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Tissue-specific profiling of membrane proteins in the salicin sequestering juveniles of the herbivorous leaf beetle, *Chrysomela populi*

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ABSTRACT

Sequestration of plant secondary metabolites is a detoxification strategy widespread in herbivorous insects including not only storage, but also usage of these metabolites for the insects' own benefit. Larvae of the poplar leaf beetle *Chrysomela populi* sequester plant-derived salicin to produce the deterrent salicylaldehyde in specialized exocrine glands. To identify putative transporters involved in the sequestration process we investigated integral membrane proteins of several tissues from juvenile *C. populi* by using a proteomics approach. Computational analyses led to the identification of 122 transport proteins in the gut, 105 in the Malpighian tubules, 94 in the fat body and 27 in the defensive glands. Among these, primary active transporters as well as electrochemical potential-driven transporters were most abundant in all tissues, including ABC transporters (especially subfamilies B, C and G) and sugar porters as most interesting families facilitating the sequestration of plant glycosides. Whereas ABC transporters are predominantly expressed simultaneously in several tissues, sugar porters are often expressed in only one tissue, suggesting that sugar porters govern more distinct functions than members of the ABC family. The inventory of transporters presented in this study provides the base for further functional characterizations on transport processes of sequestered glycosides in insects.

1. Introduction

The terrestrial ecology on our planet is shaped by the interaction of plants and herbivores. In these interactions, secondary natural products in particular take a key position: plants produce a diverse arsenal of these substances for the chemical defense against their grazers, and insects, in turn, have to develop strategies to detoxify these compounds and open up plants as a food source (Mithöfer and Boland, 2012). In insects, sequestration is one of the most widespread strategies for overcoming the chemical defenses of the plants. It involves the uptake, transfer and concentration of phytochemicals into hemolymph and/or specialized tissues (Duffey, 1980; Nishida, 2002; Opitz and Mueller, 2009). Often it is however not only a mere detoxification, but rather the targeted enrichment of plant toxins which provide low-cost metabolites for the benefit of insects in an ecological niche.

The larvae of the specialist poplar leaf beetle, *Chrysomela populi* (Chrysomelidae, Chrysomelina), even developed nine pairs of specialized defensive glands, in which the plant-derived glucoside salicin is selectively enriched and further metabolized into the volatile deterrent salicylaldehyde (Kuhn et al., 2004; Pasteels and Rowell-Rahier, 1989). These glands are composed of several secretory cells that secrete salicin and enzymatically active proteins into a chitin-coated reservoir (Gross and Schmidtberg, 2009; Noirot and Quennedey, 1974; Quennedey, 1998). In order to prevent self-poisoning, the last metabolic transformations to the aldehyde proceed in this closed reservoir (Bodemann et al., 2012; Brueckmann et al., 2002; Michalski et al., 2008), which serves as a kind of “bioreactor”.

Recent studies addressed the physiological basis of the sequestration process in the juveniles of *C. populi* (Discher et al., 2009; Kuhn et al., 2004). The results have indicated a complex influx-efflux translocation network. Accordingly, after plant metabolites, including salicin, reach the gut lumen with the diet, uptake of these compounds is mediated non-selectively into the insect blood, the hemolymph. While salicin is channeled selectively from the hemocoel into the defensive glands, unused or excessive metabolites are excreted non-selectively by the Malpighian tubules and hind-gut organs into the feces. Fat body, a prominent larval tissue with multiple metabolic functions seems to exchange plant derived compounds with the hemocoel and to be able to convert them by e.g. glycosyltransferase activity (Ahn et al., 2012; Arrese and Soulages, 2010; Kunert et al., 2008; Panini et al., 2016). Hence, fat body may also contribute to an effective sequestration of plant compounds.

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As the polar plant glucosides have negligible ability to cross phospholipid membranes by simple diffusion, they require transport proteins to govern their spatiotemporal movement (Kuhn et al., 2004). Membrane transporters are adapted to administrate the metabolic homeostasis in an organism and are therefore key players not only in fundamental physiological processes but also in the dynamics of host plant affiliation. For which transport proteins could it be demonstrated that they are functioning in the sequestration? Although many studies postulated transport systems facilitating the sequestration of secondary metabolites from plants into insects, only one transport protein has been functionally characterized to date.

