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Comparative proteomic analysis of melon phloem exudates in response to viral infection

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ABSTRACT

Phloem vasculature is the route that most plant viruses use to spread widely around the plant. In addition, phloem sap transports signals that trigger systemic defense responses to infection. We investigated the proteome-level changes that occur in phloem sap during virus infection using the 2D-DIGE technique. Total proteins were extracted from phloem exudates of healthy and Melon necrotic spot virus infected melon plants and analyzed by 2D-DIGE. A total of 1046 spots were detected but only 25 had significant changes in abundance. After mass spectrometry, 19 different proteins corresponding to 22 spots were further identified (13 of them up-accumulated and 9 down-accumulated). Most of them were involved in controlling redox balance and cell death. Only two of the differentially altered proteins had never been described to be present in the phloem before: a carboxylesterase and the fumarylacetoacetate hydrolase 1, both considered negative regulators of cell death. RT-PCR analysis of phloem sap RNAs revealed that the transcripts corresponding to some of the identified protein could be also loaded into the sieve elements. The impact of these proteins in the host response against viral infections and the potential involvement in regulating development, growth and stress response in melon plants is discussed.

Biological significance

Despite the importance of phloem as an integrative pathway for resource distribution, signaling and plant virus transport little is known about the modifications induced by these pathogens in phloem sap proteome. Only one previous study has actually examined the phloem sap proteome during viral infection using conventional two-dimensional electrophoresis. Since the major limitation of this technique has been its low sensitivity, the authors only identified five phloem proteins with altered abundance. To circumvent this issue we use two-dimensional difference in-gel electrophoresis (2D DIGE) technique, which combined with DeCyder Differential Analysis Software allows a more accurate and sensitive quantitative analysis than with conventional 2D PAGE. We identified 19 different proteins which accumulation in phloem sap was altered during a compatible plant virus infection including redox and hypersensitivity response-related proteins. Therefore, this work would help to understand the basic processes that occur in phloem during plant–virus interaction.

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1. Introduction

The phloem is a highly specialized conducting tissue of vascular plants whose major role was initially considered to be the movement and distribution of photo-assimilates, sugars and organic compounds, between autotrophic and heterotrophic tissues. It was soon evident that phloem also contained hormones and a wide range of macromolecules, including RNAs and proteins, that could act as mobile signals to regulate a variety of developmental processes and environmental responses [1–5]. The floral transition, the tuber production in potato, the systemic acquired resistance (SAR) against bacteria, fungi and viruses and the systemic wound response (SWR) against chewing insects are well-known processes triggered by phloem-mobile signals such as proteins, mRNAs and hormones [6–9]. In addition, graft-transmissible small RNAs (siRNAs and miRNAs) appear to spread systemically the so-called RNA silencing [10,11]. All these examples, and others, make clear that phloem is more than just a system for nutrient circulation in plants and, indeed, it must be considered as a complex network of integrative long-distance communication [12,13].

Most plant viruses are well known to use the phloem, mainly the sieve elements (SE), to spread throughout all parts of the plant from the roots to the leaves, fruits, seeds and even pollen grains [14,15]. Thus, interference with viral long-distance transport in the phloem could contribute to an effective reduction of disease incidence. The phloem plays a key role in plant protection producing and transporting multitudes of stress and defense proteins as well as an antioxidant defense system [16–18]. Some proteins specifically involved in viral-resistance processes have been also identified in phloem. The tobacco cadmium-ion-induced GRP protein (cdiGRP), which is localized in the cell wall of SE and companion cells (CC), blocks the entry of a tobamovirus into SE from vascular bundles by increasing callose deposition [19]. In addition, the corresponding proteins of *Arabidopsis thaliana* RTM1 and RTM2, two of the five dominant genes involved in resistance to several potyvirus, are specifically localized to SE [20]. On the other hand, phloem proteins have been also shown to facilitate the transport of endogenous [21–23], viral [24] and subviral [25–28] RNAs as well as to potentially facilitate viral transmission by aphids [29]. Thus, the understanding of many plant defense processes against viruses as well as the requirements for host susceptibility will require a detailed knowledge of which proteins are differently accumulated in the phloem during the progress of a viral infection.

The recent application of quantitative "second generation" proteomic techniques, such as differential in gel electrophoresis (DIGE), has significantly facilitated the study of plant–virus interactions through proteomic analysis (see [30] for review). By the other hand, the number of proteins identified in proteomic investigations of the phloem sap is significantly increasing [16,31–34], In this scenario, it is paradoxical that the studies dealing with the comparison of the phloem sap proteome during viral infection were limited to only one [35]. In that work, authors, using conventional 2D electrophoresis, observed that the infection of melon by *Cucumber mosaic virus* (CMV) induced the up-accumulation of a limited number of phloem proteins including the major latex protein (MLP), an enolase, a translationally controlled tumor protein homolog (TCTP), the heatshock cognate protein 70 and a fifth additional unknown protein [35]. As could be expected, all of annotated proteins were associated with stress responses.

Yield losses caused by *Melon necrotic spot virus* (MNSV) have great economic impact in melon (*Cucumis melo*)-growing regions worldwide and, at present, the only resistance found to MNSV in melon is that controlled by the single recessive gene *nsv* [36,37]. As most plant viruses, the systemic spread of MNSV in melon plants occurs through phloem tissue [14,38]. To better understand the underlying processes that are taking place during MNSV infection of melon which, at the same time, may provide new targets/markers for resistance or susceptibility processes we used a proteomic approach to study and identify phloem proteins differentially accumulated between MNSV-infected and non-infected melon plants. A total of 1046 spots were detected to match across all replicates by two-dimensional difference gel electrophoresis (2D-DIGE) [39] and 25 were statistically significant differentially accumulated. Most of them corresponded to proteins involved in redox homeostasis and cell death. The identification of these proteins offers new insights into the repertoire of mechanisms that may occur during plant–virus interaction.

2. Materials and methods

2.1. Plant material, virus inoculation and phloem sampling

C. melo L. subsp. *melo* cv. Galia plants were kept in a growth chamber, at constant temperature of 23 °C under a 16/8 h light/dark cycle. 6–10 day-old plants were infected by mechanical inoculation of fully expanded cotyledons with purified virions of MNSV-Al isolate [40]. Mock plants were inoculated with buffer 10 mM Tris–HCl pH 7.3. Phloem sap was collected from well-watered plants 15 days post-inoculation. Briefly, the main stem was cut with a sterile razor between the shoot apex and the first leaf from the top. The exudate was collected using sterile micropipette tips (10 μl) and immediately mixed with 9 volumes of phloem sap collection buffer (7 M urea, 2 M thiourea, 4% 3-[3-cholamidopropyl dimethylammonio]-1-propanesulfonate or CHAPS, and 67 mM DTT). All buffers and samples were kept on ice during phloem sap sampling.

2.2. Protein purification and labeling

Four independent assays were performed each of them including 200 MNSV-infected and 40 mock-inoculated melon plants. Phloem exudates collected in each assay were separately pooled according to its origin from healthy (H) or infected (I) plants. Finally, we obtained eight phloem sap samples (H1, H2, H3, H4, I1, I2, I3 and I4) (see experimental system in Supplementary Fig. 1). Total proteins from each pool were separately purified with 2-D Clean-Up kit (GE Healthcare Life Sciences; Piscataway, NJ, USA) to remove salts and other contaminants that could interfere with the following labeling and gel electrophoresis. Protein samples