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Cucurbita pepo contains characteristic proteins without a signal peptide in the xylem sap

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Abstract

Xylem sap is a fluid that transfers water and nutrients from the rhizosphere. This sap contains relatively low concentrations of proteins that originate from the extracellular space among the root cells. One of the characteristic proteins in the xylem sap of the Cucurbitaceae family, which includes cucumber and zucchini, is a major latex-like protein (MLP). MLPs are responsible for crop contamination through the transport of hydrophobic pollutants from the roots. However, detailed information on the content of MLPs in the xylem sap is not available. Proteomic analysis of root and xylem sap proteins from the *Cucurbita pepo* cultivars Patty Green (PG) and Raven (RA) showed that the xylem sap of cv. RA, a high accumulator of hydrophobic pollutants, contained four MLPs that accounted for over 85% of the total xylem sap proteins in this cultivar. The xylem sap of PG, a low accumulator, mainly contained an uncharacterized protein. The amount of each root protein between the PG and RA cultivars was significantly and positively correlated in spite of being with and without a signal peptide (SP). However, the amount of xylem sap proteins without an SP was not correlated. These results suggest that cv. RA is characterized by MLPs in the xylem sap.

Keywords: *Cucurbita pepo*, major latex-like protein, proteomic analysis, root protein, secretion, xylem sap protein

1. Introduction

Plant fluids, such as phloem sap and xylem sap, transport nutrients and signal molecules for healthy plant growth. Phloem sap carries assimilates produced by photosynthesis and characteristic proteins, such as phloem proteins, from the leaves to other organs (Lopez-Cobollo et al., 2016), while xylem sap transports water containing amino acids, inorganic elements, sugars, hormones, and proteins from the roots to the aboveground organs (Satoh, 2006). Proteins in these fluids are important components of the sap contents, although their concentrations are considerably different between phloem and xylem: up to 60 μg/μL in phloem sap (Walz et al., 2002) and up to 0.5 μg/μL in xylem sap (Yang et al., 2020). Phloem sap proteins are mainly categorized in the general metabolism that reflects exchange processes between companion cells and sieve elements (Rodríguez-Celma et al., 2016). Xylem sap contains pathogenesis-related (PR) proteins responsible for defense against pathogens and peroxidases related to the generation of reactive oxygen species to strengthen the cell wall (Kehr et al., 2005). Recently, small peptides have been recognized as long transport signaling molecules; CLV3/ESR-related 25 (Takahashi et al., 2018) and C-terminally encoded peptide (Okamoto et al., 2016) transmit water deficiency and nitrogen starvation signals to the upper parts of plants to close stomata and promote nitrogen uptake through xylem sap, respectively. The quality and quantity of these fluids can be controlled in response to environmental conditions. Salt-induced abiotic stresses reduced the concentrations of specific amino acids in the xylem sap of *Brassica oleracea* and induced the accumulation of enzymes related to xylem differentiation and lignification (Fernandez-Garcia et al., 2011). Nitrogen application in different forms and concentrations changed protein quality and quantity (Kasper et al., 2022). Biotic stresses also changed the amounts of PR proteins in tomato xylem sap due to infection by plant pathogens (Rep et al., 2002). Furthermore, the infection of cotton plants with *Verticillium dahliae* increased defense-related proteins (Yang et al., 2020). These reports strongly suggest that these proteins are involved in resistance against environmental stresses.

The xylem sap is an apoplastic fluid in the extracellular space. The proteins are secreted into an apoplastic space either through the conventional or unconventional pathway (Wang et al., 2018). Proteins with an N-terminal signal peptide (SP) are delivered through the endoplasmic reticulum (ER) to the Golgi apparatus and then to the extracellular space. Proteins without an SP also exist in this space, comprising up to 50–70% of the total secreted proteins (Alexandersson et al., 2013; Krause et al., 2013). These proteins are called leaderless secreted proteins (LSPs) and are secreted through several pathways, i.e., direct ER- and vacuole-plasma membrane trafficking and through multivesicular bodies and exocyst-positive organelles (Goring and Sansebastiano, 2017).

Major latex-like proteins (MLPs) have been detected in the xylem sap of members of the Cucurbitaceae family, including cucumber, pumpkin, and zucchini (Inui et al., 2013; Iwabuchi et al., 2020). MLPs do not have an SP in their N-terminals and are categorized as LSPs. *MLP* genes in roots show much higher expression than those in leaves and stems (Fujita et al., 2022a). Therefore, MLPs may be secreted from the root cells into the extracellular space via unconventional pathways before reaching the xylem vessel. The existence of MLPs in the xylem sap contributes to the transport of hydrophobic compounds from the roots to the aerial parts in the Cucurbitaceae family (Inui et al., 2013). As a result, fruits of this family are contaminated by hydrophobic pollutants. The contamination has become a serious problem because bioaccumulated pollutants show toxicity by ingestion of contaminated crops. Interestingly, this phenomenon is mainly limited to the Cucurbitaceae family (Otani et al., 2007). The possible transport activities of MLPs with respect to hydrophobic compounds have been observed in the case of *p*,*p*′-dichlorodiphenyldichloroethylene (White et al., 2003), chlordane (Incorvia Mattina et al., 2000), pyrene (Kobayashi et al., 2008), and dioxins and dioxin-like compounds (Inui et al., 2008) because these pollutants accumulate in the aerial parts of the Cucurbitaceae family. As MLPs bind to hydrophobic compounds through the internal cavity composed of hydrophobic amino acids formed in the center of MLPs, they are dissolved in xylem sap and transported via the transpiration stream (Fujita et al., 2022b; Inui et al., 2013). Despite the fact that MLPs are responsible for the accumulation of hydrophobic pollutants in the Cucurbitaceae family, no detailed information is available regarding the content of MLPs in the xylem sap and the transfer of MLPs from the roots to the xylem sap.

