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ORIGINAL ARTICLE

Susanne Hoffmann-Benning · Douglas A. Gage
Lee McIntosh · Hans Kende · Jan A.D. Zeevaart

Comparison of peptides in the phloem sap of flowering and non-flowering *Perilla* and lupine plants using microbore HPLC followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Abstract Physiological evidence indicates that flower formation is hormonally controlled. The floral stimulus, or florigen, is formed in the leaves as a response to an inductive photoperiod and translocated through the phloem to the apical meristem. However, because of difficulties in obtaining and analyzing phloem sap and the lack of a bioassay, the chemical nature of this stimulus is one of the major unsolved problems in plant biology. A combination of microbore high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was used to compare the contents of the phloem sap from flowering and non-flowering plants. Instead of using one- or two-dimensional gel electrophoresis, microbore HPLC separations allowed us to detect proteins/peptides that were very small and present at very low levels. We detected more than 100 components in the phloem sap of *Perilla ocymoides* L. and *Lupinus albus* L. Sequences for 16 peptides in a mass range from 1 to 9 kDa were obtained. Two of these could be identified, 11 showed similarity to known or deduced protein sequences, and three showed no similarity to any known protein or translated gene sequence. Four of these peptides were specific to, modified, or increased in plants that were flowering, indicating their possible role in flower induction. The sequences of these peptides showed similarities to two purine permeases, a protein with similarity to protein kinases, and a protein with no similarities to any known protein.

Keywords MALDI-TOF-MS · Peptide · *Perilla ocymoides* · Phloem sap · Proteomics

Abbreviations aa: amino acids · HPLC: high-performance (pressure) liquid chromatography · MALDI-TOF-MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Introduction

The identity of the signal for flowering has been one of the great mysteries in plant biology even before the term "florigen" was coined in 1936 (Chailakhyan 1936). Experiments using partial coverings of leaves and various dark–light regimes have shown that a particular night length, even if perceived only by a small part of one leaf, is sufficient in many species to elicit flowering. The floral stimulus is graft-transmissible in cases where functional connections can be established between graft partners (Zeevaart 1976) and it is translocated through the phloem. In some cases, it is interchangeable between different species and plants of different photoperiodic sensitivities. This indicates that it may be the same or at least chemically similar in many plants. Collection and analysis of phloem sap are complicated by the fact that, in most plants, the phloem seals itself upon wounding, and no exudate is secreted. In some plants, such as *Ricinus*, cucumber, and lupine, phloem sap can be obtained readily (Sakuth et al. 1993; Schobert et al. 1995; Marentes and Grusak 1998; Kehr et al. 1999). In other plants, aphids (Fisher et al. 1992; Nakamura et al. 1995) or exudation into EDTA to prevent sealing of the phloem (King and Zeevaart 1974) have been used. Thus far, attempts to isolate and characterize florigen have not been successful.

Dedicated to Nikolaus Amrhein, Zürich, on the occasion of his 60th birthday

S. Hoffmann-Benning · D.A. Gage · L. McIntosh
Michigan State University, MSU-DOE Plant Research Laboratory
and Department of Biochemistry and Molecular Biology,
East Lansing, MI 48824, USA

H. Kende (✉) · J.A.D. Zeevaart
Michigan State University, MSU-DOE Plant Research
Laboratory and Department of Plant Biology,
East Lansing, MI 48824, USA
E-mail: hkende@msu.edu
Fax: +1-517-3539168

As an alternative approach, researchers have tried to find flowering mutants. Such screens yielded flowering-time mutants, which were affected in the genes for photoreceptors, transcription factors, RNA-binding proteins, or meristem-identity genes (for reviews see: Hay and Ellis 1998; Koornneef et al. 1998). Up to now, none of these findings has helped to identify the chemical nature of florigen.

We decided to analyze phloem sap from *Perilla ocymoides* and *Lupinus albus*. In both cases, methods for the collection of phloem sap are well established. In lupine, a simple cut through the pedicel or into the vascular bundles of the stem allows phloem sap to be collected (Pate et al. 1974). It can then be analyzed without further preparation. However, lupine is day neutral, and only non-flowering young plants, from which it is difficult to obtain any phloem sap, could serve as controls. Therefore, we used the short-day plant *Perilla* to compare the phloem sap from induced, flowering, and non-induced vegetative plants. Results in recent years have shown that, apart from sugars, the phloem contains small molecules, peptides and proteins (Fisher et al. 1992; Sakuth et al. 1993; Schobert et al. 1995; Kühn et al. 1997; Marentes and Grusak 1998; Kehr et al. 1999; Xoconostle-Cazares et al. 1999; Haebel and Kehr 2001), and nucleic acids (Kühn et al. 1997; Ruiz-Medrano et al. 1999). The contents of the phloem are now known to be so complex that phloem transport has been called the "superinformation highway" of plants (Lucas 2000).

