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Proteomics of curcurbit phloem exudate reveals a network of defence proteins ☆

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Abstract

Many different proteins can be separated from the sap of mature sieve tubes of different plant species. To date, only a limited number of those have been identified and functionally characterised. Due to sieve tubes inability of transcription and translation, the proteins are most probably synthesised in the intimately connected companion cells and transported into the sieve elements through plasmodesmata. The specific protein composition of phloem sap suggests an important role of these proteins not only for sieve tube maintenance, but also for whole plant physiology and development.

Here we describe a comprehensive analysis of the phloem protein composition employing one- and high-resolution two-dimensional gel electrophoresis and partial sequencing by mass spectrometry. In this study more than 300 partial sequences generated by hybrid mass spectrometry were used to identify a total of 45 different proteins from the phloem exudates of cucumber (*Cucumis sativus* L. cv. Hoffmanns Giganta) and pumpkin (*Cucurbita maxima* Duch. cv. Gelber Zentner) plants. In addition to previously described phloem proteins, it was possible to localise proteins with high similarity to an acyl-CoA binding protein, a glyoxalase, a malate dehydrogenase, a rhodanese-like protein, a drought-induced protein, and a ß-glucosidase. The results indicate that the majority of the so far identified proteins are involved in stress and defence reactions.

Keywords: Cucumis sativus; Cucurbita maxima; Cucurbitaceae; Mass spectrometry; Phloem; Proteins; Stress

1. Introduction

All organs of higher vascular plants are connected by the conducting sieve elements that allow the transport of sugars and nutrients throughout the plant body. Over the past years, evidence is accumulating that sieve elements are not only competent for the translocation of small nutrients, but transport also information substances and large molecules, like proteins or even mRNAs. Since sieve elements loose ribosomes and nuclei during their specialisation for long distance transport, and are thus incapable of transcription and translation (Cronshaw, 1981), these macromolecules are likely to be imported from the adjacent companion cells through plasmodesmata (Evert, 1990; Raven, 1991). Two proteins from pumpkin phloem exudate, CmPP16

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Abbreviations: ACC-oxidase = aminocyclopropane carboxylate oxidase; ACC-synthase = aminocyclopropane carboxylate synthase; API = aspartic proteinase inhibitor (AF038166); CmPP16 = phloem protein 16-1; CmPP36 = phloem protein 36 (cytochrome b5 reductase); CSF-2 = fruit ripening and wound induced protein 2; CTI = chymotrypsin inhibitor; Cu/Zn SOD = copper/zinc superoxide dismutase; DRIP-1 = drought induced protein 1; Lox = lipoxygenase; MDH = malate dehydrogenase; MDHAR = monodehydroascorbate reductase; PP1 = phloem protein 1; PP2 = phloem protein 2; SN1 = fruit ripening protein from pepper; SWL 1 and 3 = silverleaf whitefly induced protein 1 and 3; TI = trypsin inhibitor.

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1 and 2, for example, are thought to interact with plasmodesmata in order to regulate their own cell-to-cell transport and mediate the trafficking of RNA (Xoconostle-Cazares et al., 1999). Also protein exchange between companion cells (CCs) and sieve elements (SEs) seems to be tightly controlled and selective, some proteins destined for SEs are capable of dilating the size exclusion limit of plasmodesmata (Balachandran et al., 1997; Ishiwatari et al., 1998). Chaperones and cyclophilin contained in the phloem exudate are supposed to be involved in this process and additionally ensure correct protein folding on the SE side (Schobert et al., 1995, 1998). If the proteins from the companion cells would be simply pitched in the phloem stream, they would be quickly transported to the sinks and lost at the site of production (Sjölund, 1997; Oparka and Turgeon, 1999). Therefore, most of the proteins found in phloem exudate are likely to be anchored in the cytoplasmic layer in vivo and released by the turgor drop induced by the sampling procedure. The best known example are the two structural phloem proteins PP1 and PP2, which are most probably fixed in the cytoplasm, perhaps by PP2 (Smith et al., 1987) that has been characterised as a dimeric lectin (Bostwick et al., 1992). Under certain conditions, however, PP1 and PP2 (and most probably also other phloem proteins) are able to enter the long-distance transport stream, as shown by heterografting experiments (Golecki et al., 1998).

