now possible to study global protein abundance in plants. For example, 2-DE in combination with mass spectrometric analysis can provide valuable information on changes in protein abundance in the cell infected by plant viruses. This will lead to better understanding of the global responses of plant cells to virus infections and virus-plant interactions at the protein level. Below we discuss selected global proteomics studies using different plants and viruses.

One of the most elegant examples of using proteomics tools to identify host proteins differentially regulated in plant virus infected cells was shown in the case of plant innate immunity response against TMV infection using *Nicotiana benthamiana* containing the *N* resistance gene [30]. Standard 2-DE approach using different fluorescent dyes was used to investigate differences in the level of soluble proteins from plant tissues at 0, 2, 8, 16 hours after TMV infection. In addition, another technique employing isobaric iTRAQ reagents was also applied to analyze trypsin digested total proteins from the same samples as the 2-DE approach. The advantage of iTRAQ is that the labeling reagents are maintained to the same molecular mass by adjusting with four sets of isotopic atom combinations, including ^{13}C , ^{15}N , and ^{18}O , which avoids the need for protein separation on chromatographic columns and MS analysis due to different molecular weights of the same proteins when introduced by different labeling [31]. *N. benthamiana* without the *N* gene was used as a control to exclude the changes unrelated to plant innate immunity reactions. The protein data sets from the two proteomic techniques mostly overlapped. Overall, proteins involved in cellular defense, metabolism of reactive oxygen species and hormone signaling, chaperone functions as well as cellular metabolism were identified. Down-regulation of the

expression levels of four ER chaperones identified, namely disulfide isomerases NbERp57 and NbP5, as well as Calreticulins NbCRT2 and NbCRT3, led to loss-of-resistance phenotype of N-gene carrying *N. benthamiana* against TMV. It was proposed that NbCRT2 and NbCRT3 might be involved in the induction of receptor-like kinase (IRK), which is required for innate immunity.

Another example of using 2-DE/mass spectrometry is the study on the host response to *Rice yellow mottle Virus* (RYMV), which is a single-stranded-positive-sense RNA virus. The host for RYMV was rice in these studies, since among the various model plants, rice is a useful organism for studying of virus-plant interaction due to its comparatively small genome size, which is fully sequenced [20,21]. Based on 2-DE and liquid chromatography-tandem mass spectrometric (LC-MS/MS) analysis, the authors studied differential protein expression levels to investigate the host response to RYMV infection [25]. Detailed differential protein expression levels of a susceptible and a partially resistant cultivar were analyzed 1 hour postinoculation (hpi) with RYMV, 2 day postinoculation (dpi), 5dpi and 7dpi or not challenged by RYMV. The authors identified three groups of host proteins that are regulated by viral infection, including proteins of cellular metabolism, stress-related response and host mRNA translation. It is possible that changing the host metabolism by the virus might favor the establishment of successful viral infection. Accordingly, systematic genome-wide screens for host factors affecting replication of *Tomato bushy stunt virus* (TBSV) [32], *Brome mosaic virus* (BMV) [33] and influenza virus strain H1N1 and H5N1 [34] also led to the identification of cellular metabolism pathways, indicating the importance of cellular metabolism for virus infections.

The abundance of abiotic stress-induced proteins, including heat shock proteins, salt-stress-induced proteins, or biotic stress-induced proteins, like pathogenesis-related (PR) proteins and ethylene-inducible proteins, has changed during viral infection as shown in the above proteomics study [25]. Heat shock protein 70 (Hsp70) and Hsp90 are well-studied proteins for their roles during plus-strand RNA virus infection. For example, Hsp70 was shown to be required for the assembly of the tombusvirus replicase complex based on yeast, a model host, and *in vitro* studies [3,7,35,36]. In addition, Hsp90 was shown to be involved in flock house virus protein-A translation in *Drosophila* cells [37]. Salt-stress-induced proteins are proteins induced by high salt concentration in soil or low water supply. Identifying of this kind of proteins, such as dehydrin, in RYMV infected rice could be due to induction of this pathway in response to different stresses. Interestingly, salt-stress-induced proteins were also identified in another proteomics study based on virus-plant interaction [28]. PR proteins are host defense proteins involved in innate immunity to pathogen invasion. PR-10A, which contains a ribonuclease domain [38], was up-regulated up to 23-fold at 7dpi in the incompatible rice cultivar. Also the expression of ethylene-inducible proteins was highly down-regulated in the susceptible cultivar. Altogether, the stress-induced proteins could serve different roles during virus infection, including facilitating virus replication, promoting host defenses against the virus and reducing damage caused by the induction.