We attempted to identify and compare peptides and small proteins in both, flowering and non-flowering *Perilla* and *Lupinus* plants using a mass spectrometry-based proteomics approach. The plan was to identify differences in the composition of phloem exudate between induced and non-induced or flowering and vegetative plants. Proteomics implies separating complex protein mixtures using one- or two-dimensional gel electrophoresis, followed by mass spectrometric analysis of individual bands or spots. Algorithms have been developed for the identification of proteins from databases using mass spectrometric data. However, this approach presents several problems for the analysis of phloem peptides. Smaller proteins and peptides are often lost during electrophoresis. Molecules present in very small amounts will not be visible in gels or detectable by mass-spectrometric analyses of crude extracts. Complex mixtures can lead to signal suppression during MALDI-MS analysis, which can result in several masses not being detected. In addition, few DNA-deduced protein sequences are available for *Perilla* and lupine, complicating sequence identification. Direct analysis of lupine phloem sap using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has already been reported by Marentes and Grusak (1998), who successfully determined the masses of several compounds. Our approach of microbore-HPLC separation prior to MALDI-TOF-MS allowed us to detect more peptides than were found in previous studies because of a reduction of suppression effects. It also greatly increased the resolution and sensitivity and allowed us to detect the presence of more than 100 molecules between 1 and 15 kDa in lupine as well as in *Perilla*, including those identified by Marentes and Grusak. Some of the most intriguing proteins and peptides, including several that appear to be present specifically in the phloem exudates from flowering plants, were purified and sequenced.

Materials and methods

Plant material

Green-leaved *Perilla ocymoides* L. plants (Zeevaart 1985) were raised from seed (propagated in our greenhouse) under long-day conditions as described by King and Zeevaart (1974). To induce flowering, plants were moved to short-day conditions (16-h night, 20 °C) at 6–8 weeks of age. Control plants received the same 16-h dark period, but with a 15-min night interruption. Phloem sap was typically collected 21 days after the start of the inductive dark periods.

Lupinus albus L., cv Ultra (Lupin-Triticale Enterprises, Perham, Minn., USA) plants were raised from seeds under the same long-day conditions as *Perilla ocymoides* L. Lupine phloem sap was collected by either cutting the pedicel or by nicking the vascular bundles with a razor blade at the flowering stage, which was approximately at 8 weeks of age (Pate et al. 1974). Sap was collected and used immediately or frozen (–80 °C) for later use. HPLC chromatograms of lupine sap collected by either method showed only minor differences.

Collection of phloem exudates from *Perilla*

Leaves were cut and recut in 20 mM K_2-EDTA, pH 7.0, and placed in a beaker containing a shallow layer of a solution of 20 mM K_2-EDTA in a humid atmosphere and in the dark. After 1.5 h, the solution was discarded. The cut surfaces of the petioles were rinsed thoroughly with water, and the exudates were collected in deionized water for ca. 10 h. They were then lyophilized and used immediately or stored dry at –80 °C.

Analysis of phloem exudates

Phloem exudate was analyzed using an Ultrafast Microprotein Analyzer system with a peptide microtrap and a C_{18} reverse-phase column (all from Michrom BioResources, Auburn, Calif.). The gradient was from 5% acetonitrile/0.1% trifluoroacetic acid in water to 65% acetonitrile/0.1% trifluoroacetic acid in water in 45 min. HPLC fractions were collected by peak (absorbance at 214 nm), except for small peaks, in which case they were collected by time.

Mass spectra were obtained using a MALDI-TOF Elite or a DE-STR (both by PerSeptive Biosystems, Framingham, Mass.). Samples were prepared by drying 1 µl of the HPLC fractions on the plate prior to adding 0.5 µl matrix solution (α-cyano-4-hydroxy cinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid), and spectra were acquired in linear or reflectron mode. Purified peptides/proteins were submitted to Edman sequencing at the Michigan State University Molecular Structure Sequencing and Support Facility. Partial sequences were used for a database (mostly NCBlnr or SwissProt) search. The search engine was Protein Prospector (http://prospector.ucsf.edu) from the University of California, San Francisco, developed by Peter Baker and Karl Clauser. This program performs pairwise comparisons and does not allow for gaps. To do a search, we entered the Webpage and went to "MS-Pattern". There, we used the default settings expected for the following: (1) database: NCBlnr.8.17.2002, Genpept., or

Swissprot. (2) Cys modified by: unmodified (3) Regular expression: amino acid sequence for our peptide, and (4) Max. no. of mismatched AA's: 50% of the number of AA typed under "regular expression". To qualify as significant, a minimum match of 60% of the amino acids was required. In addition, even though we searched all species, matches with plant proteins were weighed higher than those with bacterial or animal proteins.