Besides the obvious function of structural proteins, evidence is accumulating that SEs are not only a passive transport system for solutes and information molecules, but are an active component of whole plant physiology. In this context various enzyme activities could be detected in the phloem sap (Kennecke et al., 1971; Eschrich et al., 1972; Eschrich and Heyser, 1975; Geigenberger et al., 1993; Yoo et al., 2002). More recent results suggest that SEs even contain complete sets of enzymes to perform complex biosynthetic reactions. For example, the major enzymes involved in the production of ascorbic acid (Hancock et al., 2003), jasmonic acid (Hause et al., 2003), or alkaloid biosynthesis (Bird et al., 2003) were found in sieve elements. In addition, the presence of a complete and functional antioxidant defence system in the SEs of cucurbits could be demonstrated (Walz et al., 2002).

In this paper, we describe the attempt to comprehensively analyse the proteins present in exudate of mature SEs from cucumber and pumpkin plants, in order to (i) compare the phloem proteins of both cucurbits, (ii) identify new phloem proteins and thus (iii) shed light on further possible enzymatic pathways present in the phloem stream.

2. Results

2.1. Separation of proteins from sieve tubes of cucurbits

Cucumber and pumpkin phloem exudates were collected from stems of 6–8 weeks old plants. To collect samples, plants were wounded with a sterile needle, the

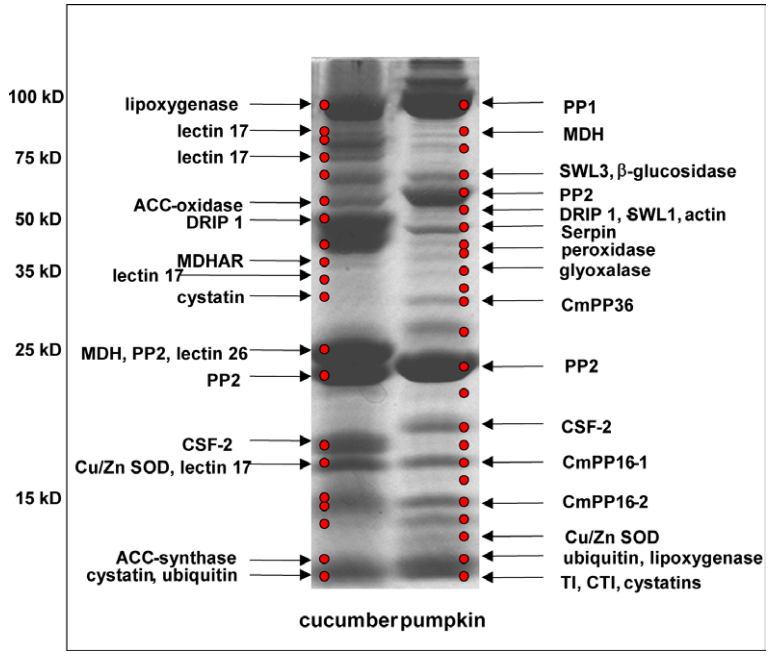

Fig. 1. 1D PAGE and identification of proteins from cucumber and pumpkin plants. 50 μg protein were separated and stained with colloidal coomassie. Protein bands were cut out, digested in gel using trypsin, analysed by mass spectrometry and subsequent database searches. Using this approach, partial sequences were deduced and several proteins could be identified and are indicated at the appropriate bands.

first droplet was discarded and the exuding sap was collected. This needle technique is limited to certain appropriate plant species. In this study, cucumber and pumpkin were the plants of choice, because of the high phloem protein content and the ease of the sampling technique. The detected phloem protein concentrations were approximately 60 μg/μl in cucumber and 35 μg/μl in pumpkin (Walz et al., 2002). 50 μg of these phloem proteins were separated by one-dimensional polyacrylamide gel electrophoresis (1D PAGE) and stained with colloidal Coomassie blue. Phloem exudate of cucumber and pumpkin plants showed about 30 different protein