In this study, the xylem sap and root proteins of two cultivars of *Cucurbita pepo* were subjected to proteomic analysis. The *C. pepo* L. subspecies *ovifera* cultivar Patty Green (PG) and ssp. *pepo* cv. Raven (RA) were selected as representatives of low and high accumulators of hydrophobic pollutants, respectively (Matsuo et al., 2011). The differences in the content and quantity of proteins between low and high accumulators and between xylem sap and roots were revealed to be a specific accumulation of uncharacterized proteins (UCPs) and MLPs in the xylem sap, despite the LSPs.

2. Materials and methods

2.1. Plant cultivation and collection of xylem sap and root proteins

Seeds of *C. pepo* L. ssp. *ovifera* cv. PG and ssp. *pepo* cv. RA (Johnny’s Selected Seeds, Albion, ME, USA) were peeled and immersed in tap water overnight at 4 °C. Seeds were sown in pots supplemented with soil (Hyponex Japan Corp., Ltd., Osaka, Japan) and cultivated for three weeks under a 16-hour light and 8-hour dark cycle at 26 °C. The plants were sufficiently watered 2 hours before stem cutting. The stem below the cotyledons was cut by a fresh scalpel blade, and the exudation of solution was observed on a root-side cut surface within several minutes. To avoid contamination of phloem sap and other living cells, the cut surface was gently washed with ultrapure water 4–5 times. During washing, the pH was checked using pH test paper. After the pH of the solution decreased below 6, the collection of naturally exuded xylem sap was started in a plastic tube. The volume of collected xylem sap was measured after overnight collection (Fig. S1A). The roots were washed with tap water to remove soil particles and ground with a mortar and pestle using liquid nitrogen. The fresh weights of the aerial parts, including the stems, leaves, and roots, were measured (Fig. S1B and Fig. S1C).

2.2. Preparation of protein samples

Frozen root powder was suspended in ice-cold extraction buffer (50 mM potassium phosphate buffer [pH 7.0], 10 mM EDTA, 0.1% [v/v] Triton-X100, 0.1% [v/v] *N*-lauroylsarcosine sodium salt, and 10 mM 2-mercaptoethanol). The suspension was centrifuged for 10 min at 20,700×*g* at 4 °C to separate soluble proteins from insoluble debris. Protein concentrations were measured using the Bradford method (Bradford, 1976). Protein samples were subjected to 15% SDS–PAGE, and proteins were detected by silver staining and immunoblotting using anti-actin, anti-MLP-PG1, and anti-MLP-GR3 antibodies (Fig. S2) (Goto et al., 2019). The resulting supernatants from the roots and xylem sap were lyophilized. These samples were then used for reduction, alkylation, and digestion.

2.3. Protein reduction, alkylation, and in-solution enzymatic digestion

The samples were reduced by the addition of 3 mM tris(2-carboxyethyl)phosphine (TCEP) for 45 min at 25 °C and then alkylated with 15 mM iodoacetamide for 60 min in the dark at room temperature. Proteolytic digestion was performed with the addition of 500 ng Trypsin/Lis-C solution (Promega Corp., Madison, WI, USA), and the mixture was incubated overnight with shaking at 500 rpm at ambient temperature. Digestion was stopped by the addition of formic acid (1% final concentration), and the resulting solution was centrifuged at 15,000×*g* for 2 min. The supernatant, containing approximately 100 mg of digested proteins, was desalted on disposable TopTip C-18 columns (Glygen, Athens, GA, USA) and dried by vacuum centrifugation.

2.4. Liquid chromatography and mass spectrometry (LC–MS/MS)

All experiments were performed using an Orbitrap Fusion (Thermo Fisher Scientific, Waltham, MA, USA) coupled with an Ultimate3000 nanoRLSC (Thermo Fisher Scientific) according to a previous report (Risha et al., 2020). Peptides were separated using an in-house packed column (Polymicro Technologies, 15 cm × 75 μm internal diameter, supplemented with Luna C18(2), 3 μm, 100 Å [Phenomenex Inc., Torrance, CA, USA]) with a water/acetonitrile/0.1% formic acid gradient. The samples were loaded onto the column for 105 min at a flow rate of 0.30 μL/min. Peptides were separated using 2% acetonitrile for the first 7 min, followed by a linear gradient of 2–35% acetonitrile for 68 min, 35–98% acetonitrile for 5 min, and 98% acetonitrile for 10 min, followed by a gradient of 98–2% acetonitrile for 10 min. The column was then washed for 10 min with 2% acetonitrile. Eluted peptides were directly sprayed into a mass spectrometer using positive electrospray ionization (ESI) at an ion source temperature of 250 °C and an ion spray voltage of 2.1 kV. The Orbitrap Fusion Tribrid was programmed in the data-dependent acquisition mode. Full-scan MS spectra (m/z 350–2000) were acquired at a resolution of 60,000. Precursor ions were filtered according to monoisotopic precursor selection, charge state (+2 to +7), and dynamic exclusion (30 s with a ± 10 ppm window). The automatic gain control settings were 5e5 for full scans and 1e4 for MS/MS scans. Fragmentation was performed by collision-induced dissociation in a linear ion trap. The precursors were isolated using a 2 m/z isolation window and fragmented with a normalized collision energy of 35%.