A flow chart of the phloem sap preparation and analysis is shown in Fig. 1.

Enzymatic digests

Carboxypeptidase Y sequencing was performed using a Sequazyme C-Peptide Sequencing Kit (PerSeptive Biosystems).

Tryptic digests were performed using 13 ng/µl trypsin (Promega, Madison, Wisc.) in 100 mM ammonium bicarbonate (pH 8.0) at 37 °C overnight. The digests were then separated using reverse-phase HPLC and sequenced at the Michigan State University Molecular Structure Sequencing and Support Facility.

Results and discussion

Establishing the optimal method for analysis of peptides and small proteins in phloem sap of lupine

Marentes and Grusak (1998) demonstrated that it is possible to analyze crude lupine phloem sap. Their MALDI-TOF-MS determinations showed the presence of approximately ten proteins, as well as differences in protein profiles at various stages of plant development. To increase the resolution and detect more peptides or proteins, we added a reverse-phase microbore HPLC step as an additional dimension prior to MALDI-TOF-MS analysis. This not only allowed us to separate peptides into various fractions, but also enabled us to detect more compounds in the MALDI-TOF-MS since there was less signal suppression, which often happens as a result of interactions in complex mixtures. In addition, we concentrated and desalted the phloem sap using a C_{18} peptide microtrap. The trap also limited the maximum size of proteins that could be detected. It allowed us to see very small amounts of peptides and proteins. Several of these proteins were present in such small amounts that they would not be visible on silver-stained gels, or they could be lost because of their small size. Table 1 shows a list of the peptides/proteins we detected in phloem sap of lupine. At the time of flower emergence, we detected not only the five substances isolated by Marentes and Grusak (1998), but an additional 100 small proteins and peptides. In addition, separating the original five phloem components showed that two of the MALDI-TOF-MS signals belonged to the single- and double-charged ions of the same molecule. Several of the detected masses also appeared in phloem sap of other plant species (*Perilla*, cucumber) at the same retention time. One of those was sequenced and identified as ubiquitin.

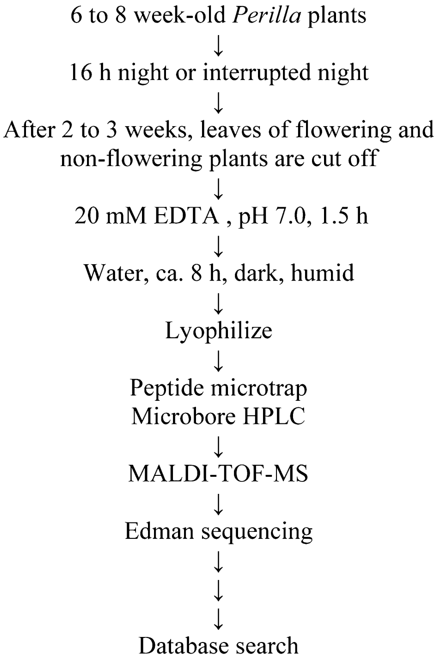

Fig. 1. Flow chart of phloem sap exudation and subsequent analysis. For some samples, tryptic digestion or CPY digestion were performed and analyzed using MALDI-TOF-MS

Analysis and comparison of the phloem exudates of flowering and non-flowering *Perilla* plants

We used the method established for lupine to analyze *Perilla* phloem exudates. Figure 2 shows reverse-phase HPLC chromatograms of phloem exudates from plants with emerging flower buds (trace A), from flowering (trace B), and from non-flowering (trace C) plants. Under these conditions the chromatograms displayed only minor differences, although the sap contained a complex mixture of substances. MALDI-TOF-MS analysis showed that most of the HPLC fractions contained multiple compounds. As in lupine, sap from *Perilla* contained more than 100 compounds between 1 and 21 kDa, which eluted at a concentration of 10 to 40% acetonitrile (Table 2). Some of these compounds are likely to be small proteins. Others may be fragments of larger proteins resulting from degradation during collection or from proteolytic processing required for entry into the phloem translocation pathway (Xoconostle-Cazares et al. 2000).