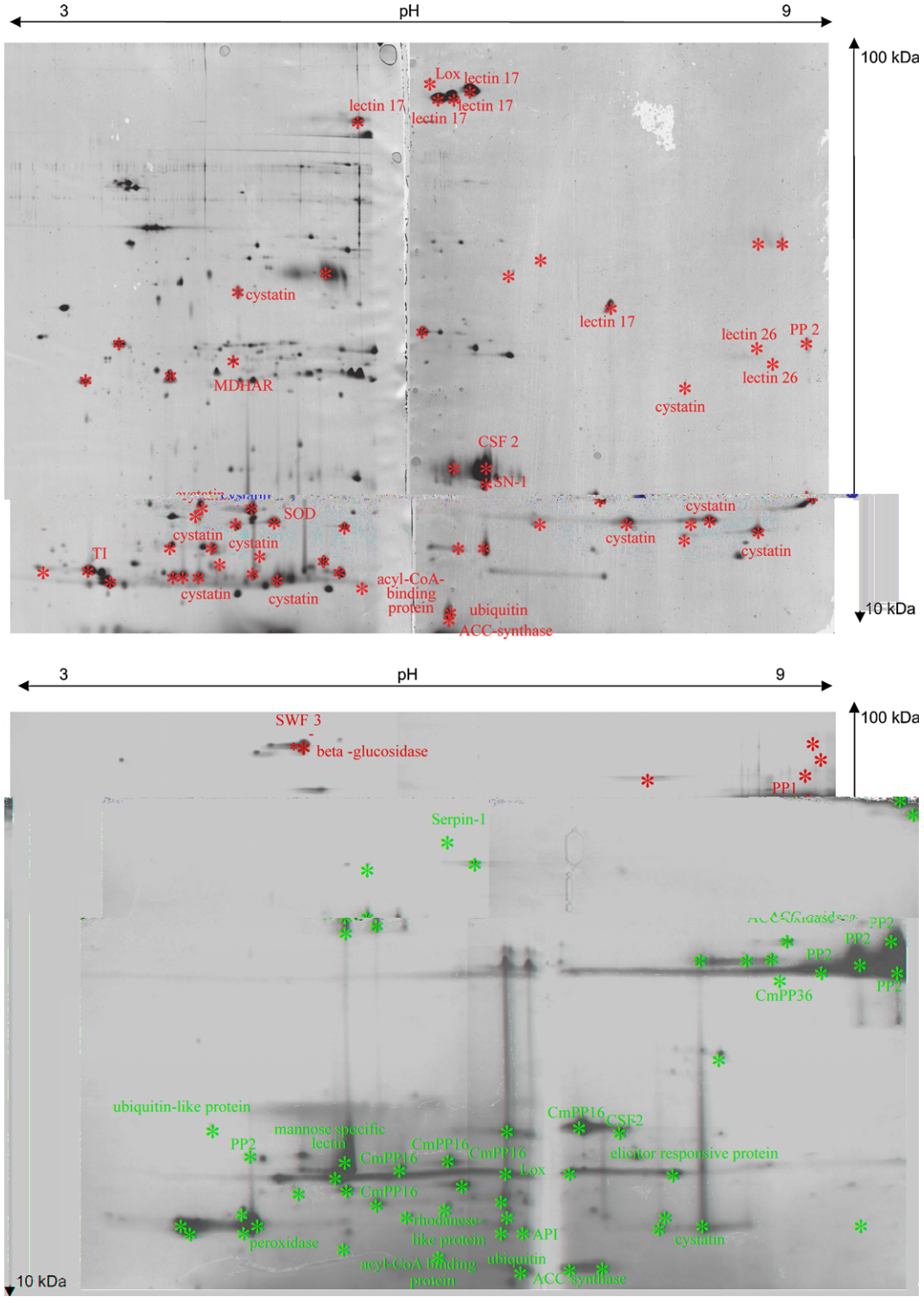

Fig. 2. High resolution 2D PAGE and identification of phloem proteins from cucumber and pumpkin plants. (a) Cucumber, (b) pumpkin. 750 μg protein were separated and stained with colloidal Coomassie. For further information and abbreviations see Fig. 1.

bands, which were used for subsequent mass spectrometric analysis (Fig. 1; Walz et al., 2002).

To increase the resolution achieved by 1D PAGE, a high-resolution two-dimensional (2D) PAGE protein separation system (Klose and Kobalz, 1995) was employed. Here, 750 μg of phloem proteins as a starting material were used for protein separation. The prepared 2D gels, which were stained with a mass spectrometry compatible silver staining protocol according to Shevchenko et al. (1996), allowed to resolve about 350–400 different protein spots from each of the two phloem exudates. 80 of the major protein spots from each species were than further processed for mass spectrometric analysis (Fig. 2).