2.5. Peptide and protein identification, data processing, and statistical analysis

MS raw files were analyzed using MaxQuant, version 2.0.1.0 (Cox and Mann, 2008). The Cucurbitaceae UniProt FASTA database was used to search for the peptides with the Andromeda search engine (Cox et al., 2011) integrated into MaxQuant. Oxidation and N-terminal acetylation were set as variable modifications, whereas carbamidomethyl was fixed. Trypsin was chosen as the digestion enzyme with a maximum of two missed cleavages. The identified peptides had an initial precursor mass deviation of up to 10 ppm and fragment mass deviation of 0.5 Da. The false discovery rate (FDR) for peptides (minimum of 6 amino acids) and proteins was 5%. A reverse sequence database was used to determine the FDR. Only unique peptides were considered for label-free protein quantification. A contaminant database provided by the Andromeda search engine was used. All proteins matching the reverse database or labeled contaminants were filtered. Label-free protein quantification (LFQ) values were obtained using MaxQuant quantitative label-free analysis (Cox and Mann, 2008).

LFQ values were used as approximations of the relative protein abundance in the biological samples. The log2-fold-change calculation and unpaired two-sample test for Volcano plots were based on the LFQ intensity values for which missing values were imputed from a shifted normal distribution (shift = 1.8 sd, width = 0.3 sd). The revised intensity was obtained by dividing each protein value by the sum of all intensities. The molecular weights of the proteins were obtained from MaxQuant tables based on their amino acid sequences, and the presence of signal peptides was predicted using SignalP version 5.0. All data visualizations were generated using Microsoft Excel 16.64 or R version 4.1.1.

3. Results and discussion

3.1. Protein detection in the roots and xylem sap of C. pepo

The volume of xylem sap and weight of the aerial parts of the cv. RA were significantly higher than those of cv. PG (Fig. S1A and Fig. S1B), although root weight was not significantly different between the cultivars (Fig. S1C). The root proteins of cv. PG showed a band pattern similar to that of cv. RA (Fig. S2A), while the band patterns of xylem sap proteins differed (Fig. S2B). The major bands of the PG and RA cultivars were approximately 9 kDa and 17 kDa, respectively. The number of proteins detected in the roots of the PG and RA cultivars was 215 and 372, respectively, whereas those in the xylem sap were 91 and 114, respectively (Table 1). These results suggest that the roots contain more proteins than the xylem sap, and some root proteins had translocated to the xylem sap. The samples from xylem sap did not show the bands derived from actin proteins, whereas those from roots had the bands, meaning that contamination of cytosolic proteins in xylem sap is quite limited (Kato et al., 2022).

3.2. Proteins with and without an SP in the roots and xylem sap of C. pepo

While 3.5–4.2% of root proteins had an SP predicted by SignalP, compared to the root proteins, xylem sap proteins showed high ratios of proteins with an SP (24–32%). Alvarez et al. and Ligat et al. predicted that 97% and 88% of xylem sap proteins are secreted proteins in maize and *Brassica oleracea*, respectively (Alvarez et al., 2006; Ligat et al., 2011). One of the possibilities for the low ratios of proteins with an SP in *C. pepo* is that protein degradation occurs by SP peptidases during the collection of xylem sap. SPs located at the N-terminus of proteins are essential for xylem sap proteins to be secreted outside the cells through conventional protein secretion mechanisms (Ding et al., 2014). Secreted proteins have also been reported to exist without an SP. These are called LSPs. More than 65% of the xylem sap proteins did not contain an SP and were translocated to the xylem sap (Table 1). This proportion is comparable to that reported in previous studies (Alexandersson et al., 2013; Krause et al., 2013). The roots and xylem sap revealed 88 and 26 commonly reported proteins in the PG and RA cultivars, respectively. To evaluate the quantitative differences in common PG and RA proteins in the roots and xylem sap, fold-change and p values were visualized using a volcano plot (Fig. 1, Table S1). In the roots, three proteins were significantly more abundant in RA (V-type proton ATPase catalytic subunit A, calreticulin, and sucrose synthase), while PG returned five (mainly major latex-like protein [MLP-GR3] and triosephosphate isomerase) (corrected $p < 0.05$). Among xylem sap proteins, six were significantly more abundant in RA, including the major latex-like proteins MLP-GR3, MLP-GR1, and expansin-like B1, and three were more abundant in PG: putative chymotrypsin protease inhibitor, expansin-like A2, and superoxide dismutase [Cu-Zn]. The content of proteins with an SP in the xylem sap of both cultivars showed a peak at 30–40 kDa (Fig. 2), whereas proteins without an SP had a peak at 10–20 kDa. These results suggest that xylem sap proteins with smaller molecular weights are translocated into xylem sap, even if they do not have an SP. Ten percent and 3.2% of proteins overlapped between roots and xylem sap in cv. PG and RA, respectively. A total of 4.5% and 17% of overlapping proteins were predicted to be secreted.

3.3. Main proteins in the roots and xylem sap of *C. pepo*

The intensity of each protein, as determined by MaxQuant, represents the quantity of proteins. Each protein intensity was revised by the sum of all protein intensities in the roots and xylem sap. Table 2 shows the 10 main proteins detected in high amounts in the roots and xylem sap. Proteins consisting of cell skeletons such as actin and tubulin were detected in the roots of both cultivars. Two ATP synthase subunits, along with enolases, heat shock proteins, plasma intrinsic proteins, and eukaryotic translation initiation factors, were detected in the roots of both cultivars. This suggests that similar proteins were produced in both PG and RA. This is supported by the fact that their amounts were significantly correlated between the cultivars (Fig. 3A). Although calmodulin-7-like and patellin 1 were detected in the 10 main proteins in cv. PG, they were also detected in out of the 10 main proteins of the cv. RA. Similarly, glyceraldehyde-3-phosphate dehydrogenase and adenosylhomocysteinase, which were detected in the main 10 proteins of the roots of cv. RA were also detected in those of cv. PG, but not in the main 10. The main 10 proteins in the roots of both cultivars accounted for only 36% of the total proteins (Fig. 4A and Fig. 4B, insets), suggesting that various types of proteins exist in the roots. This finding was confirmed by multiple protein bands separated by SDS–PAGE (Fig. S2A). There were no root proteins with an SP in the main 10 (Table 2).