In Strauss et al. (2013), we described the role of ATP binding cassette (ABC) transporters in the sequestration by means of *Cp*MRP identified from larval *C. populi*. Localization studies revealed that *Cp*MRP was exclusively localized in the intracellular vesicular structures of the secretory cells responsible for the irreversible shuttling of pre-filtered compounds into defensive secretions. Functional assays revealed *Cp*MRP as a transporter for salicin, the naturally sequestered host-plant precursor of *C. populi*, but also for other plant derived glucosides. The transporter seems to have hence a broader substrate spectrum. Given the energy dependent intracellular shuttling, this high capacity ABC transporter provides a sink of deterrent precursors in the defensive glands, which may feedback to all other translocation processes (pacemaker function of *Cp*MRP).

Owing to the often broad substrate spectrum and ubiquitous occurrence of ABC pumps (Dassa, 2011; Holland, 2011), other members of this superfamily could play a role in the sequestration process. Indeed, this superfamily has already been in the focus of a transcriptomics approach that resulted in a catalogue of putative ABC transporters from *C. populi* (Strauss et al., 2014). However, this study was limited to primary-active transporters and did not include other gatekeepers of small molecules such as secondary-active (allow uphill transport) or passive transporters (allow transport down an electrochemical gradient) (Hediger et al., 2013). Further, though transcriptomics studies are indispensable for the understanding of gene functioning, studies of the cellular proteome donates valuable aspects about the end products of complex gene expression cascades. For this reason, we describe in the present paper the inventory of membrane transporters from juvenile *C. populi* based on a proteomics screening strategy. We have analyzed the membranous fraction of different larval tissues governing the sequestration process of salicin. After their identification we further investigated the putative integral transport proteins regarding their distribution in the different larval tissues. Our findings pave the way for further physiological studies of spatiotemporal accumulation of metabolites in specialist herbivores.

2. Experimental section

2.1. Beetle collection and culture

C. populi (L.) was collected near Dornburg, Germany (+51°00′52.00″, +11°38′17.00″), on *Populus maximowiczii* x *Populus nigra*. Adult beetles and all developmental stages were kept in the lab in a light/dark cycle of 16 h light and 8 h darkness at 18 °C ± 2 °C in light and 13 °C ± 2 °C in darkness.

2.2. Tissue dissection and sample preparation

Approximately 100–200 mg of the defensive glands, gut tissue, Malpighian tubules and fat body tissue of *C. populi* larvae of the 3rd instar were dissected and collected in differential centrifugation buffer (250 mM sucrose, 20 mM HEPES pH 7.4, 10 mM KCl, 1,5 mM $MgCl_2$, 1 mM DTT, 1 x Protease inhibitor cocktail (SERVA)) in innuSpeed Lysis tubes P (Analytic Jena AG, Jena, Germany). Cell lysis was performed using the GenoGrinder 2000 (SPEX CertiPrep Group L.L.C., Metuchen, Netherlands) for two times 40 s and once 1 min at 1200 rpm under cooling conditions (4 °C). Nuclei and cell debris were discarded through centrifugation for 10 min at 1200 × *g* and 4 °C. The supernatant was then used for subsequent differential centrifugation (Graham, 2002; Paulo et al., 2013; Völkl, 2001) (Fig. S-1). All centrifugation steps were performed at 4 °C. Mitochondrial and nuclear proteins were pelletized by centrifugation for 30 min at 10,000 × *g*. A further centrifugation for 1 h at 100,000 × *g* (Optima MAX E Ultracentrifuge, Beckman Coulter, Brea, USA) separated proteins of the membrane and membrane-associated fractions from the cytosolic proteins in the supernatant. The membrane and membrane-associated proteins were resuspended in resuspension buffer (100 mM potassium phosphate buffer pH 7.4, 20% (v/v) glycerol, 1x Protease inhibitor cocktail (SERVA)) and centrifuged again for 1 h at 100,000 × *g* (Lu et al., 2008; Speers and Wu, 2007). The resulting pellet contained the membrane proteins whereas the supernatant included the membrane-associated proteins. The supernatant of the cytosolic and membrane associated proteins were precipitated over night at −20 °C with 100% ethanol (sample:ethanol 1:2). All pellets were resuspended in SDS-loading buffer (250 mM Tris-HCl, 10% SDS, 30% Glycerol, 5% β-mercaptoethanol, 0.02% bromphenol blue) and incubated at 70 °C for 15 min. Proteins were then separated by SDS-PAGE (Any kD Precast Gel, BioRad, Hercules, CA) and stained with Roti® Blue quick (Carl ROTH GmbH & Co, Karlsruhe).