2.2. Identification of phloem proteins by mass spectrometry

To identify proteins from the various PAGE gels, tryptic peptides of the separated protein spots/bands were prepared and then analysed using electrospray ionisation quadrupole-time–of-flight tandem mass spectrometry (ESI-Q-TOF-MS/MS; Shevchenko et al., 1997). After desalting and concentration, as many tryptic peptides from each sample as possible were selected and submitted to collision-induced fragmentation. The resultant fragmentation spectra were than used to deduce amino acid sequence stretches guided by a special software (Walz et al., 2002).

From the analysed samples, 70% resulted in clearly interpretable fragmentation spectra, while 30% contained either no peptides, peptides at too low concentrations or peptides exhibiting unfavourable fragmentation behaviour.

The resulting partial sequence information was subsequently used for database searches with the short sequence BLAST algorithm that is freely available at the NCBI web site via the internet (http://www.ncbi.nlm.nih.gov/). Only peptides showing a very high sequence identity to database entries were regarded as identified.

Table 1
Summary of phloem proteins which could be identified in the present study (The sequences can be accessed as supplementary material (Table 2))

No.	Protein ID	Functional category	Identified in	
			C. sativus	*C. maxima*
1	ACC-oxidase	3	+ (2)	+ (1)
2	ACC-synthase	3	+ (1)	+ (3)
3	Actin	5		+ (2)
4	Acyl-CoA binding protein	3	+ (1)	+ (2)
5	Aspartic proteinase inhibitor	2		+ (11)
6	Beta-glucosidase	4		+ (5)
7	Chymotrypsin inhibitor	2		+ (2)
8	CmPP16-1	6		+ (5)
9	CmPP16-2	6		+ (8)
10	Cm lectin 17	4, 5	+ (22)	
11	Cm lectin 26	4, 5	+ (16)	
12	CSF-2	4	+ (11)	+ (3)
13	Cystatin	2	+ (7)	+ (6)
14	Cytochome b5 reductase CmPP36	6		+ (7)
15	DRIP-1	1	+ (3)	+ (3)
16	Elicitor responsive/phloem-like protein	3		+ (4)
17	Glyoxalase 1	1		+ (3)
18	Lectin, mannose-binding	4, 5		+ (3)
19	Lipoxygenase	3	+ (11)	+ (4)
20	Malate dehydrogenase	1	+ (2)	+ (4)
21	Monodehydroascorbate reductase	1	+ (7)	
22	Peroxidase	1		+ (4)
23	Phloem protein 1	5		+ (16)
24	Phloem protein 2	5	+ (3)	+ (25)
25	Rhodanese-like protein	3		+ (4)
26	Serpin-1 phloem	2		+ (40)
27	SWF-1 (peptidase)	4		+ (4)
28	SWF-3 (ß-glucosidase)	4		+ (11)
29	SN-1	4	+ (4)	
30	SOD, Cu/Zn	1	+ (3)	+ (1)
31	Trypsin inhibitor	2	+ (1)	+ (3)
32	Ubiquitin	5	+ (4)	+ (4)
33	Ubiquitin-like protein	4, 5		+ (1)

Phloem proteins are listed in alphabetical order, possible functions are indicated by numbers 1–6 (1 = antioxidant defence, 2 = proteinase inhibitors, 3 = phytohormones and signalling, 4 = other defence proteins, 5 = structure, 6 = transport). + indicate that this protein was present in the respective species. The number of different peptides that matched to the identified protein with high similarity is given in brackets.

According to this criterion, more than 300 of the partial sequences closely matched to distinct proteins in the database. Compared to other proteomic studies, the success rate of protein identification was rather low, although most of the peptides resulted in high quality fragmentation spectra. This low success rate is a general problem observed for species like cucurbits, from which genomic sequence information is rarely available.

In summary, it was possible to identify 16 different phloem proteins from cucumber and 29 different phloem proteins from pumpkin (Figs. 1 and 2, Table 1).