3.4. Possible functions of proteins in the roots and xylem sap of C. pepo

In the xylem sap of the cv. PG, the UCP (UniProt ID, A0A0A0KR65) with a 9-kDa molecular weight (estimated based on its amino acid sequence) was detected, and its amount accounted for approximately 40% of the xylem sap proteins of that cultivar (Table 2, Fig. 4C, inset). UCP detected in the xylem sap of the cv. PG were not detected in the xylem sap of cv. RA. SDS–PAGE analysis of cv. PG xylem sap showed a major band at approximately 9 kDa, which was likely a UCP (Fig. S2B). Both cultivars contained three peroxidases (Table 2). Peroxidases have been detected in the xylem sap of wheat (Zeng et al., 2022), *Solanum lycopersicum* (Ceballos-Laita et al., 2020), and *B. napus* (Lee et al., 2022). These are related to lignification of cell walls and defense responses to pathogens. Superoxide dismutase is a reactive oxygen scavenging enzyme related to plant defense against pathogens that has been detected in the apoplast of *Zea mays* (Nogueira-lopez et al., 2018). Cysteine-rich repeat secretory protein 38-like and eukaryotic translation initiation factor 5A were detected in both cultivars, and the latter was detected in the roots. Cysteine-rich repeat secretory proteins in wheat are responsible for defending against infection by two fungal pathogens (Guo et al., 2020). This protein in tomatoes might be involved in systemic resistance signaling after infection with fungal pathogens (Rep et al., 2003). The putative chymotrypsin protease inhibitor and aspartyl protease AED3 were detected in the xylem sap of cv. PG, but not that of cv. RA. Proteases in the xylem sap play a role in defense reactions by specific degradation of polypeptides (Buhtz et al., 2004) and programmed cell death (Fernandez-Garcia et al., 2011). These results indicate that xylem sap contains many defense-related proteins, with no distinction between cultivars.

3.5. MLPs in the roots and xylem sap of C. pepo

Four MLPs (MLP-GR3, CmMLP2, MLP-GR1, and CfMLP2) were detected in the main 10 xylem sap proteins of cv. RA (Table 2, Fig. 4D). The amount of MLP-GR3 was 5.5 times higher than that of CmMLP2 (Fig. 4D). The *MLP-GR1* and *MLP-GR3* genes were cloned from *C. pepo*, whereas the *CmMLP2* and *CfMLP2* genes were cloned from *C. moschata* and *C. ficifolia*, respectively (Inui et al., 2013; Iwabuchi et al., 2020). This result suggests that several *MLP* genes are simultaneously expressed because the *C. pepo* genome contains 21 *MLP* homolog genes (Fujita et al., 2022b). Surprisingly, the sum of the four MLPs accounted for over 85% of the total xylem sap proteins in cv. RA, and these MLPs did not have an SP (Table 2, Fig. 4D, inset). Since MLPs are at approximately 17 kDa, the major band at 17 kDa in SDS–PAGE of the xylem sap of cv. RA was related to MLPs (Fig. S2B). Peroxidases and cysteine-rich repeat secretory protein 38-like possessed an SP in the main 10 proteins in both cultivars. There were significant positive correlations in xylem sap proteins with an SP between the cultivars (Fig. 3B, blue solid line). However, no correlation was observed for proteins without an SP (Fig. 3B, red dotted line). These results suggest that each cultivar is characterized by xylem sap proteins without an SP secreted through unconventional protein secretion mechanisms.

MLP-GR3 and CmMLP2 had higher accumulations in the xylem sap of cv. RA than in that of cv. PG (Fig. 5A and Fig. 5B). These MLPs were also detected in the roots, but the accumulation patterns differed between the cultivars, with high accumulation of MLP-GR3 and CmMLP2 in PG and RA, respectively. Immunoblotting showed that the roots of both cultivars contained MLPs detected using anti-MLP-PG1 and anti-MLP-GR3 antibodies (Fig. S2D). MLP-GR3 and

CmMLP2, as well as MLP-GR1, which differ from MLP-PG1 by one amino acid, have previously been detected by anti-MLP-GR3 and anti-MLP-PG1 antibodies, respectively (Iwabuchi et al., 2020). Therefore, the bands detected by anti-MLP-GR3 and anti-MLP-PG1 antibodies in the roots of cv. PG were those of MLP-GR3 and MLP-GR1, respectively (Fig. 5A and Fig. 5C). Bands corresponding to MLPs detected by these two MLP antibodies were also detected in the roots of several *C. pepo* cultivars, including RA and PG (Goto et al., 2019). The roots of the cv. RA contained MLP-GR3 and CmMLP2, although MLP-GR1 was not detected (Fig. 5A–C). The protein bands in the roots of the cv. RA detected by the anti-MLP-PG1 antibody could be MLPs, although these were not detected by proteomic analysis. The xylem sap of cv. RA contained three MLPs detected using anti-MLP-PG1 and anti-MLP-GR3 antibodies (Fig. 5A–C, Fig. S2E). The higher accumulation of MLPs in the xylem sap of cv. RA than in that of cv. PG has been previously reported, and the amounts of these MLPs in the xylem sap are significantly and positively correlated with the bioconcentration factors of hydrophobic pollutants (Goto et al., 2019).