2.3. nanoUPLC-MS/MS analysis

All protein bands of the membranous fraction were cut from the gel matrix and tryptic digestion was carried out as described (Shevchenko et al., 2006). For LC-MS analysis the extracted tryptic peptides were reconstructed in 50 µL aqueous 0.1% formic acid. 1 µL of the peptide mixture was injected onto an UPLC M-class system (Waters) online coupled to a Synapt G2-si mass spectrometer equipped with a T-WAVE-IMS device (Waters). Samples were first online preconcentrated and desalted using a UPLC M-Class Symmetry C18 trap column (100 Å, 180 µm × 20 mm, 5 µm particle size) at a flow rate of 15 µL min-1 (0.1% aqueous formic acid). Next, peptides were eluted onto a ACQUITY UPLC HSS T3 analytical column (100 Å, 75 µm X 200, 1.8 µm particle size) at a flow rate of 350 nL/min with the following gradient: 1–9% B over 10 min, 9–19% B over 10 min, 19–32% B over 10 min, 32–48% B over 10 min, 48–58% over 5 min, 70–95% over 5min, isocratic at 95% B for 4 min, and a return to 1% B over 1 min (phases A and B composed of 0.1%FA and 100% acetonitrile in 0.1% FA, respectively). The analytical column was re-equilibrated for 9 min prior to the next injection. The eluted peptides were transferred into the mass spectrometer operated in V-mode with a resolving power of at least 20,000 full width at half height (FWHM). All analyses were performed in a positive ESI mode. A 100 fmol/µL human Glu-Fibrinopeptide B in 0.1% formic acid/acetonitrile (1:1 v/v) was infused at a flow rate of 1 µL min-1 through the reference sprayer every 45 s to compensate for mass shifts in MS and MS/MS fragmentation mode.

During $HDMS^E$ analysis, a wave height of 40 V was applied in IMS past of TriWave, and the traveling wave velocity was ramped from 1000 m/s to 600 m/s. Wave velocities in the trap and transfer cell were set to 311 m/s and 175 m/s and wave heights to 4 V and 4 V, respectively. For fragmentation, the collision energy was linearly ramped in the Transfer region of TriWave from 20 to 45 V. The acquisition time in each mode was 0.5 s with a 0.05-s interscan delay.

$HDMS^E$ data were collected using MassLynx v4.1 software (Waters).

2.4. Data processing

Data analysis was performed using ProteinLynx Global Server (PLGS) version 2.5.2 (Waters). The thresholds for low/high energy scan ions and peptide intensity were set at 150, 10 and 750 counts, respectively. The processed data were searched against the *C. populi* protein subdatabase constructed from in-house transcriptome-database by their translation from all six reading frames combined with a

database containing common contaminants (human keratins and trypsin). The database searching was performed at a False Discovery Rate (FDR) of 2%, following searching parameters were applied for the minimum numbers of: fragments per peptide (2), peptides per protein (2), fragments per protein (7), and maximum number of missed tryptic cleavage sites (1). Searches were restricted to tryptic peptides with a fixed carbamidomethyl modification for Cys residues. Identified hits were classified using in-house made script based on classification described for PAnalyzer software (Prieto et al., 2012). According to this algorithm proteins were divided into four groups: conclusive, non-conclusive, indistinguishable and ambiguous group. Proteins classified as conclusive hits (proteins with at least one unique peptide) and group with homologous proteins (indistinguishable hits) were considered as confident matches.

2.5. Computational analysis

Integral membrane proteins (IMPs) were identified through detection of predicted transmembrane α-helices (TMHs) and the protein grand average of hydropathicity (GRAVY) values. The analysis of the predicted TMHs was done by the TMHMM algorithm (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001) using a web-based agent. GRAVY values of the proteins were calculated as described from Kyte and Doolittle (1982) (Kyte and Doolittle, 1982) using again a web-based agent (Mbeunkui and Goshe, 2011). For further analysis we defined IMPs as proteins with at least 6 TMHs that exhibited in addition a positive GRAVY value. The resulting IMPs of all tissues were functionally characterized by BLAST analysis using the Uniprot analysis tool (http://www.uniprot.org/blast/) against Arthropoda. An E value of 10 was set as cutoff. In order to select proteins with putative transport activity, the description of functions in cellular processes of the BLAST results was used to categorize IMPs manually into transport, enzymatic activity, cell-cell interaction & intracellular trafficking, DNA replication & protein expression, other, and uncharacterized (sequences of all IMPs are provided as fasta file). Those proteins sorted into the category transport were further classified by the Transporter classification database (TCDB) (http://tcdb.org/) (Saier et al. 2014, 2016). In addition, another classification of the protein families was performed for all IMPs by using the web server tool PfamScan (https://www.ebi.ac.uk/Tools/pfa/pfamscan/) (Bateman et al., 2018). The resulting Pfam annotations were than used to countercheck the assignment of the TCDB classification (Table S2). The calculation of the transcript expression levels using RNA-seq reads was performed according to Strauss et al. (2014).