17 identified proteins could be assigned to 14 bands from the 1D PAGE cucumber phloem gels and 22 identified proteins to 16 bands from the 1D PAGE separated pumpkin phloem protein gels (Fig. 1). From the 2D PAGE separated phloem samples, it was possible to identify proteins in 27 spots from cucumber (Fig. 2(a)) and 28 spots from pumpkin (Fig. 2(b)). Some of the identified proteins were present in more than one band or spot, as apparent from Figs. 1 and 2. This observation of multiple protein spots for similar or even identical gene products is also of great interest, since it suggests that these proteins are either products from different genes and therefore provide a high heterogeneity of the detected protein, or they arose from different post translational protein modifications. The second assumption opens the question if these proteins are modified in CCs and transported into the SEs in the modified form, or if they are modified in the SEs and therefore the modifying factors must also be present in this compartment. Both assumptions underline the complex composition of the examined system.

In pumpkin, sequences highly similar to almost all known phloem proteins from cucurbits were obtained (Figs. 1 and 2, Table 1). In addition to the, already previously described, major phloem proteins PP1 and PP2 (Bostwick et al., 1992), the RNA-binding proteins CmPP16-1 and CmPP16-2 (Xoconostle-Cazares et al., 1999), the housekeeping protein ubiquitin (Schobert et al., 1995), an aspartic proteinase inhibitor, a trypsin inhibitor, a chymotrypsin inhibitor, cystatin and serpin (Murray and Christeller, 1995; Christeller et al., 1998; Schobert et al., 1998; Yoo et al., 2000) were found. Moreover, several enzymes from antioxidant defence systems (Haebel and Kehr, 2001; Walz et al., 2002), the fruit ripening and wound induced protein CSF-2 (Haebel and Kehr, 2001), the cytochrome b5 reductase CmPP36 (Xoconostle-Cazares et al., 2000) and a lipoxygenase (Avdiushko et al., 1994) could be identified in the phloem exudate of pumpkin. Additionally, we found indications for the presence of new proteins, including a malate dehydrogenase, an acyl-CoA binding protein, an actin, an elicitor responsive/CmPP16-like protein, a drought induced protein DRIP-1, a glyoxalase 1, a rhodanese-like protein, a mannose specific lectin, an ubiquitin-like protein from phloem exudate of pumpkin. Furthermore we could identify a ß-glucosidase, the two silverleaf whitefly induced proteins SWF-1 and SWF-3, and two ethylene biosynthesis enzymes aminocyclopropane-carboxylate (ACC)-synthase and ACC-oxidase in pumpkin (Table 1, Figs. 1 and 2).

In addition to the known phloem proteins lipoxygenase (Avdiushko et al., 1994), several antioxidant defence enzymes (Walz et al., 2002) and the 17 and 26 kD lectins (Dinant et al., 2003), we could show the presence of a trypsin inhibitor, a fruit ripening protein SN-1, and supposedly more than one cystatin-like protein in the phloem sap of cucumber. Furthermore, the existence of several phloem proteins only known from pumpkin could be also confirmed in cucumber, like PP2, CSF-2, ubiquitin. A malate dehydrogenase, an acyl-CoA binding protein, DRIP-1, ACC-synthase and ACC-oxidase we found in the phloem of both species in this study (Table 1, Figs. 1 and 2).

Taken together, we could identify 45 different proteins from the phloem sap of the two cucurbits investigated. The results show that many of the proteins analysed occur in both species.

3. Discussion

Mature SEs are largely degenerated cells and often regarded as passive transport pipes. Although incapable of transcription and translation, we show that several hundred protein spots can be separated by high-resolution two-dimensional gel electrophoresis (Fig. 2). Together with previous studies, this indicates that the protein furniture of fully differentiated SEs is unexpectedly complex.

In this study, all major protein bands/spots from 1D- and 2D PAGE separated phloem saps were partially sequenced by tandem mass spectrometry. In this way, the presence of several previously described proteins was confirmed, while a set of new, previously unknown, phloem proteins could be identified (Table 1). While some of the proteins are involved in structure and maintenance (PP1 and PP2, ubiquitin, ubiquitin-like protein, lectins, actin) or transport processes (CmPP16 1 and 2, CmPP36), the vast majority of the identified proteins can be linked to direct and indirect stress- and defence responses.