4. Conclusion

In this study, proteomic analysis revealed that the xylem sap of *C. pepo* cultivars contained higher numbers and amounts of proteins without an SP than with an SP. It has been suggested that proteins without an SP play a significant role in the functioning of xylem sap. As the amounts of proteins without an SP in the xylem sap of PG and RA cultivars were not correlated, cultivar differences might have been factors. Furthermore, UCPs and four MLPs were the characteristic proteins of the Cucurbitaceae family, and these proteins have not been reported in the xylem sap of other plant families. Therefore, these proteins may be related to the specific functions of the

Cucurbitaceae family. The cv. RA is a high accumulator of hydrophobic pollutants and accumulates a large number of MLPs in the xylem sap (Goto et al., 2019; Matsuo et al., 2011). In high-accumulators, MLPs are responsible for crop contamination (Fujita and Inui, 2021). As the functions of UCPs have not been reported to date, they should be clarified in future studies. In addition, the dynamics of MLPs in xylem sap, such as adsorption and passing through the vessel wall, should be researched to reveal the distribution of MLPs in the upper parts of plants.

Author contributions

HI, ZM, NH, KF, PS, and IK performed the experiments and collected and analyzed the data. HI, ZM, NH, KF and IK wrote the manuscript. HI and IK conceptualized the overall work.

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Highlights

- Proteomic analysis revealed characteristic proteins in the *Cucurbita pepo*.
- Major latex-like proteins were detected in a high accumulator of pollutants.
- An uncharacterized protein was contained in a low accumulator of pollutants.
- There were many leaderless secreted proteins in the xylem sap of *C. pepo*.
- Xylem sap proteins characterized a high accumulation of pollutants in *C. pepo*.

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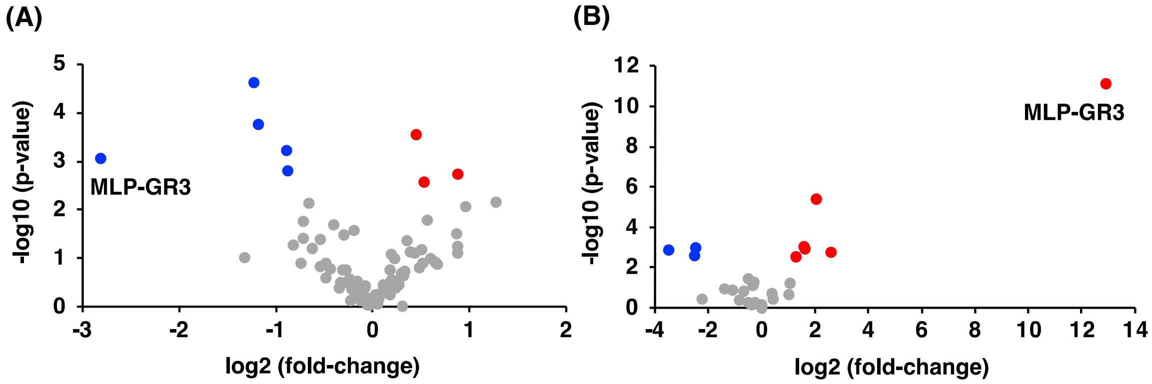

Fig. 1. Volcano plots of protein abundances comparing the *Cucurbita pepo* cultivars Patty Green (PG) and Raven (RA) in roots (A) and xylem sap (B).

Proteins significantly more abundant in RA are indicated in red and those more abundant in PG in blue. P values were obtained by a two-sample *t* test, and significance was assigned based on a threshold of $p < 0.05$ after Benjamini–Hochberg correction.

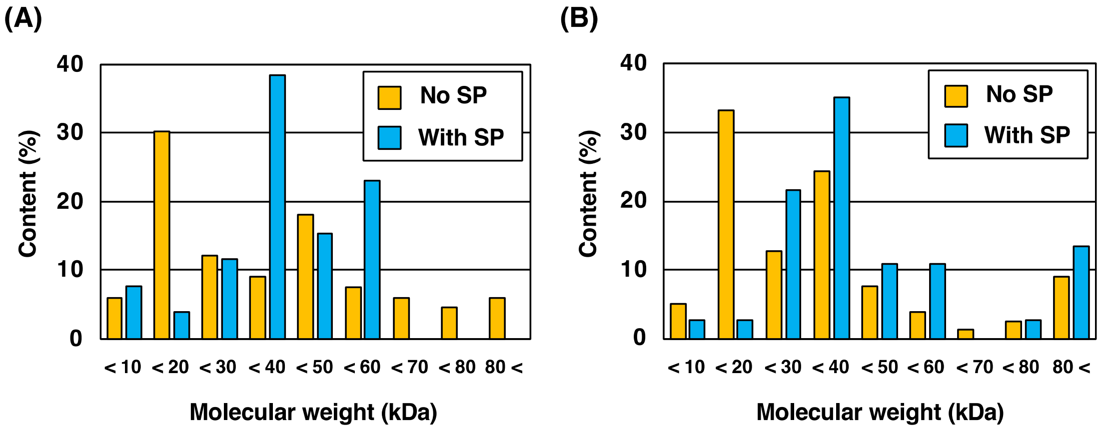

Fig. 2. Protein contents in xylem sap of the *Cucurbita pepo* cultivars Patty Green (A) and Raven (B).

Xylem sap was collected from the cut stem of *C. pepo* cultivated for three weeks in pots supplemented with soil. Xylem sap was lyophilized and subjected to liquid chromatography with mass spectrometry (n = 6). Detected proteins were divided into their molecular weights. Signal peptides (SPs) in xylem sap proteins were predicted using the SignalP 5.0 program.