The above research on RYMV infection has also shown altered levels of expression for proteins involved in translation and protein degradation pathways, including protein ubiquitination, in rice. The translation machinery may be involved in viral protein translation, stability [6] or viral polymerase activity [39]. As shown by study from other viruses, the proteins in host translational pathways have strong associations or played important functions in plant virus replication. Eukaryotic translation elongation factor 1A is a host factor in protein synthesis pathway and have strong association with tobamovirus [40] or tombusvirus replicases [6]. Another example is Eukaryotic translation initiation factor 3 (eIF-3), which can be co-purified and enriched together with BMV [39] or (TMV) [41]

RNA-dependent RNA polymerases. Further study showed that eIF-3 contribute to enhance BMV minus-strand RNA synthesis [39].

Another proteomics study on virus-plant interaction [28], based on 2-DE analysis to identify differentially expressed host proteins, also used both compatible and incompatible interactions. Three functional protein groups were identified in this study: antioxidant enzymes, aminopeptidases and pathogenesis-related proteins elicited by TMV infection.

Differential regulation of the antioxidant proteins suggests that the tomato fruits react to scavenge reactive oxygen species (ROS), which were likely induced by TMV replication. Aminopeptidases also showed increased expression levels, among which expression of DIP-1 peptidase increased dramatically. DIP-1, as a peptidase, was also induced by drought stress in watermelon. This study together with the previously discussed proteomics study on RYMV infected rice resulted in identification of several drought-stress-induced proteins [25]. Thus, emerging evidence suggests that drought-stress and some viral infections induce similar pathways in plants. Beta-1,3-Endoglucanase (GLU) and Chitinase (CHI), known as PR proteins, were induced by TMV infection in tomato fruits. GLU and CHI are plant-secreted enzymes against fungal infections by digesting the fungal cell wall. The mechanism of their actions against viral infections is unclear, however, GLU and CHI have also been found in response to many abiotic or biotic stresses, including viral infections [43,44]. The green alga virus, Chlorella virus, CVK2, encodes and uses chitinase and chitosanase to aid its infection, mostly likely by helping the digestion of the cell wall and/or release viral particles [45,46]. Research on CVK2 chitinase and chitosanase might help further understand the function of chitinase in higher plants during viral infection.

While the above described examples were based on global proteomics analysis of total protein extracts, thus testing whole cellular protein profiles, it could be useful to examine virus-plant interactions based on purified host organelles or different cellular fractions. This approach could zoom in to specific host pathways and to specific cell locations, and also reduce the protein complexity in the samples. As an example, a proteomics study was performed to show the effect of Tobamovirus infection on plant photo-system II electron transport, including the detailed changes of PsbO and PsbP protein levels [27]. To observe PsbO and PsbP levels, which are located in chloroplast stromal thylakoid membranes, the authors purified the thylakoid membrane that helped reduce the number of proteins on the 2-DE gels. The thylakoid membranes were isolated from *N. benthamiana* infected with *pepper mild mottle virus* (PMMoV) at different days post inoculation. The authors found that protein accumulation of PsbP isoforms was greatly inhibited by PMMoV infection, while PsbO level was reduced slightly [47].

A similar approach was used to study nuclear proteins in purified nuclei from TMV infected hot pepper plant by 2-DE [48]. Six nuclear proteins were identified based on their increased accumulation after TMV infection. These proteins are 14-3-3 protein, 26S proteasome subunit RPN7, mRNA-binding protein, Rab11 GTPase, Ubiquitin extension protein and a hypothetical protein. Additional analysis confirmed the increased level for RPN7 in plants inoculated with TMV-P₀, an incompatible pathogen of hot pepper, but not with the compatible TMV-P_{1,2}. Subcellular studies on RPN7 overexpressed in hot pepper suggested that RPN7 might be involved in triggering programmed cell death in the hot pepper plant.

Overall, the advantage of using 2-DE in combination with mass spectrometric analysis is the somewhat unbiased identification of host proteins, whose expression are changed during infection. The 2-DE/mass spectrometric analysis promises a window to look at the whole picture of virus-host interplay [25,26,28]. Many different cell responses are induced by virus infections, including hypersensitive reaction [49,50] and activation of the RNA silencing

machinery [51]. Viruses also change the host cellular functions to favor infection, viral entry, translation and replication, such as induction of host membrane proliferation [52], unsaturated fatty acid production, sterol synthesis [9,53], the expression of heat shock proteins [54,55]. However, not all host proteins can be studied using 2-DE. For example, 2-DE can frequently lead to the identification of relatively abundant proteins with pI 4-7 and molecular weight from 10 kDa to 100 kDa. This limited a study to about 1000 rice proteins during RYMV infection, which is only ~2% of total proteins [25]. Detection limit for minor proteins is also a disadvantage leading to underestimation of the roles of low abundant proteins in viral complexes or in general, in virus infections.