Proteins/peptides from several fractions were purified and sequenced (Table 3). Several of these fractions contained substances whose levels were consistently higher in exudates from plants with buds or flowers. The fraction at a retention time of 15–16 min contained a peptide with a molecular mass of 2,162 Da (MH^+ at *m/z* 2,163). A molecule with the same mass was present in the corresponding HPLC fraction from non-induced plants, but at much lower concentration. Hence, the level of this compound appeared to increase during flower induction. The partial sequence showed no similarity to any known protein. However, "similarity " may be hard to define since the sequence could have origi-

Table 1. Molecular masses of compounds identified in the phloem sap of lupine collected at the time of inflorescence emergence. Microtrap/microbore HPLC separation followed by MALDI-TOF-MS of individual fractions was used. The data are compared with compounds identified from crude phloem sap at the time of inflorescence emergence (Marentes and Grusak 1998; third column). Masses in *bold* correspond to compounds detected by Marentes and Grusak (1998). Listed compounds were detected in at least three different exudate separations

Retention time (min)	Masses of detected compounds (Da)	Compounds detected by Marentes and Grusak (Da)
< 8.0	2,060; 3,007	
8.0–10.0	1,746; 2,031; 2,316; 2,597	
10.0–15.0	1,098; 2,046; 2,329; 2,629; 8,597	
15.0–16.5	1,848; 1,946; 2,207; 2,683; 2,706; 2,985; 3,002; 4,999	
16.5–17.0	1,734; 2,174; 3,345; 2,695; 3,738; 4,779	
17.0–18.0	2,491; 2,552; 2,749; 2,911; 3,402; 3,544	
18.0–19.0	2,996; 4,027	
19.0–19.5	2,410; 2,791; 6,532	
19.5–20.0	1,849; 2,116; 3,807	
20.0–21.0	3,254; 3,756; 4,211	
21.0–22.0	3,046; 3,638; 5,623; 6,461; 6,874	
22.0–23.0	1,072	
23.0	2,845; 3,199; 3,642; 7,480	
23.0–24.0	1,519; 2,867	
24.0–24.5	2,713; 3,360; 3,905	
24.5–25.0	1,391; 1,872; 3,058; 3,807; 4,573; 5,904; 9,098	
25.0–29.0	3,312; 6,587; 6,928; 7,450; 8,932; 9,061; **9,215**; 9,632	9,210
29.0–30.0	1,424; 7,745; 8,457; 9,375; 9,995; 11,347; 15,327	
30.0–35.0	**6,654**; 7,119; **7,528**; 7,835; 10,150; 10,315; 14,506; 19,105	6,653; 7,528
35.0–36.0	5,235; 5,800; **4,260–8,517**[a]; 9,501; 10,060; 11,185; 11,405	4,265–8,515
> 36.0	2,930; 4,329; 6,444; 7,752; 8,188; 8,474; 9,443; 10,002; 27,887	

[a] The compound with the mass of 8,517 Da was digested with trypsin, the fragments separated by HPLC, and one fragment sequenced using Edman sequencing. It was identified as ubiquitin

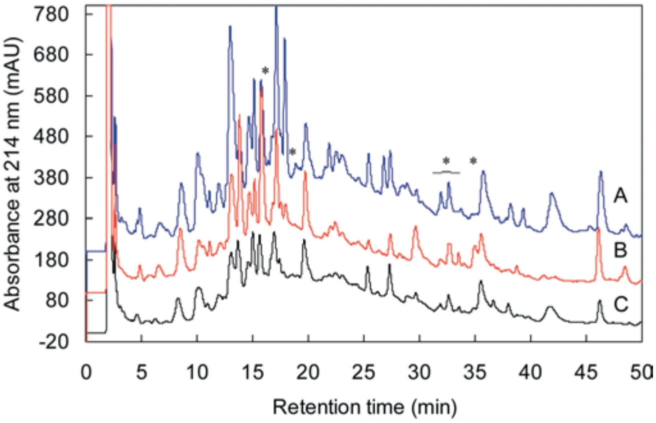

Fig. 2. HPLC chromatogram of *Perilla* phloem exudate from plants with opening flower buds (trace **A**), flowers (trace **B**), and non-flowering plants (trace **C**). Fractions marked with an *asterisk* indicate induced compounds

nated from a non-conserved region of a protein. In this case, similarity to proteins from other plant species could not be found. Alternatively, some of the short sequences may show similarity to the products of multiple genes.