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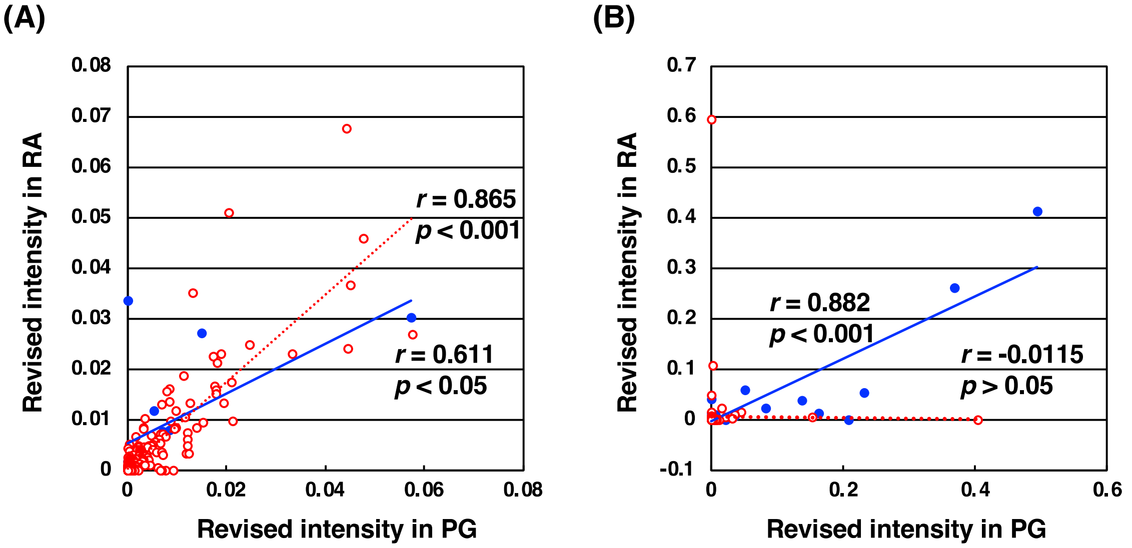


Fig. 3. Correlation between protein levels of the *Cucurbita pepo* cultivars Patty Green (PG) and Raven (RA) in roots (A) and xylem sap (B).

Closed blue and open red circles represent revised intensity of proteins with a signal peptide (SP) (root, $n = 15$; xylem sap, $n = 47$) and proteins without an SP (root, $n = 445$; xylem sap, $n = 122$) from cells, respectively. Possession of an SP was estimated using the SignalP 5.0 program. Blue solid and red dotted lines represent regression lines for proteins with and without an SP, respectively. The revised intensity of proteins with an SP in roots and xylem sap was 3- and 8-times magnified to describe the graphs with that of proteins without an SP, respectively.

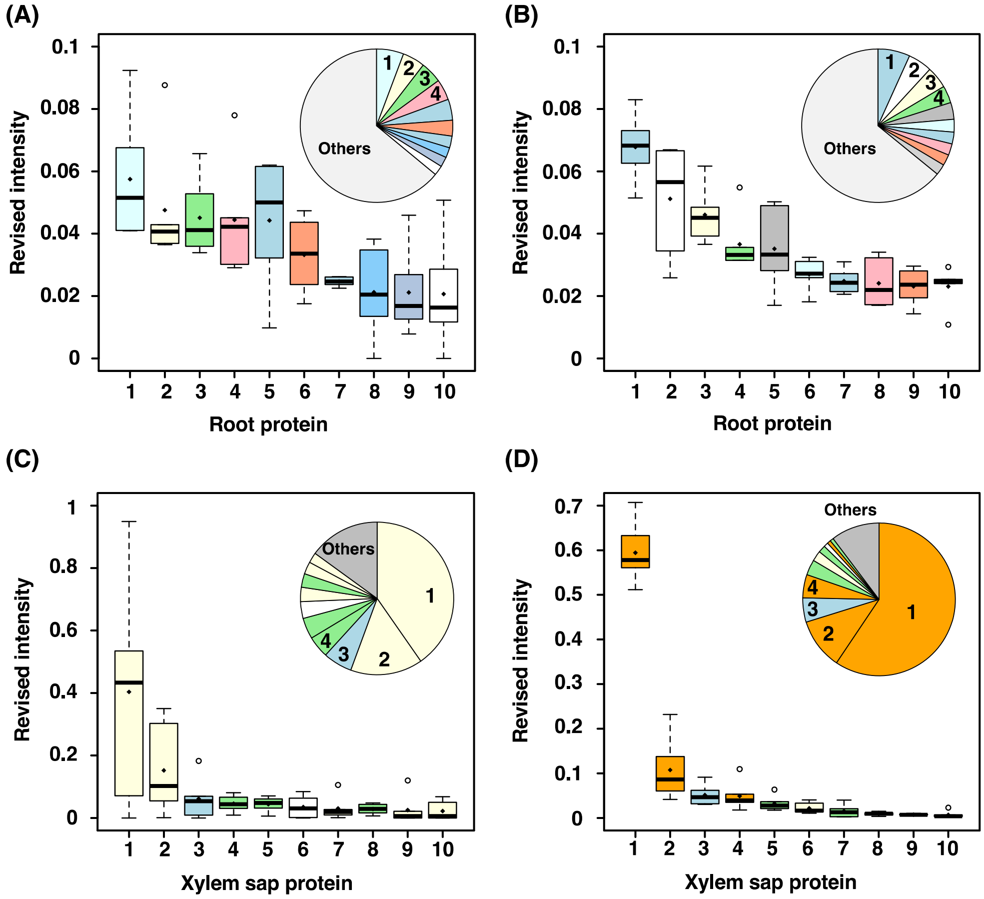


Fig. 4. Protein levels in roots (A, B) and xylem sap (C, D) of the *Cucurbita pepo* cultivars Patty Green (A, C) and Raven (B, D).

Root proteins were extracted from *C. pepo* cultivated for three weeks in pots supplemented with soil. Root extracts and xylem sap collected from the cut stem were lyophilized and subjected to liquid chromatography with mass spectrometry (*n* = 6). The main 10 proteins showing high amounts in roots and xylem sap were described. Pie charts in the graphs show the proportion of each protein in all detected proteins. The numbers below the graphs and in the pie charts show proteins described in Table 2.