Proteomics studies in general produce a list of proteins. However, the subsequent in-depth analysis to demonstrate the significance of the identified proteins during viral infections is frequently missing or incomplete as in the case of the above studies on RYMV and TMV [25,28,56]. Therefore, researchers performing global proteomics studies should perform meaningful functional studies with individual host proteins during viral infections. The functional and mechanistic studies with the identified host proteins could lead to evidence for global mechanisms used by some plant viruses to infect cells.

3. Composition of viral protein complexes based on 2D-gel electrophoresis and mass spectrometric analysis

Plant RNA viruses assemble ribonucleoprotein (RNP) or protein complexes to facilitate the infection process. Purification and subsequent proteomics analysis of these complexes can reveal the host and viral protein components that are recruited to form the given complex. The identified host proteins likely interact with the viral proteins or the viral RNA. The control experiments performed in the absence of the viral proteins should be useful to identify those host proteins, which contaminate the purified preparations.

This approach was used successfully to identify host and viral components within the functional replicase complex of a tombusvirus. Tombusviruses, which are single-stranded-positive-sense RNA viruses, can assemble the viral replicase complex and replicate a viral replicon RNA in yeast cells [57,58]. Yeast is a great model host due its small genome size, limited gene redundancy, high level of conserved genes with plants genes, as well as the availability of straightforward genetic tools and genome-wide mutant libraries [32,33]. Accordingly, the solubilized active replicase complex of *Cucumber necrosis virus* (CNV) was purified via two-step affinity purification [3], followed by 2-DE and mass spectrometry. This analysis led to the identification of the two viral proteins and three host proteins in the viral replicase complex, namely Ssa1/2p (Heat shock protein 70, Hsp70), Tdh2/3p (glyceraldehyde-3-phosphate dehydrogenase, GAPDH), and Pdc1p (pyruvate decarboxylase). The 2-DE analysis also revealed 3-to-7 additional host proteins, which were strongly associated with the viral replicase complex. Two out of these host proteins were later identified as Cdc34p and Tef1/2p by protein array and co-immunoprecipitation experiments (see below) [4,6]

Additional in-depth studies with the identified host proteins in the tombusvirus replicase complex confirmed that the yeast Ssa1/2p and Tdh2/3p or their plant homologues are indeed resident proteins in the viral replicase complex and play functional roles during various steps of replication [5,35]. For example, Hsp70, which is a molecular chaperon in the cell cytosol, has been shown to co-localize with the tombusvirus replication complex to host peroxisome membrane when co-expressed with the viral replication proteins, p33 and p92^{pol}, and the viral replicon RNA [7]. By using temperature-sensitive (ts) mutant of Hsp70, the authors demonstrated that Hsp70 is critical for the localization of p33 replication protein to the peroxisome membrane, which is the site of viral replication [36]. Biochemical data indicate

that Hsp70 might function during the insertion of the replication proteins into intracellular membranes. In vitro assembly of the tombusvirus replicase complex using purified recombinant viral replication proteins, the replicon RNA and Hsp70 together with the membranous fraction of a yeast cell free extract revealed the essential role of Hsp70 in the assembly of the tombusvirus replicase complex [35]. The host metabolic protein GAPDH was also studied in some details for its role in tombusvirus replication [5]. Subcellular localization of GAPDH via confocal microscopy showed that GAPDH is recruited from the cytosol to the peroxisome membrane. Tombusvirus replication studies using yeast or plants expressing reduced level of GAPDH revealed that GAPDH is likely involved in retaining the minus-stranded viral replication intermediate in the replicase complex [5].

To identify host factors associated with a potyvirus RNA dependent RNA polymerase (RdRp), an improved tandem affinity purification (NTAPi)-tagged RdRp of *Turnip mosaic virus* (TuMV) was expressed in *Arabidopsis thaliana*. The TuMV RdRp was then purified by tandem affinity purification, while the NTAPi-tagged GFP was used as a control. Mass spectrometric analysis identified three host proteins present in the TuMV RdRp preparation, namely heat shock cognate 70-3 (AtHsc70-3), poly(A)-binding (PABP) proteins, and translation elongation factor eEF1A [59,60]. Interaction of AtHsc70-3 and PABP with TuMV RdRp protein was also confirmed *in vitro*. Interestingly, redistribution of AtHsc70-3 and PABP to large perinuclear ER-derived vesicles was observed in the presence of overexpressed TuMV 6K-VPg-Pro replication protein, which was shown to interact with TuMV RdRp and possibly induce the ER association of the RdRp during TuMV infection [60]. The authors proposed that the interaction of TuMV RdRp with the host AtHsc70-3 and PABP proteins would likely take place in the ER derived replicase complex, albeit their roles in potyvirus replication need further studies.