The HPLC fraction at a retention time of 18–19 min contained a mixture of substances. These included a peak representing a peptide with a mass of 3,388 Da, which was specific to induced plants (Table 2, Fig. 3C), and five sets of peaks, which increased in mass from 3,388 Da to 4,300 Da in non-induced plants (Fig. 3A). The mass difference between the sets of peaks in non-induced plants was 162, which could correspond to the addition of a hexose residue. Additional peaks showed mass differences of 42, 161, and 203, which would be characteristic for acetyl, hexosamine, and N-acetylhexosamine, respectively. These findings are indicative for the presence of multiple glycoforms. Treatment of the fraction from non-induced plants with proteinase K led to the disappearance of these peaks while the compounds with masses at 2.6, 2.9, and 5.1 kDa were not digested (Fig. 3B). This indicates that the compounds ranging from 3,388 Da to 4,300 Da were indeed proteinaceous. Thus, it appears that this protein is glycosylated in non-flowering plants. Partial sequences of this peptide using either carboxy-peptidase Y sequencing or tryptic digestion followed by Edman sequencing showed similarity to Ser/Thr protein kinases as the only proteins with similarity to both sequences. Protein kinases play an important role in signal transduction pathways. Nakamura et al. (1995) previously detected three proteins with kinase activity in rice phloem sap, one of which had a calcium-dependent kinase activity. In addition, Lee and Lucas (2001) suggested that trafficking, at least via plasmodesmata, may be regulated at the level of protein phosphorylation. Thus, the role of this kinase-like protein may be to activate other proteins necessary for flower induction or to "mark" them for transport. Tryptic digestion of the same fraction, followed by Edman sequencing, revealed a second sequence with similarity (60% identity in 14 aa) to a putative *Arabidopsis thaliana* purine permease and other membrane proteins.

A fraction with the retention time of 32–34 min contained multiple components, one of which (6.7 kDa) could not be detected in non-flowering plants (Fig. 4). Attempts to perform carboxy-peptidase Y sequencing yielded only one amino acid plus a group of compounds at about *m*/*z* 4,000 that showed a mass difference of 80

Table 2. Molecular masses of compounds identified in phloem exudates of flowering *Perilla* plants after separation using a microtrap/microbore HPLC followed by MALDI-TOF-MS of individual fractions. Substances printed in *bold* were further purified and sequenced. Listed compounds were detected in at least three separate experiments

Retention time (min)	Mass of detected compounds (Da)
< 13.0	2,000-group; 2,038; 2,961; 4,057; 4,125; 4,410
13.0–15.0	2,344; 2,431; 3,850; 4,292
15.0–16.0	1,320; 1,523; 1,650; 1,754; 1,946; **2,162**
16.0–17.0	1,895; 3,116; 3,400; 3,561; **5,075/5,167**
17.0–18.0	2,021; 2,204; 2,624; 2,757; 2,993; 3,021; 3,189; 3,435; 3,750; 4,211; 5,014; 8,598
18.0–19.0	2,295; **3,388**; 5,224; 7,776; 9,449
19.0–20.0	1,685; 1,738; 2,129; **2,370**; 4,812; 11,063
20.0–22.0	1,685–1,728–1,772-polymer; **2,336**; 3,285; 4,282; 4,839
22.0–24.0	**2,449**; 2,851; 3,594; 3,653; 3,987; 4,009
24.0–27.0	2,393; **2,643**; 2,766; 7,412; 8,951; 9,536
27.0–30.0	**2,050**; 2,405; 2,803; 2,932; 3,055; 3,282; 3,446; 3,758; 4,839; 7,275; 7,468; 7,772; 9,254
30.0–32.0	1,033; 1,921-polymer; 2,650; 3,397; 3,853; **4,300**; 7,589
32.0–34.0	3,610; **6,700**; 8,177; 8,601; 8,832
34.0–36.0	2,181; **8,525**; 11,094; 11,509
36.0–37.0	1,577; 2,579; 8,210; 8,459; 10,489
> 37.0	2,403; 3,027; 4,545; 5,136; 5,268; 10,755; 21,454

Table 3. Summary of peptides detected in *Perilla* phloem exudate. Peptides not detected or modified in non-induced plants (*bold letters*) potentially play a role in flowering. Sequences showing similarities are printed in *italics*. *A.t. Arabidopsis thaliana*