3.1. Antioxidant defence related proteins

As a general reaction to stress and pathogen attack, plants produce reactive oxygen species. To avoid damage of endogenous components, antioxidant defence proteins and metabolites are essential to maintain cellular functions. This holds true especially for the SEs, because of their unusual long lifetime (Raven, 1991). The existence of a complete set of functional antioxidant

defence proteins in the phloem of cucurbits, including a copper/zinc superoxide dismutase (Cu/Zn-SOD), a peroxidase, a monodehydroascorbate reductase (MDHAR), a dehydroascorbate reductase (DHAR), and a glutathione reductase, has previously been demonstrated (McEuen and Hill, 1982; Alosi et al., 1988; Walz et al., 2002) and is confirmed in the present study (Fig. 2(b), Table 1).

Apart from a fully functional enzymatic antioxidant defence system, several non-enzymatic radical scavengers are required. The most common ones, ascorbate and glutathione have been shown to be present in the phloem sap (Franceschi and Tarlyn, 2002; Alosi et al., 1988). Moreover, a recent study provides strong evidence that ascorbic acid cannot only be imported, but it also can be produced in the phloem itself (Hancock et al., 2003). A similar scenario can be envisaged for glutathione. This radical scavenger is probably not synthesised in the phloem, but since two enzymes, namely a glutathione reductase (Alosi et al., 1988) and a glyoxalase 1 (identified in the phloem of pumpkin, Table 1), are present in the phloem sap, recycling of reduced glutathione is likely to occur (Veena et al., 1999).

In cucurbits, the unusual amino acid citrulline constitutes another component of antioxidant defence, since it scavenges hydroxyl radicals with high efficiency (Yokota et al., 2002). Citrulline occurs at high concentrations in the phloem of cucurbits (Mitchell et al., 1992). The drought induced N-acetyl glutamate transferase-like protein DRIP-1 (Kawasaki et al., 2000) identified in the analysed phloem exudates from both plants, catalyses a step in citrulline biosynthesis by delivering the carbon skeleton of glutamate into the urea cycle (Yokota et al., 2002). Thus, DRIP-1, could also support defence reactions by providing citrulline. The carbon source for synthesising amino acids like citrulline could be provided by citrate cycle reactions. This assumption is supported by the detection of proteins with high similarity to cytosolic malate dehydrogenases (Table 1) and the presence of malate, citrate, succinate and fumarate (Richardson et al., 1982) in the phloem of cucurbits. Nonetheless, sequences similar to other enzymes from the citrate cycle could not be found and activities could not be detected for all enzymes (Lehmann, 1973), questioning the functionality of a complete citrate cycle in SEs.

3.2. Proteinase inhibitors

Another central defence reaction in plants is provided by the production of proteinase inhibitors. Proteinase inhibitors are supposed to protect plants from herbivores, phloem-invading pathogens or insect attack (Christeller et al., 1992; Ryan, 2000). For example, phloem-sucking insects that nourish on amino acids from degraded plant proteins also ingest the phloem proteinase inhibitors that interfere with their digestion (Christeller et al., 1998). The present study provides additional evidence for the presence of a huge number of different proteinase inhibitors in the phloem of both species investigated. Some of these proteins are existing in more than one form. In addition to the phloem-serpin-1 (Yoo et al., 2000), aspartic, tryptic and chymotryptic proteinase inhibitors (MacGibbon and Mann, 1986; Murray and Christeller, 1995; Christeller et al., 1998; Schobert et al., 1998; Haebel and Kehr, 2001) we further found several different proteins with high similarity to cystatins in the phloem of cucumber and pumpkin (Table 1). This stable pool of proteinase inhibitors inside the long-distance transport system not only allows a protection of transported and functional proteins inside the SEs, but it also provides the machinery for fast and efficient defence reactions against invading enemies.

3.3. Phytohormones and signalling

The synthesis of proteinase inhibitors and other defence proteins is initiated by ethylene- or jasmonic acid/systemin-dependent pathways (Ryan, 2000). It is an appealing assumption that these phytohormones are not only transported, but also synthesised in the phloem. Recently, lipoxygenase and other enzymes of the jasmonic acid biosynthesis pathway could be localised to SEs in tomato (Hause et al., 2003). In this study, lipoxygenase could be also identified in the phloem of both cucurbits analysed (Table 1), indicating that the competence for jasmonic acid biosynthesis might be a common feature of phloem cells in different plant species.