Another major viral RNP complex is the virion, containing the encapsidated viral RNA. A proteomics approach was also used to test the proteins present in the purified RYMV virion preparation. First, virion preparation was purified via size exclusion chromatography, since the virion is a large, but uniformly-sized RNP complex. However, it is important to note that size exclusion chromatography may not be suitable to remove all the contaminating host proteins that could be present in the same fraction as the virions. Interestingly, many host proteins involving those involved in host metabolism, transcription, translation, and host defense pathways were found in the RYMV virion fraction. Further experiments will be needed to show if the identified host proteins bind directly to the virion [28,39–41].

4. Identification of host proteins binding to viral proteins or the viral RNA based on protein microarrays

Protein microarray is another proteomics approach for studying virus-host interactions. Protein microarrays contain purified, highly condensed, covalently attached host proteins on glass surfaces. Protein microarray can be used for global screening to identify proteins interacting with other proteins, RNA or DNA, protein kinase substrates and finding small molecules targeted by proteins [61]. Accordingly, protein microarray has been used to identify host proteins binding to the viral RNA or viral proteins [4,6,29].

A yeast protein microarray was used for screening for BMV RNA-yeast protein interactions [29]. About five thousands yeast proteins was laid onto FAST slides. A small segment of the BMV RNA3 containing a clamp adenine motif (CAM) at the 3' untranslated region was labeled with cy3, while a mutant version of this RNA region was labeled with cy5. The 3' untranslated region of BMV RNA was chosen since it is involved in minus-strand viral RNA synthesis [62], translation regulation [63] and virion assembly [64]. The mutant version is debilitated in these functions, thus is a good control for finding specific host

factors involved in these functions. Both the wt and the mutated RNAs were analyzed for their abilities to bind to host proteins based on the yeast microarray by determining the ratio of cy3/cy5. Among 12 top candidates from the screen, pseudouridine synthase Pus4p, actin patch binding protein App1p and UDP-N-acetylglucosamine pyrophosphorylase Qri1p were the best in binding to the wt RNA segment. These proteins were further tested for their roles in BMV replication in plants. The authors found that Pus4p and App1p modestly reduced plus-strand accumulation, but they dramatically inhibited the systemic spread of BMV through in *Nicotiana benthamiana*. Also, Pus4p inhibited the encapsidation of BMV RNA by competing with the BMV coat protein. This research demonstrated that a yeast protein microarray could provide valuable information about the host players in virus-host interactions, and the data can be further used to study virus infections in higher plants.

Identification of host factors affecting tombusvirus replication was also assisted by the use of the yeast protein microarray. TBSV replicase proteins were tested for interaction with 4088 purified yeast proteins on protein microarray chip [4]. The approach led to the identification of over 50 yeast proteins interacting with the full-length TBSV p33 replication protein, or its truncated versions, such as p33N82 harboring the N-terminal 82aa of p33; p33C containing the C-terminal RNA-binding and protein interaction domains of p33; as well as p92C, which is a truncated p92^{pol} replication protein having RNA-dependent RNA polymerase motifs. The authors found 58 yeast proteins that interacted with p33, while 29 of the 58 host proteins interacted with p33C, and 8 of the 58 host proteins interacted with p33N82. Moreover, 33 host proteins bound to p92C. The identified yeast proteins included protein chaperones, ubiquitin-associated proteins, translation factors and RNA-modifying enzymes. The binding of p33 to 19 of the above host proteins was confirmed with protein pull-down assay and a split-ubiquitin-based yeast two-hybrid assay.

Among the identified proteins were Cdc34p and Rsp5p, which were further studied for their roles in tombusvirus replication. Cdc34p is a ubiquitin-conjugating enzyme and a catalytic subunit of SCF ubiquitin-protein ligase complex [65]. Cdc34p regulates degradation of many cell proteins to control the cell cycle [66]. In yeast, a model host for studying plant virus-host interaction [67], Cdc34p was found to be a component of the purified tombusvirus replicase complex. Down-regulation of Cdc34p expression led a 5-fold decrease in tombusvirus repRNA accumulation, while overexpression of Cdc34p increased viral repRNA accumulation by 2-fold. Interestingly, Cdc34p ubiquitinated p33 in vitro, and it was proposed that in vivo ubiquitination of p33 could affect the assembly of the replicase complex or recruitment of host factors into replication. Rsp5p is a member of the Nedd4 family of E3 ubiquitin ligases, which can ubiquitinate target proteins involved in many cellular processes [68–70].