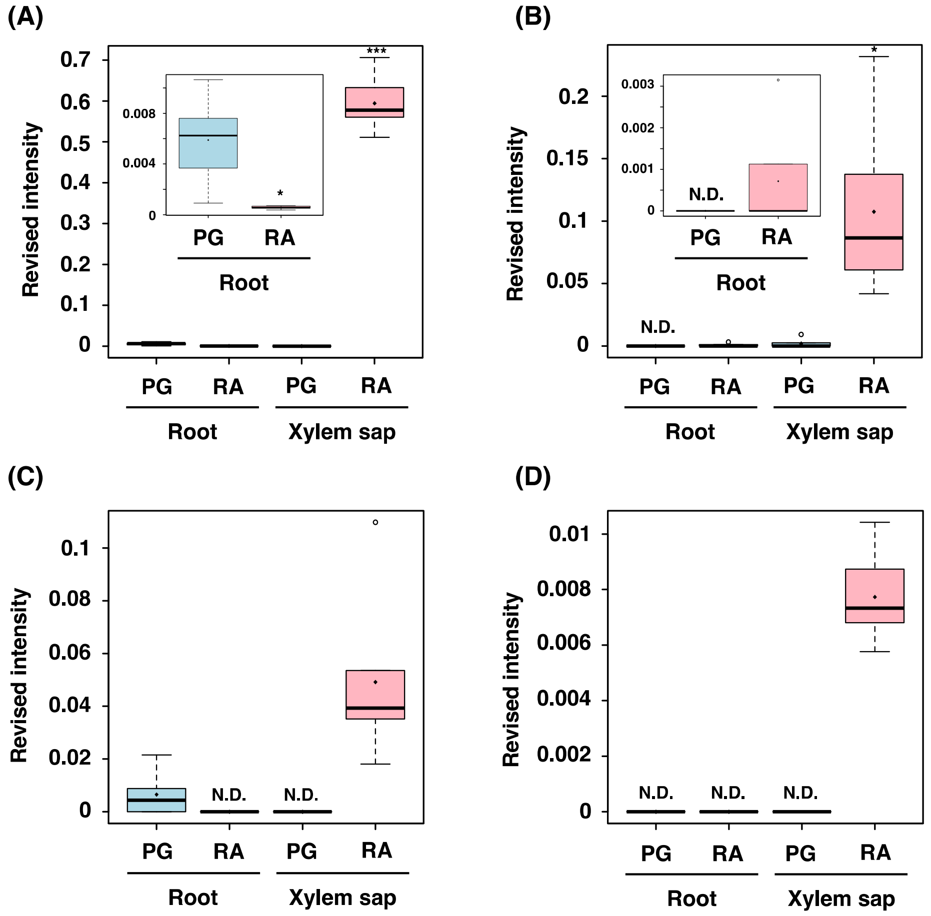


Fig. 5. Protein levels of major latex-like proteins (MLPs) in roots and xylem sap of the *Cucurbita pepo* cultivars Patty Green (PG) and Raven (RA).

Root proteins were extracted from *C. pepo* cultivated for three weeks in pots supplemented with soil. Root extracts and xylem sap collected from the cut stem were lyophilized and subjected to liquid chromatography with mass spectrometry ($n = 6$). Protein levels of MLP-GR3 (A), CmMLP2 (B), MLP-GR1 (C), and CfMLP2 (D) are described. Insets (A, B) show the magnified graphs for root proteins. Asterisks indicate significant differences compared to the values of PG (*$p < 0.05$; ***$p < 0.001$; Student's *t* test). N.D., not detected.

Table 1
Total number of root and xylem sap proteins detected in the *Cucurbita pepo* cultivars Patty Green and Raven

Cultivar	Number of root proteins			Number of xylem sap proteins		
	Signal peptide[*]		Total	Signal peptide[*]		Total
	–	+		–	+	
Patty Green	**206**	**9**	**215**	**69**	**22**	**91**
Raven	**359**	**13**	**372**	**77**	**37**	**114**

[*] Signal peptides of root and xylem sap proteins were predicted using SignalP 5.0 program.

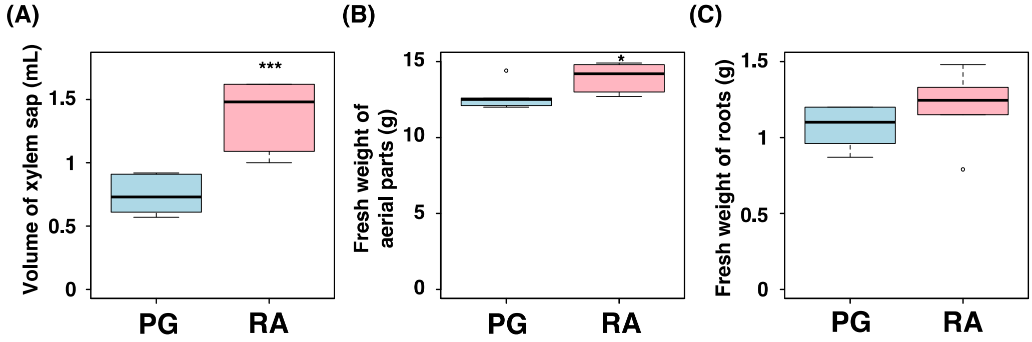
Table 2
Root and xylem sap proteins detected in the *Cucurbita pepo* cultivars Patty Green and Raven.

