Indications of lipids / lipid signalling in the phloem exudates of *Arabidopsis thaliana* and *Perilla ocymoides*

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Introduction

The role of the phloem has changed from that of simple assimilate transport to a trafficking system for pathogen response and developmental regulators (Citovsky and Zambryski, 2000; Wu et al., 2002; Ding et al., 2003, Haywood et al., 2005). It is crucial for the transport of mineral nutrients, plant viruses, virus-induced silencing, defense and resistance against pathogen infection and signaling of environmental conditions. The phloem contains a multitude of compounds: small molecules (Corbesier et al., 2003), peptides and proteins (Fisher et al. 1992; Hoffmann-Benning et al., 2002; Giavalisco et al., 2006), nucleic acids (Ruiz-Medrano et al., 1999; Haywood at al., 2005), and lipids (Madey et al., 2002). Moreover, interactions between proteins or RNA and proteins to facilitate or regulate phloem transport appear more and more likely (Xoconostle-Cazares et al., 1999; Yoo et al., 2004; Aoki et al., 2005). The contents of the phloem are now known to be so complex that phloem transport has been called the "superinformation highway" of plants (Lukas, 2000). Since the phloem appears to lack any capacity for transcription and translation, the close association of sieve elements with companion cells is crucial for its function and any macromolecule identified within the sieve tube system is probably derived from companion cells through plasmodesmata.

With analytical methodology getting increasingly sensitive, the complexity of the phloem becomes even more apparent. The next logical step to elucidate the functions of all phloem macromolecules is the establishment of compehensive databases of vascular genes and phloem-mobile transcripts, proteins and metabolites. This paper represents first steps towards this goal. We used two plant species: (I) *Perilla ocymoides* because it is a strict short-day plant, which allows us to determine changes in the phloem composition during different photoperiodisms and (II) *Arabidopsis thaliana,* whose genome is completely sequenced allowing for easy identification of proteins and transcripts. Our data show that a combined approach can yield new aspects of phloem translocation. We have detected fatty acids, lipids, and proteins, which could function in lipid binding/transport or metabolism within the phloem sap and will now work towards establishing their role.

Extraction and analysis of phloem exudate

Collection and analysis of the phloem sap are complicated by the fact that, in most plants, the phloem seals itself upon wounding, and no exudates are secreted. To prevent this, *Arabidopsis* and *Perilla* petioles were incubated for one hour in EDTA, followed by collection of the exudate into water using a method modified from the one previously described for *Perilla* (King and Zeevaart 1974). The resulting phloem exudate was lyophilized and further analyzed as described in figure 1:

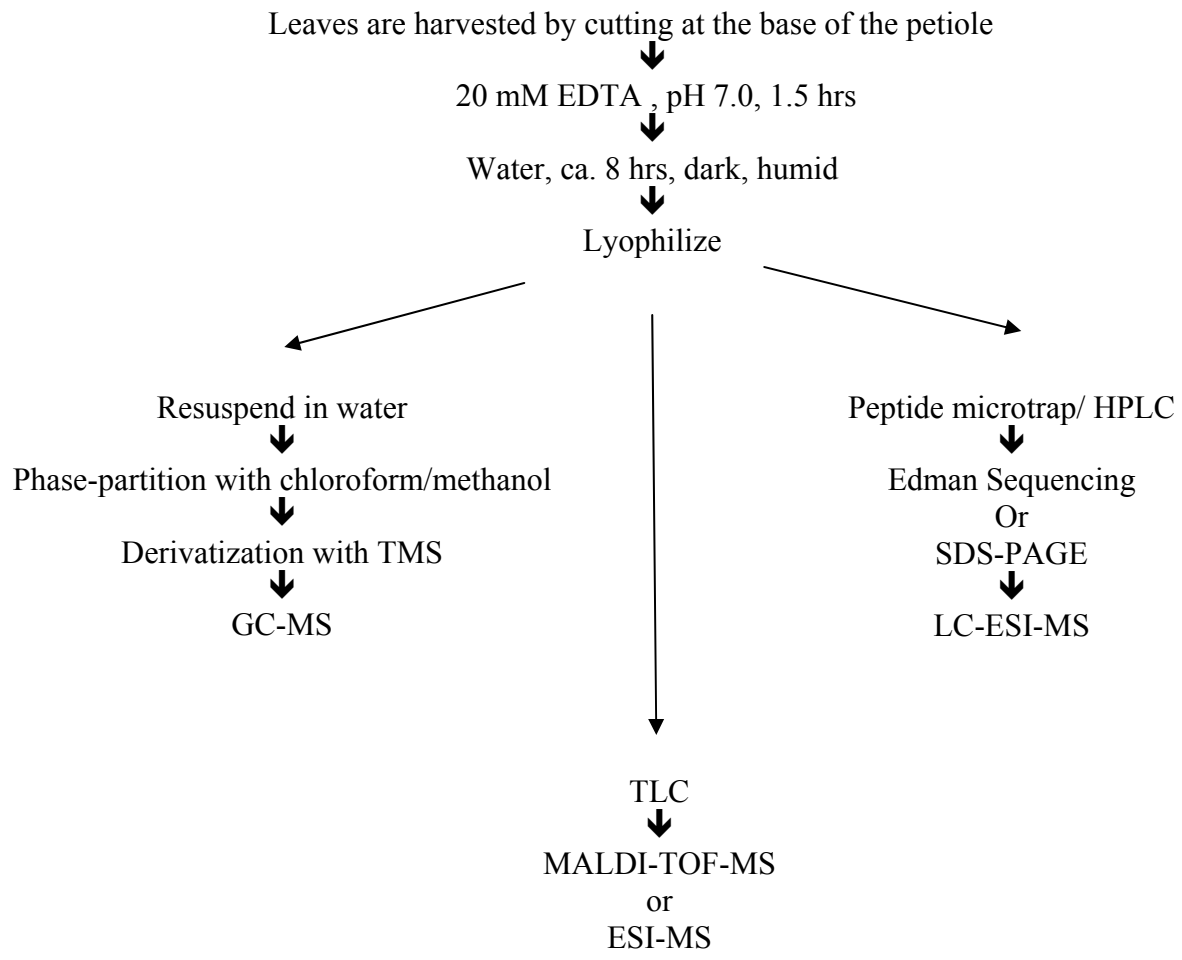

Figure 1: Collection and Extraction of *Perilla* or *Arabidopsis* Phloem Sap

Characterization of metabolites present in the phloem exudate

GC-MS analysis revealed the presence of at least 121 and 179 metabolites in the phloem exudate of *Arabidopsis* and *Perilla* respectively. Of these, about 50% were distinct spectra of unknown identity. The remainder consisted mostly of amino acids (11% / 6%), sugars and sugar derivatives (14% / 26%), fatty acids and their derivatives (13% / 4%), and organic acids and phosphates (6% / 7%). Both, amino acids and sugars (monosaccharides and disaccharides) are assimilates expected to be present in the phloem. However, we were also able to detect a significant number of fatty acids and fatty acid derivatives. In addition, we observed differences in the pattern of polar lipids when we compared lipids from leaves of the two plant species, between leaves and phloem exudates, and between flowering and non-flowering plants. Only one paper has previously reported the presence of lipids/fatty acids in the phloem (Madey et al., 2002). They detected small amounts of lipids and large amounts of free fatty acids in the phloem of canola. Like Madey et al (2003) the fatty acids we detected included short and medium-length, saturated fatty acids, including odd-chain-length fatty acid. These could simply be transported in the phloem, or play a role in pathogen response or signaling.