In contrast to jasmonic acid biosynthesis, there was so far no evidence that other phytohormones can also be produced in the phloem. In the present study we found proteins similar to two key-enzymes of ethylene biosynthesis pathway in cucumber and pumpkin phloem sap, namely an ACC-synthase and an ACC-oxidase (Table 1). This observation indicates that, in addition to jasmonic acid biosynthesis, at least some steps of ethylene production can also occur in the phloem of cucurbits. A further hint indicating a possible ethylene biosynthesis in the phloem comes from the presence of key enzymes needed for the detoxification of problematic by-products of this pathway, like cyanide (Kende, 1993). For the efficient detoxification of the accumulating cyanide, sulfurtransferases and ß-cyano-L-alanine synthase are suggested to be necessary enzymes (Meyer et al., 2003). Interestingly, we could identify a rhodanese (thiosulfate:cyanide sulfurtransferase)-like protein in the phloem of pumpkin (Fig. 2(b), Table 1). Supplementary investigations confirmed the presence of sulfurtransferase activities in the phloem of cucumber and pumpkin (data not shown).

Apart from the beforehand mentioned hormonal signalling molecules, another protein that could be involved in long distance signalling was identified by partial sequences in the phloem of both species analysed. This uncovered protein has high similarity to an acyl-CoA-binding protein-like protein. Cytosolic acyl-CoA-binding proteins are short, highly conserved proteins that bind acyl-CoA molecules. Although this kind of proteins are mostly functioning in developmental processes (Hill et al., 1994), a participation in the regulation of the acyl-pool of the plasma membrane, involved in acyl-CoA signal transduction, has been supposed (Chye et al., 1999).

Last but not least, the presence of an elicitor-responsive protein in the phloem of pumpkin (Table 1) further suggests the occurrence of additional signal perception systems in the phloem that probably will be discovered in the future.

3.4. Other defence proteins

We could identify several other proteins in the phloem exudates of healthy cucurbit plants that have been described to react to stress or pathogen attack, although their exact function in these reactions is not well understood.

We found two proteins in pumpkin phloem that were previously characterised as silverleaf whitefly-induced proteins (SWF 1 and 3; van de Ven et al., 2000) in leaves of *Cucurbita pepo*. SWF-1 was described as regulated via an abscisic acid (ABA) dependent pathway and functions as a peptidase, whereas SWF-3 was regulated by an ABA-independent signalling pathway and shows similarity to a ß-glucosidase (van de Ven et al., 2000). In addition to SWF-3, we found another protein with high similarity to different ß-glucosidases in phloem sap of pumpkin. ß-glucosidases can affect plant defence reactions, e.g. by catabolising glucolised forms of phytohormones (Dietz et al., 2000; Mainguet et al., 2000; Sue et al., 2000; van de Ven et al., 2000; Gerardi et al., 2001).

CSF-2, described as a wound- and ripening-induced protein in cucumber fruits (Suyama et al., 1999), could be identified in the phloem of cucumber and pumpkin. This protein is expressed during fruit development and growth and was previously localised to phloem sap of pumpkin by Haebel and Kehr (2001). Furthermore, a protein with similar function and sequence was present in the phloem of cucumber, SN-1. The expression of SN-1 increased during wounding in pepper (Pozueta-Romero et al., 1995).

In addition to structural support, the two major cucurbit phloem proteins PP1 and PP2 also function in stress and pathogen responses. While normally fixed in the cytoplasm by PP2 (Smith et al., 1987) at least a part of these proteins is able to translocate under certain conditions (Golecki et al., 1998). This ability is likely to be connected with their function in sealing of sieve pores upon wounding (Alosi et al., 1988). PP2 has been characterised as a dimeric lectin (Bostwick et al., 1992). Besides PP2, several other lectin-like proteins could be found in the phloem sap of both cucurbits, especially in cucumber (Table 1). This finding is supported by the high number of lectins described by Dinant et al. (2003) in the phloem of different species. Phloem lectins could aid defence by recognising and binding invading pathogens.

4. Conclusions

The results of this study enhance the current knowledge about the protein composition of cucurbit phloem sap. We could identify 45 proteins, whereof 23 previously had not been assigned to the phloem of the species analysed here.