Rsp5p can interact with the tombusvirus p33 and the central portion of p92^{pol} replication protein via its WW domain [71]. In a genetic study, Rsp5p overexpression led to reduced TBSV RNA accumulation, while its downregulation increased TBSV accumulation. The function of Rsp5p in TBSV replication might be due to the role of Rsp5p in degradation of the p92^{pol} replication protein [71].

Another application for the yeast protein microarray was the identification of host proteins binding to the tombusvirus RNA as compared with the BMV RNA1 [6]. This work led to the identification of Gcd2p, the delta subunit of the translation initiation factor eIF2B; Sec62p, a subunit in the Sec63 complex; Deg1p tRNA pseudouridine synthase; the nucleolar protein Utp7p; Dbp2p ATP-dependent RNA helicase; Tef2p translation elongation factor eEF1A; RPL8A subunit of the large (60S) ribosomal, Pus4p pseudouridine synthase, to name a few that specifically bound to the TBSV genomic RNA. Further in vitro and in vivo binding assay confirmed the protein microarray results. In-depth analysis of Tef2p revealed

its functional relevance in TBSV replication. Tef2p is eukaryotic translational elongation factor EF-1 alpha (eEF1A) that is also coded by the *TEF1* gene. eEF1A helps protein translation by facilitating ribosome reception of aminoacyl-tRNA [72], and may also be involved in tRNA nuclear-cytoplasmic trafficking [73]. RNA binding studies revealed that eEF1A bound to an important *cis*-acting element, called replication silencer element in the 3' untranslated region of TBSV repRNA, confirming the microarray assay and in vivo RNA-protein co-purification experiments [6]. In addition to its TBSV RNA binding activity, eEF1A was found to be part of TBSV replicase complex, affects the assembly of the viral replicase and the stability of the viral replication protein as well as promotes minus-strand synthesis (Li, Esposito, Tupman, Kinzy and Nagy, PLoS Pathogens, *in press*). Different mutations of *tef2* in *tef1* deletion yeast strain significantly affected TBSV repRNA accumulation and replicase protein stability, giving evidence of multiple roles of eEF1A in tombusvirus replication [6]. Additional studies with other viruses, like West Nile Virus (41, 42), Q-beta virus (43) and TMV (15) indicate that eEF1A is part of viral replicase complexes and it might be a common host factor.

5. Identification of host protein interacting with viral proteins based on yeast two-hybrid screens

Due to vast literature for yeast two-hybrid (Y2H) screens, we will only give examples instead of covering the whole research field. The Y2H screens are based on cDNA libraries prepared from various hosts. Since the cDNA libraries may not represent all the genes expressed in the host due to differential gene expression in different parts of plants or due to different stresses the plant exposed to, most cDNA libraries are somewhat biased. Nevertheless, Y2H screens nicely complement other proteomics screens, such as the protein microarray approach, since Y2H is a reliable method and the interaction takes place in the cellular environment that likely provides suitable conditions, such as pH, salt concentration, presence of protein chaperons and subcellular membranes and organelles for cellular localization of proteins. However, Y2H screens are the most suitable for identification of cytosolic (soluble) host proteins. Moreover, due to protein overexpression in yeast, some of the interactions identified could be serendipitous (false positives).

Y2H screening of a cDNA library prepared from *Nicotiana benthamiana* for interaction with *Bamboo mosaic virus* (BaMV) RNA-dependent RNA polymerase (RdRp) protein domain [74] led to the identification of a putative methyltransferase protein (PNbMTS1). Overexpression of PNbMTS1 inhibited the accumulation of BaMV coat protein in protoplasts. Deletion of the putative signal peptide sequence, the transmembrane domain or the AdoMet-binding domain of PNbMTS1 inhibited BaMV replication. In contrast, silencing of PNbMTS1 led to 6-fold increased BaMV RNA accumulation, suggesting that PNbMTS1 might play a role in host defense against BaMV.

Y2H screen was also used successfully to find several host proteins binding to the helicase domain of the TMV 126K replication protein based on screening an *Arabidopsis* cDNA library. Among the identified host proteins, further studies were performed with Aux/IAA protein PAP1 [75–77] and a NAC domain transcription factor ATAF2 [78]. PAP1 is a negative regulator of auxin response factors. The authors found that a TMV mutant in the helicase domain of TMV 126K protein, which could not interact with PAP1, caused attenuated disease symptoms in *Arabidopsis*. In addition, knock down of PAP1 mRNA level resulted in attenuated symptoms in TMV infected plants, suggesting that PAP1 is a major player in symptom manifestation. Moreover, TMV infection was found to alter the accumulation and subcellular localization of PAP1. In addition, 30% of those *Arabidopsis* genes that are induced or inhibited by TMV infection contain multiple auxin response

promoter elements. Overall, TMV might interact with PAP1 to attenuate its function as a transcription factor and alter disease symptoms [75,78].