Characterization of proteins present in the phloem exudate

To investigate a possible role of lipid signaling and/or metabolism in the phloem exudates, we analyzed the proteins present in the phloem sap within the sieve elements. We were able to

distinguish 128 proteins present in Arabidopsis phloem exudate. Twenty of these proteins were of unknown functions. 28 proteins fell into no larger category and included proteins involved in amino acid metabolism, cytoskeleton, phosphate homeostasis, ribosomal proteins, and more. We also detected 18 proteins involved in protein modification, folding, and turnover, 5 receptors, 21 proteins important for the response to pathogens or oxidative stress, and 12 proteins necessary for carbohydrate metabolism. Twenty-six of the proteins we detected contained nucleotide, DNA, or RNA binding sites, many of those being RNA binding proteins or transcription factors.

We were also able to detect 11 proteins that play a role in lipid/fatty acid metabolism (aspartic protease, 3-ß-hydroxy dehydrogenase, sqd1, glycerophosphodiester diesterase, lipase, long-chain fatty acid CoA ligase) and storage (glycine-rich protein), binding (glycine-rich protein7, annexin, lipid-associated family protein, GRP17) and signaling (annexin, putative lipase, glycerophosphodiester diesterase). Very little has been reported about lipids and fatty acids in the phloem. Madey et al. (2002) found the presence of lipid particles in canola phloem sap. Those particles showed a fatty acid composition different from those typically found in membranes, namely mostly short- and medium-chain and odd-carbon number fatty acids. This suggests that the lipids found are not artifacts from membranes but indeed intrinsic to phloem exudate.. One of their possible roles could be that of lipid signaling. Only annexin and a lipid transfer protein (LTP) had previously been reported in the phloem exudate. The LTP was shown to play a role in systemic acquired resistance (Maldonado et al., 2002) and Annexin, has been shown to bind phospholipids and is involved in Ca2+-signalling as well as callose formation (Andrawis et al., 1993). We also detected a putative lipase and glycerophosphodiester diesterase. Lipases have been implicated in salicylic acid signaling (Feys et al., 2001) and wound response (Guan and Nothnagel, 2004). Glycerophosphodiester diesterase is a little described enzyme likely involved in phospholipid metabolism. Its induction in response to phosphate limitation may suggest a role in phosphate sensing (van der Rest et al., 2004). In *Perilla*, we were only able to detect one protein related to lipid synthesis and one related to fatty acid transport. This may be due to the fact that the *Perilla* genome is not sequenced and that proteins can, thus, only be identified if large homologies exist.

Our results indicate that lipids and lipid signaling may play a larger role in the phloem than previously thought and should be further studied.

Conclusions and perspectives

We showed that it is possible to obtain phloem exudate from *Arabidopsis thaliana* and *Perilla ocymoides* leaves in sufficient amounts for metabolite, protein, and lipid analysis and without major contamination from companion cells. Since the complete *Arabidopsis* genome is sequenced and publicly available, this will allow us to better identify proteins and mRNA in the phloem and follow up with physiological experiments to establish their function. We have detected/ identified 11 lipid binding/metabolism-related proteins in *Arabidopsis* plus several fatty acids and lipids. Our results indicate that there may be an independent lipid transport and signaling system present in the phloem. This has been suspected before (Madey et al.2002; Maldonado et al. 2002), yet many protein-components necessary are described here for the first time.

Several of the lipids present in the phloem of *Arabidopsis* and *Perilla* are different from leaf lipids. In addition, there are differences in the lipid composition of flowering and non-flowering plants. They will be identified and their function analyzed. It is intriguing to think that these lipids may play a role in signaling.

Perilla plants are known to contain omega-3 fatty acids in their seeds and are appreciated in Asian countries for their high nutritional value. Perilla oil is also sold as dietary supplement to improve eyesight and heart health and as preventative for some cancers. However, the seeds scatter easily and are hard to harvest. Engineering a plant that produces large amounts of

nutritionally important oil in the leaves and transports it throughout the plant as well as stores it in the leaves may be desirable.

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