The composition of phloem proteins within the cucurbit family seems to be quite conserved, since 12 of the identified proteins were present in both species examined. Most of the so far identified proteins can be related to stress- and defence reactions, indicating a pivotal role of the phloem in plant responses to biotic and abiotic challenges. These findings allow the assumption, that SEs are not only transporting signals, but probably they might be involved in generating these signals themselves. The activity of such and other complex biochemical pathways in the phloem would demand a high degree of spatial organisation and coordinated action of the participating enzymes, an aspect that has not been in the focus of phloem protein analyses yet. Thus, further investigations will be necessary to fully understand the function, the organisation and the contribution of the phloem to whole plant defence and whole plant physiology.

5. Experimental

5.1. Plant material

Seeds from *Cucurbita maxima* Duch. cv. "Gelber Zentner" and from *Cucumis sativus* L. cv. "Hoffmanns Giganta" (Treppens, Berlin, Germany) were grown in commercially available pot soil under controlled conditions (16 h light, 8 h darkness; 22 °C at day, 18 °C at night; 60% relative humidity; light intensity 250 $\mu mol\, m^2\, s^{-1}$).

5.2. Phloem sampling

Phloem exudate was obtained by wounding 6–8 weeks old adult cucurbit plants with a sterile needle. After the first droplets were discarded, plastic micropipettes

(Eppendorf, Hamburg, Germany) were used to collect phloem sap exuding from cucumber and pumpkin stems.

Depending on the plant and the sampling site, between 5 and 20 μl samples could be collected from one puncture. The samples were directly expelled into the appropriate solutions for further analysis (see below).

5.3. Determination of protein concentrations

Protein concentrations were determined with the BioRad protein assay kit (BioRad, Munich, Germany) with the microassay procedure using 0.5 μl of phloem sap following the instructions of the supplier.

5.4. Sample pre-treatment and gel electrophoresis

For 1D PAGE, 1 μl phloem sample was immediately mixed with 14 μl sample buffer [50 mM Tris, adjusted to pH 8.0 with HCl, 1 mM EDTA, 2.5% (w/v) SDS, 5% (w/v) mercaptoethanol, 15% (v/v) glycerol and 0.05% (w/v) bromophenol blue]. Furthermore the conditions and performance of 1D PAGE are described by Walz et al. (2002).

For two dimensional gel electrophoresis, nearly 200 μl of phloem samples were collected on ice and proteins were purified and concentrated by treatment with 300 μl 0.1 M HCl followed by the addition of 1.5 ml 100% cold acetone. After centrifugation for 15 min at 14000 rpm, the pellet was washed two times with 100% acetone and air dried.

5.5. High-resolution 2D PAGE

The protein pellets were resuspended in 100 μl first dimension buffer [2 M thiourea, 7 M urea, 4% 3-[(3-chloramidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and 10 mM DTT]. The following protein separation was performed in a two dimensional gel electrophoresis system described previously in Giavalisco et al. (2003). For this purpose 15 μl (750 μg) cucumber and 27 μl (750 μg) pumpkin were separated in 40 cm times 1.5 mm tube gels for the isoelectrical focussing and $23.2\times30\times0.1$ cm SDS–PAGE gels.

After protein separation the gels were stained with a mass spectrometry compatible silver staining protocol according to Shevchenko et al. (1996).

5.6. In-gel tryptic digestion

For in-gel digestion, stained protein bands and spots were excised, transferred to 0.5 ml siliconised reaction tubes (Ambion, Huntingdon, UK), digested over night with 0.01 μg/μl Trypsin at 37 °C and extracted as described by Walz et al. (2002).

5.7. Mass spectrometry

The tryptic peptides were analysed with a quadrupole time-of-flight hybrid mass spectrometer (Q-TOF, Micromass, Altrincham, UK), equipped with a z-spray source. Conditions for the measurements, instrument settings and description of the procedure are specified in Walz et al. (2002).

5.8. Database search

The database searches using partial sequences from tryptic peptides were done with the short sequence blast algorithm available in the internet (http://www.ncbi.nlm.nih.gov/) and were limited to green plants. Additional amino acid sequence alignments were performed using a freely accessible align program (http://www2.igh.cnrs.fr/bin/align-guess.cgi).

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