The interaction of helicase domain of TMV 126K replication protein with ATAF2 was also confirmed by immunoprecipitation assay [78]. ATAF2 overexpression decreased TMV accumulation and the expression level of ATAF2 was up-regulated in TMV inoculated leaves, albeit not in the systemically infected leaves. Furthermore, ATAF2 was shown to regulate the expression of the host pathogenic-related (PR) protein. This study indicated that replication protein-ATAF2 interaction plays a role for virus by avoiding the host PR protein induction, thus could promote systemic infection by TMV.

Using cDNA libraries prepared from leaves and roots of sugar beet (*Beta vulgaris L.*), a Y2H screen against the p25 pathogenecity factor of *Beet necrotic yellow vein virus* (BNYVV) [79] identified 36 sequences representing host proteins, and 27 of those proteins have known functions in plants [80]. The identified host proteins can be grouped into several functional categories, such as Ubiquitin-proteasome system, stress-related proteins, cell cycle, translation and post-translational modification, metabolism and gene expression. The interaction of 10 of the identified host proteins with p25 was also confirmed in planta by bimolecular fluorescence complementation assay (BiFC). Dissection of the functions of the host proteins in BNYVV infection will need further studies.

Y2H screen was also useful to identify pathogenic determinants for geminivirus infections, which are occasionally associated with DNA- satellite. The DNA- encodes only one gene,

C1, which is important for symptom development and enhances the symptoms caused by geminiviruses in plants [81]. A screen for host proteins binding to C1 coded by DNA- associated with *Cotton leaf curl Multan virus* (CLCuMV) was based on Y2H screen, which identified a host ubiquitin-conjugating enzyme (SIUBC3) [82]. Interestingly, DNA- coding for a mutant version of C1 that lacks the SIUBC3 binding domain cannot induce the DNA- specific symptom in plants. In addition, expression of C1 in tobacco plants reduced the level of polyubiquitinated proteins. Overall, these findings supported the hypothesis that DNA- specific symptoms are due to hindering the activity of SIUBC3, which leads to malfunction of the host protein degradation and protein modification pathways.

Another study based on Y2H screens identified host proteins binding to *Potato virus X* (PVX) movement protein p12, which is required for intercellular movement [14] by enlarging the plasmodesmal size exclusion limit (SEL) [13]. Three gene products that are homologues of tobacco ankyrin repeat-containing protein HBPI, namely TIP1, TIP2 and TIP3, can specifically bind to PVX p12. These TIPs were found to interact with plasmodesmal SEL regulator, -1,3-gucanase. Agroinfiltration of both TIP1 and p12 expression plasmids induced co-localization and accumulation of these two proteins to the cellular periphery, which may suggest that TIPs are the proteins that recruit the PVX movement proteins to -1,3-gucanase and thus regulate the SEL.

Since many host protein-viral replication protein interactions are expected to occur on membrane surfaces and lipids within the membranes could affect membrane-bound protein-protein interactions, it is important to perform MYTH (membrane yeast two-hybrid) split-ubiquitin assay to identify these interactions. The traditional proteomics assays might not be optimal for these studies as demonstrated by the above examples from Y2H screens and the previous global proteomics approach using the yeast protein array that are underrepresented for membrane proteins [4]. Thus, to complement the previous protein array approach that targets mostly soluble host proteins, the membrane-based global protein-protein approach, termed MYTH was used to identify host proteins interacting with the tombusvirus replication proteins [83]. Briefly, the split-ubiquitin assay is based on the ability of N-

terminal (NubG) and C-terminal (Cub) halves of ubiquitin to reconstitute a functional protein [84,85]. NubG and Cub (fused separately to interacting proteins) are brought to close proximity by the interacting proteins, resulting in reconstitution of a functional ubiquitin protein. This leads to the cleavage of the reconstituted ubiquitin by endogenous ubiquitin specific proteases, resulting in the release of the transcription factor, which allows growth on selective media.