Mass (Da)	Sequence	Non-flowering	Sequence similarities (%)
2162	GPPKYXGSASEIA	Detected	No similarities found
5075/5167	KAVIVPILANLVLVHyp, *kaaivpglvslivvp* *maailpdlatqvlp* *faviveipfslrlvp*	Detected	A.t. 2-oxo-glutarate/malate translocator-like protein AF370494 (60%), Rice pyrophosphatase ovp1, AB012765 (60%), *Trillium* maturase, AB017379 (60%), phosphorylmutases in bacteria
1860	LDVTLGSDDGGLVFIPQN, *ldvllggddgslafipgn* *ldvllgsddgglafvpnn*	Not determined	Potato leaf EST425039, BE921270 (72%) with similarity to plastocyanin from, e.g., *Mercurialis perennis*
3388	VYVHGN FTTGSGHNS	Modified	Possibly glycosylated, similarity to protein kinases
3388–2	KILPIAVVVGI/LLSG *rlliaavivgilsg*	Not detected	Putative A.t. purine permease, AF078531(64%) and other hypothetical membrane proteins
2336	ALGPIKVGSAVAG	Detected	Ubiquitin thiolesterase 19 (O94966)
2465	PLIANILPHISD *plianpsplish* *pllaniypyisf*	Detected	Putative protein in A.t., NM_121313 (67%), *Festuca pratensis* Glu1, AJ295946 (67%)
2448	VDAGDIIGGIATILPHypIAV	Detected	Low similarity to a mouse protein and transporters
2370	GIIIPHypGGFGGYGGG LGGGGVIVGGGGGGI	Detected	Glycine-rich (cell wall) proteins (contains hydroxyproline)
2643	SIEPPIGFPIRGGRL CCLGVIVDEIDVA	Detected	No similarities found
1271	VKALVALLNSGEG *vka-vavlnsseg* *vaalvaltnsgrg*	Not determined	Poplar cytosolic superoxide dismutase, AF016892 (77%), rice hypothetical proteins, AL606627 (77%) with similarity to cellulose
4300	PEAASAPGSGN *peaasapgsgn* *peaasaagaga*	Not determined	H.s. "survival enhancing peptide"/ DCD-1, AY044239 (100%), putative receptor kinase in rice, AF128457 (73%)
6700	SLIPLLVGILEG *slipvlvgivvg*	Not detected	Purine permeases in bacteria, P41006 (75%),
6700–2	EPVPFLLHGQGGDGG *epvpavlqgqggdgg*	Not determined	Putative protein expressed in young rice flowers / Osc6, C73664/S28608 (80%)
8525	KIFVKTLTGKTITLEV *QIFVKTLTGKTITLEV*	Not detected	Ubiquitin (e.g., rice, D46280, 93%)

between the MALDI peaks (not shown). This could indicate multiple phosphorylation sites. We could only purify several hundred fmoles of material, which yielded a 12 aa sequence with similarity to bacterial purine permeases (75% in 12 aa). The two potential purine permeases found in *Perilla* phloem sap, though similar, are clearly distinct from each other. Purine permeases have been found associated with sieve elements and are thought to transport nucleic acid bases across the plasma membrane (Gillissen et al. 2000). There is also evidence that purine permeases are subject to in vivo phosphorylation in yeast (Pinson et al. 1996). Purine permeases are important for nucleotide and ATP synthesis, but may also play a role in the transport of cy-