Root protein							
Patty Green				Raven			
	UniProt ID	Protein name	Signal peptide		UniProt ID	Protein name	Signal peptide
1*	E5GBH5	Tubulin alpha chain		1*	A0A5A7TSB1	ATP synthase subunit beta	
2	A0A5D3DQ90	Actin 7 isoform 1		2	A0A5D3D8W1	Eukaryotic translation initiation factor 5A	
3	A0A411D493	Enolase		3	A0A5D3DQ90	Actin 7 isoform 1	
4	Q8GSN4	Non-cell-autonomous heat shock cognate protein 70		4	A0A411D493	Enolase	
5	A0A5A7TSB1	ATP synthase subunit beta		5	E1B2J6	Glyceraldehyde-3-phosphate dehydrogenase	
6	V5RFY5	Plasma intrinsic protein 1-2		6	E5GBH5	Tubulin alpha chain	
7	D5I3E1	ATP synthase subunit alpha		7	D5I3E1	ATP synthase subunit alpha	
8	A0A5A7UIG9	Calmodulin-7-like		8	Q8GSN4	Non-cell-autonomous heat shock cognate protein 70	
9	Q2Q0V7	Patellin 1		9	V5RFY5	Plasma intrinsic protein 1-2	
10	A0A5D3D8W1	Eukaryotic translation initiation factor 5A		10	E5LG77	Adenosylhomocysteinase	
Xylem sap protein							
Patty Green				Raven			
	UniProt ID	Protein name	Signal peptide		UniProt ID	Protein name	Signal peptide
1*	A0A0A0KR65	Uncharacterized protein		1*	N0DK07	Major latex-like protein (MLP-GR3)	
2	Q6DKU8	Putative chymotrypsin protease inhibitor		2	A0A1V1G4S5	Major latex-like protein (CmMLP2)	
3	A0A5D3C568	Cysteine-rich repeat secretory protein 38-like	✓	3	A0A5D3C568	Cysteine-rich repeat secretory protein 38-like	✓
4	A0A0A0LWG8	Peroxidase	✓	4	N0DK19	Major latex-like protein (MLP-GR1)	
5	A0A0A0LP01	Peroxidase		5	A0A0A0LWG8	Peroxidase	✓
6	A0A5D3D8W1	Eukaryotic translation initiation factor 5A		6	A0A5D3E742	Expansin-like B1	
7	W0FW16	Superoxide dismutase [Cu-Zn]		7	A0A0A0LP01	Peroxidase	

8	**A0A5D3DV43**	**Peroxidase**	✓	**8**	**A0A5D3D8W1**	**Eukaryotic translation initiation factor 5A**	
9	**A0A1S3BGB2**	**Aspartyl protease AED3**	✓	**9**	**A0A1V1FRQ1**	**Major latex-like protein (CfMLP2)**	
10	**A0A5D3DQ90**	**Actin 7 isoform 1**		**10**	**A0A1S3C8W0**	**Peroxidase**	✓

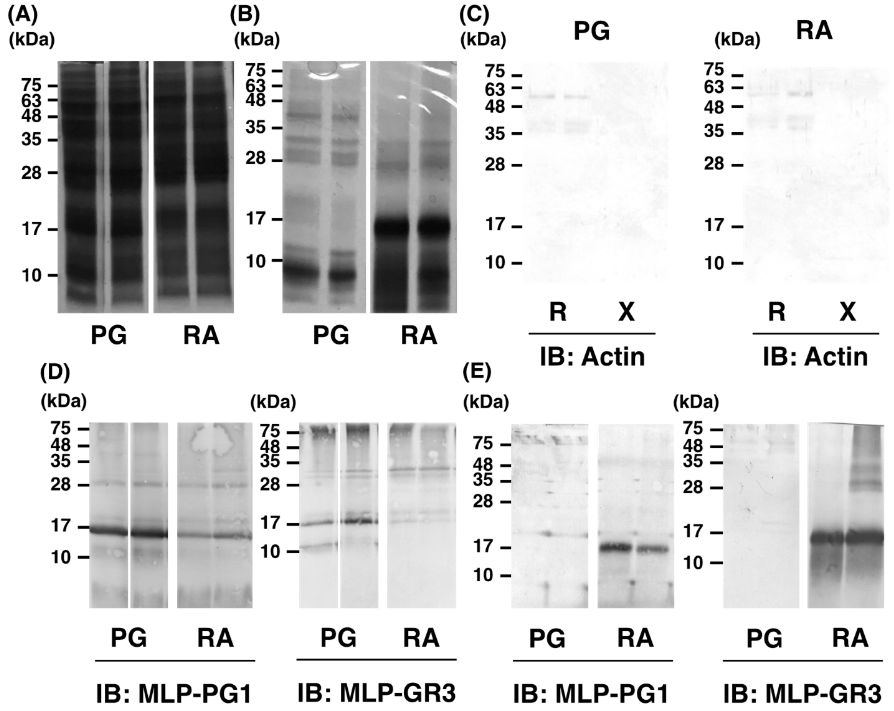
The top 10 proteins are shown.

[*] Numbers represent proteins described in Fig. 4.



Supplementary Figure 1 Volume of xylem sap (A), weight of aerial parts (B), and weight of roots (C) of the *Cucurbita pepo* cultivars, Patty Green (PG) and Raven (RA).

The *C. pepo* cultivars PG and RA were cultivated for three weeks in pots supplemented with soil ($n = 6$). Xylem sap was collected for one day, and their volumes were measured. The aerial parts were weighed after the stems were cut. Soil particles were removed from the roots using tap water and the roots were weighed. Asterisks indicate significant differences compared to the values of PG (*$p < 0.05$; ***$p < 0.001$; Student's *t* test).



Supplementary Figure 2 Root and xylem sap proteins of the *Cucurbita pepo* cultivars, Patty Green (PG) and Raven (RA).

The *C. pepo* cultivars PG and RA were cultivated for three weeks in pots supplemented with soil (*n* = 6). Representative root extracts (A, C, D) and xylem sap (B, C, E) collected from cut stems were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were detected by silver staining (A, B), and immunoblotting (IB) using anti-actin antibody (C) and anti-MLP antibodies (D, E). R, root extract; X, xylem sap