The MYTH screens led to the identification of 57 yeast proteins that interacted with the replication proteins of two tombusviruses [83]. Among the identified host proteins were Tef1p translation elongation factor 1A and GAPDH (Tdh2p and Tdh3p in yeast), which have previously been shown to be permanent residents in the tombusvirus replicase complex [3,5,6]. The identification of these host proteins in the MYTH screens suggests that this approach is useful to find relevant interactors of membrane-bound viral replication proteins. In addition, the role of Cpr1p (Cyclosporin A-sensitive proline rotamase 1), which is a member of the cyclophilin family of proteins, was further studied [83]. Cyclophilins are ubiquitous, highly conserved proteins having prolyl isomerase (PPIase or rotamase) activity. Cyclophilins are involved in catalyzing *cis-trans* isomerization of the peptidyl-prolyl bonds that could alter the structure, function or localization of the client proteins [86] [87]. Overall, isomerization of the peptidyl-prolyl bonds are frequently required for protein refolding following traffic through cellular membranes and PPIases play a global role in facilitating correct protein folding and conformational changes [87]. Interestingly, Cpr1p was shown to bind to the viral p33 protein and inhibited TBSV replication in yeast based on deletion and over-expression analysis, suggesting that cyclophilins might be part of the innate response of the host against some viruses. Since prolyl isomerases are conserved and ubiquitous proteins, their role could be common against viruses [83]. Overall, the MYTH screens, by focusing on protein-protein interactions taking place in the intracellular membranes, can be more powerful to identify protein interactions in association with cellular proteins and lipids than traditional proteomics approaches.

6. Additional approaches to identify host proteins interacting with viral proteins

In addition to the above approaches, there are many more proteomics-based methods that can be suitable to identify host factors involved in plant virus – host interactions. For example, antibody-based co-immunoprecipitation was used to identify host factors in the partially purified TMV and BMV replicase complexes. Using the membrane fraction of the vacuole-depleted tobacco BY-2 protoplasts expressing the FLAG-tagged 180K replicase protein of *Tomato Mosaic virus* (ToMV), the authors affinity-purified the replicase complex [40]. Several antibodies were used to identify co-purified host proteins, which included Hsp70, eEF1A, Tom1, Tom2 and eEIF-3 [40,41]. Co-immunoprecipitation was also used to demonstrate that eEIF-3 interacted with the BMV replicase [39]. Further studies will be needed to dissect the functions of these host proteins in TMV and BMV replication.

7. Bioinformatical analyses of global proteomics data obtained from plant virus infections

Collecting global proteomics datasets from virus-infected cells is just a start rather than an end of studying of host-virus interactions. Important functions of host proteins during viral infections should be characterized in depth. Viruses are known to co-opt host proteins and host pathways to establish successful infections [5,7,32–34,39,67,88]. Also, host proteins involved in defense against plant viruses as well as damage control proteins should be characterized in order to better understand virus-host interactions. Global proteomics studies

usually result in large data sets. By using bioinformatics tools could be helpful to formulate working models on the possible roles of host proteins during virus infections or group the host proteins based on their functions to streamline in-depth studies.

STRING, which is a database of protein interaction including biochemical interactions and genetic interactions, can be used to filter out some important host protein networks [22]. In a genetic screen focusing on human proteins important to influenza virus replication [34], STRING was used to assess important host protein-protein interactions involved in influenza infection, by using the interaction network from the screened host proteins. The major protein-protein interactions from the positive hits of this study provided evidence that many host functions are used or modulated by influenza virus infection, like RNA splicing, vacuolar ATPases function, nuclear transport, membrane transport and translation. These host proteins might be important to influenza virus to establish efficient infection. Proteomics study can also be benefited from this host protein interaction network database, since STRING could highlight the important interactions, including one or two core proteins.

Analysis of host pathways involved or recruited for viral infection gives an alternative view on the role of host proteins. REACTOME is a host pathway database covering many organisms [23]. As proteomics data can provide candidate host proteins, which have correlation with viral infection, analysis using REACTOME could tell which host pathways are modified by viral infection, as it been used in the genetics study of influenza virus infection [34,89], West Nile Virus, and Dengue Virus [89].

We performed bioinformatical analysis on host proteins interacting with TBSV replication proteins or the viral RNA [4,6] using STRING and REACTOME databases (Fig. 1 and Fig. 2). Host protein interaction network analyzed by STRING showed that many identified host factors are in the center of the network. In the map of the network (Fig. 1), Rsp5 ubiquitin ligase has the largest number of connections to the other identified host factors, and seems to be an important host factors affecting tombusvirus replication. Accordingly, Rsp5p was found to play an important role by inhibiting tombusvirus replication *in vitro* and *in vivo* [71].