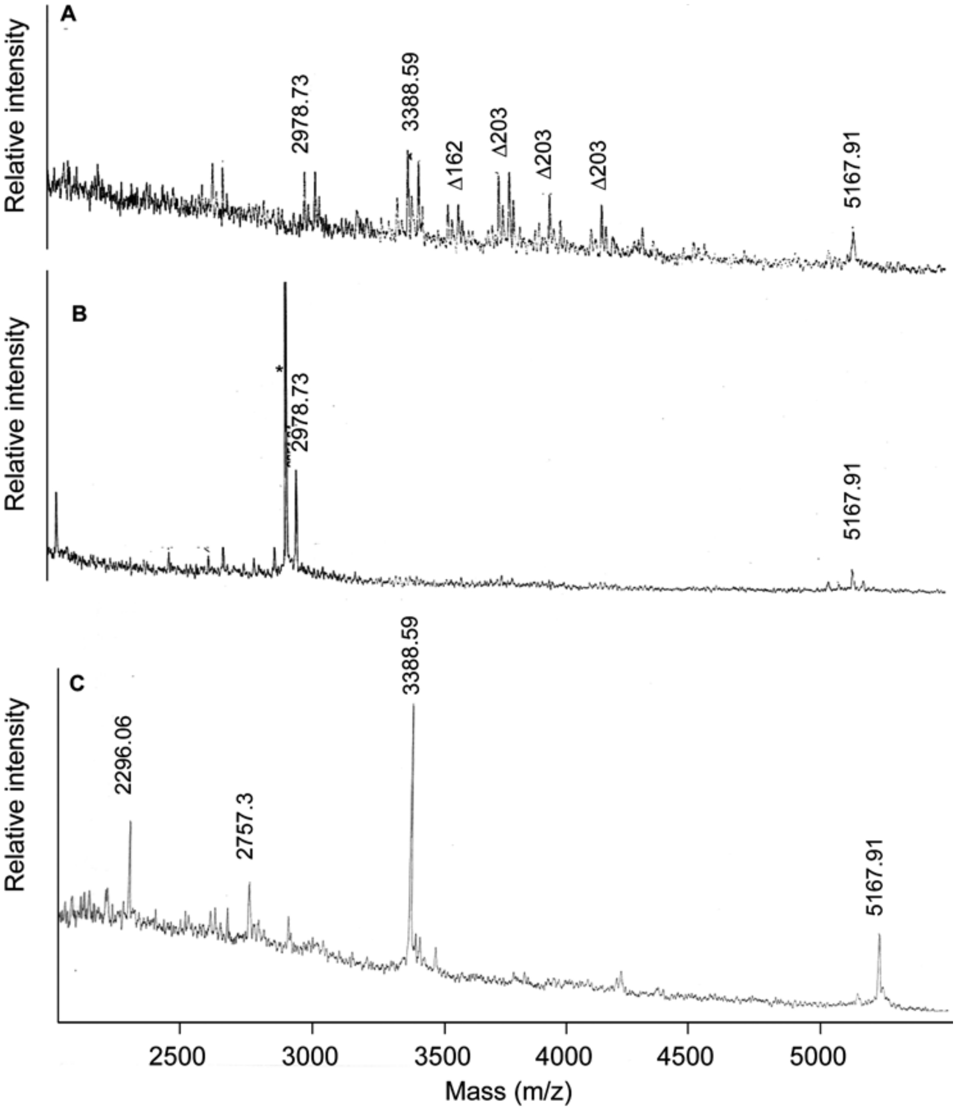

Fig. 3. MALDI-TOF-MS spectrum of the fraction collected at a retention time of 18 to 19 min from exudate of non-flowering *Perilla* plants (**A**) before and after (**B**) treatment with proteinase K (*). **C** The equivalent fraction in flowering plants

tokinin or purine-related alkaloids, such as nicotine and caffeine, which are graft-transmissible. It is also conceivable that they could bind and stabilize nucleic acids.

Both the 3.3- and the 6.7-kDa proteins are smaller then the known proteins they show similarity to. This could be due to non-specific degradation during the long preparation procedure. However, it could also be a result of specific proteolytic processing necessary for the proteins to enter the phloem/sieve elements. A third possibility is that the *Perilla* peptides are novel peptides with similarities to certain functional areas of known proteins.

The major signal in the fraction at 34–36 min arises from a 8.5-kDa protein. Purification and sequencing showed it to be ubiquitin (93% identity in 16 aa to maize and *Arabidopsis* ubiquitin). Ubiquitin has been previously described in phloem sap (Schobert et al. 1995; Haebel and Kehr 2001). This is particularly interesting since one of the other proteins found in the phloem sap shows similarity to a human ubiquitin thiolesterase 19-like protein, an enzyme that, together with ubiquitin, is involved in the ubiquitin-dependent proteolytic pathway in conjunction with the 26S proteasome. An additional large protein found in the phloem exudate also showed similarity to a ubiquitin-activating protein (not shown). The ubiquitin and proteasome-dependent proteolytic pathway is a highly conserved system and is important for the removal of abnormal proteins. However, it is also necessary for the specific degradation of cell-cycle-regulating proteins and of transcription factors and, thus, for the regulation of developmental and stress responses (for a review see Ingvardsen and Veierskov 2001). It has been shown that ubiquitination is a prerequisite for the correct formation of the vascular system (Seufert and Jentsch 1992). From our results, it appears that at least three components of the ubiquitination pathway are present in the phloem sap. Even though evidence of the 26S proteasome has not been found in the phloem (Schobert et al. 1995), selective ubiquitination could still be necessary to control phloem protein degradation or to facilitate movement into or within the phloem.