REACTOME database was also used for analysis of proteomics data sets for tombusviruses. We found that 6 of the cellular processes are over-represented from the tombusvirus proteomics datasets, including gene expression, protein, lipid and carbohydrate metabolisms as well as telomere maintenance and DNA replication (Fig. 2). Interestingly, the identified cellular processes from the tombusvirus proteomics studies partially overlap with a genome-wide study on influenza virus on human cell [34], which also identified gene expression and carbohydrate metabolism processes. This indicates, that these cellular processes might have some common roles in virus-host interactions. It is likely, that these types of bioinformatics analyses are valuable for designing experiments to investigate mechanism of viral infection and the response by the host defenses.

7. Summary of the characterized host factors for plant viruses from proteomics studies

Proteomics approaches are revolutionizing how we study plant virus-host interactions. Global or specialized proteomics approaches have already led to the identification of a large number of host factors involved in plant virus infections. In combination with extensive genomics datasets, the proteomics datasets provide a broad view on virus-plant interactions [3,4,6,25,26,28,29,32,33], and bioinformatics analysis of these datasets frequently results in testable models. However, the proteomics approaches do not usually identify the molecular

mechanism of the identified host factors during viral infections or how viruses co-opt host cellular functions to facilitate infection and disarm host defense pathways. Therefore, to answer these major questions raised by proteomics studies, additional experiments using genetics, biochemistry, cell biology and other approaches should be performed to dissect the functional roles of specific host proteins during plant virus infections. These future results promise better understanding of virus-host interactions and will likely lead to the development of novel antiviral approaches to combat the damage caused by infectious viral diseases in agriculture.

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Fig. 1. Physical and genetic protein interaction map of candidate host factors from proteomics studies of tombusviruses, using the STRING database. Circles represent different host proteins, while the thickness of lines indicates strength of the interaction between two proteins.

Fig. 2. Highlighted cellular pathways based on the identified host proteins in proteomics studies of tombusviruses. Each arrow represents a cellular pathway generated from published datasets or bioinformatics analysis. We highlighted six cellular pathways based on the input datasets of the identified host proteins from proteomics screens, which are considered to be involved in virus-host interactions.

TABLE 1

A brief list of characterized host proteins and their functions in plant virus replication.

Virus	Protein name	Function Description	Proposed function	references
TBSV	Cdc34	Ubiquitin-conjugating enzyme (E2) and catalytic subunit of SCF ubiquitin-protein ligase complex	component of the viral replicase complex; ubiquitinate p33 to enhance TBSV replication.	[4]
TBSV	Tef1/2	Translational elongation factor EF-1 alpha	component of the viral replicase complex; mutation of tef1/2 reduces TBSV replicase protein stability in vivo and viral replication.	[6]
TBSV	Tdh2/3	Glyceraldehyde-3-phosphate dehydrogenase	component of the viral replicase complex; helps the asymmetric replication of TBSV.	[3,5]
TBSV	Ssa1/2	ATP binding protein involved in protein folding and vacuolar import of proteins; member of heat shock protein 70 (HSP70) family	component of the viral replicase complex; assists TBSV p33 replication protein insertion to host membrane.	[3,7,35]
TBSV	Rsp5	a member of the Nedd4 family of E3 ubiquitin ligases	Interacts p33 and p92 replicase protein via WW domain; decreases p92 expression while not p33; inhibit virus replication	[71]
BMV	Pus4	Pseudouridine synthase;	increases the minus-strand RNA synthesis by 30%, and decreases viral positive-strand RNA synthesis; helps viral systemic movement in plant.	[29]
	App1p	Protein of unknown function, interacts with Rvs161p and Rvs167p	inhibits the viral RNA synthesis and viral systemic movement	[29]
	Qri1p	UDP-N-acetylglucosamine pyrophosphorylase	inhibits the viral RNA synthesis	[29]
TMV	NbCRT2, NbCRT3	Calnexin; integral membrane ER chaperone involved in folding and quality control of glycoproteins	Silencing leads to partial loss of N immune receptor mediated defense to TMV; Induces IRK expression.	[30]
	NbERP57, NbP5	Protein disulfide isomerase, multifunctional protein resident in the endoplasmic reticulum lumen	Silencing leads to partial loss of N immune receptor mediated defense to TMV	[30]
	RPN7	26S proteasome subunit	TMV Induced overexpression of RPN7 leads to the programmed cell death, and causes symptom of TMV.	[48]
BaMV	PNbMTS1	putative methyltransferase	inhibits BaMV coat protein accumulation; silencing of PNbMTS1 stimulates viral RNA accumulation suggesting its antiviral function	[48,74]
ClCuMV- C1	SIUBC3	ubiquitin-conjugating enzyme	interacts with viral C1 protein and causes C1 specific symptom	[82]
PVX	TIP1	homologues of tobacco ankyrin repeat-containing protein	Interacts with PVX p12 movement protein and regulates cell-to-cell movement	[14]