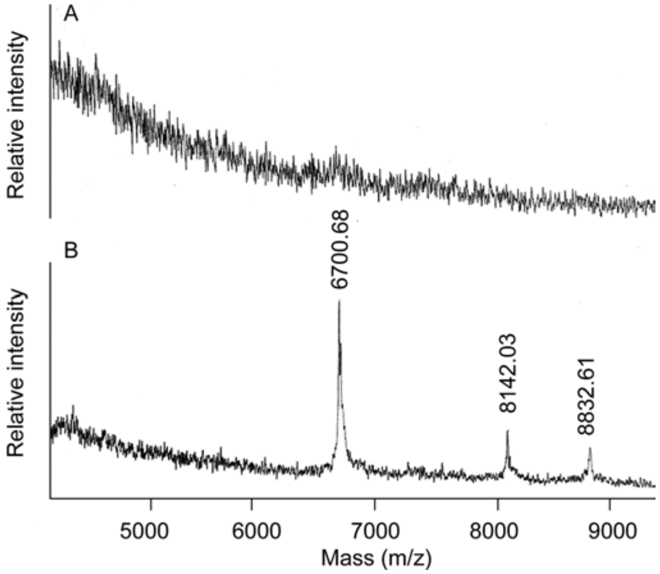

Fig. 4. MALDI-TOF-MS spectrum of the fraction collected at a retention time of 32 to 34 min from exudates of **A** non-flowering and **B** flowering *Perilla* plants of the purified fraction containing the compound with the mass 6700

The three peptide/proteins with the masses 3.3, 6.7, and 8.5 kDa described above have also been observed at the same retention times in phloem exudates from flowering lupine. The identity of the 8.5-kDa compound as ubiquitin has been confirmed in lupine sap. Partial sequencing of the lupine 6.7-kDa protein has also revealed a short sequence identical to the *Perilla* protein. This points at the importance of these compounds not only for metabolic processes in the phloem, but possibly also for the induction of flowering.

We also sequenced several other proteins found in *Perilla* phloem exudate (marked in bold in Table 2). Two of these showed no or very low similarity to any known protein. We found a peptide with similarity to the derived aa sequence of a putative plastocyanin potato leaf expressed sequence tag (EST; 72% identity in 18 aa). One sequence displayed similarity to a human "survival-promoting peptide" with phosphatase activity (100% identity in 11 aa; Cunningham et al. 1998). Two other peptide sequences have similarities to an *Arabidopsis* protein with unknown function (67% identity in 12 aa) and to the deduced protein product of a rice gene, which is highly expressed in young flowers (80% identity in 15 aa). Additional sequences have low similarity to cytosolic superoxide dismutase (77% identity in 13 aa) and a translocator-like protein (60% identity in 16 aa). We also found a protein with similarity to glycine-rich cell wall proteins and glycine-rich RNA-binding proteins. None of these appear to be specific to flowering plants.

Conclusions

We demonstrated that there are alternatives to analyzing phloem sap directly by MALDI-TOF-MS (Marentes and Grusak 1998), or by gel electrophoresis followed by mass spectrometry (Haebel and Kehr 2001). It is possible to separate phloem components using peptide microtrap/microbore HPLC prior to mass spectrometric analysis. This enabled us to identify and characterize a larger number of small proteins, many of which occur at low concentration in phloem exudate.

We used this technique to compare the peptide and protein components of the phloem sap from flowering and non-flowering plants. We detected more than 100 peptides and proteins in the phloem exudates of both *Perilla* and lupine, including three proteins necessary for the ubiquitination pathway, which has been shown to be involved in the regulation of developmental processes. This approach also allowed us to identify four small proteins that potentially play a role in the induction of flowering. One of these showed no similarity to any known protein sequences. A second small protein in phloem exudates of induced plants was similar to Ser/Thr receptor-like protein kinases. Two additional protein sequences appeared to be related to two different purine permeases. These may be important for the transport of nucleic acid bases or related signaling molecules, such as cytokinins, into and within the sieve elements. They could also play a role in binding and stabilizing nucleic acids. It is not known whether the floral stimulus is composed of one or more components. Thus, the kinase-like protein and the purine permeases may act in concert to induce flowering directly or to facilitate the transport of the signal. The fact that they are present not only in induced *Perilla*, but also in flowering lupine, further supports their possible role in flower induction.

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