In addition the transport proteins of all tissues were used to perform the localization of the matched peptides within the whole protein sequences. For that the Boyer-Moore string search algorithm was used to determine the position of a matched peptide within the corresponding protein sequence. Afterwards, the position of each peptide was compared with the site of TMHs of the corresponding protein that was predicted by the TMHMM algorithm (http://www.cbs.dtu.dk/services/TMHMM/). Through this analysis we were able to determine if a peptide is localized within a membrane-spanning region or if the according sequence is inside or outside of the cell.

3. Results and discussion

3.1. Identification of integral membrane proteins in tissues from juvenile C. populi

In our study, we aimed to address the distribution of potential transport proteins in different larval tissues. We were particularly interested in the identification of transport proteins that could be important in the sequestration process of plant derived glycosides. At the beginning of our study, we analyzed membranous tissues fractions by using LC-MS/MS. The computational evaluation of all data sets revealed a total of 5759 unique protein sequences. These were distributed differently to the respective tissues so that 3506 possible proteins could be identified from the intestinal tissue, 2494 from Malpighian tubules, 2485 from fat body tissue and 1748 from defensive glands.

Table 1
Distribution of all identified proteins as a function of the number of transmembrane α-helices (TMHs) from different tissues of juvenile *C. populi*.

Larval tissues	Number of proteins							
	total	with n TMHs						
		0	1	2	3	4	5	≥6
Gut	3506	1649	646	379	233	162	105	332
Malpighian tubules	2494	1203	437	262	153	132	58	249
Fat body	2485	1094	446	275	187	126	84	273
Defensive glands	1748	812	359	196	131	73	54	123
All tissues	5759	2759	1000	597	383	270	166	584

Next, we predicted the secondary structure of the identified proteins by using the number of TMHs as an indicator of transport/integral membrane proteins. From this analysis we found that nearly half of the proteins in all tissues do not have TMHs (Table 1). All other proteins were characterized by 1–31 predicted TMHs. A more detailed analysis of these proteins showed that the majority possess less than 6 TMHs (82% in the gut, 81% in the Malpighian tubules, 80% in the fat body and 87% in the defensive glands). As transport proteins for the translocation of low molecular weight molecules however contain in general at least 6 TMHs (Cura and Carruthers, 2012; Dahl et al., 2004; Rees et al., 2009; Tan et al., 2008; ter Beek et al., 2014), we have sorted the proteins by the predicted TMHs which led to a reduction of the possible transport proteins with ≥6 TMHs to 332 in the gut, 249 in the Malpighian tubules, 273 in the fat body and 123 in the defensive glands.

In addition to sorting by the TMHs, the GRAVY value is also often used for the identification of membrane proteins (Lin et al., 2013; Masuda et al., 2008). Proteins that are integrated into a membrane should exhibit a positive GRAVY value, which shows hydrophobicity of a protein, whereas hydrophilic proteins are characterized by negative GRAVY values. It is known that the GRAVY value correlates with the amount of TMHs of a protein (Kyte and Doolittle, 1982), because the hydrophobic amino acids in the membrane-spanning regions result in general in positive GRAVY values of the whole protein. To validate our results we calculated the GRAVY value for all identified proteins using 6 TMHs as cut-off (Table 2). As expected, proteins with at least 6 TMHs were often characterized by higher GRAVY values, but a few of them exhibited also negative GRAVY values. Therefore, a combination of both sorting methods was used for the identification of transmembrane proteins, meaning that IMPs were defined as proteins with both at least 6 TMHs and a positive GRAVY value. Sorting by this definition led to an effective reduction of the resulting protein candidates to 247 IMPs in the gut, 197 in the Malpighian tubules, 202 in the fat body and 96 in the defensive glands.

The quality of the identification of IMPs by the membrane proteomic analysis was further characterized through analyzing the number of matched peptides of the resulting IMPs. From this analysis we found that more than 95% of the IMPs in each tissue were identified by more than one peptide (Fig. S-2). The majority of the IMPs in our study were identified by 2–20 peptides (85% in the gut, 82% in the Malpighian tubules, 83% in the fat body and 90% in the defensive glands) and there were also IMPs that were identified by more than 20 peptides (14% in the gut, 14% in the Malpighian tubules, 15% in the fat body and 6% in the defensive glands). The identification of a protein by a single peptide is in general designated as an insufficient identification method (Blackburn et al., 2010; Mbeunkui and Goshe, 2011; Schirle et al., 2003). Therefore, the identification of proteins by multiple peptides provides more serious results. The results from our analysis and also the peptide-to-protein ratios of the IMPs: 12.6 for the gut, 12.0

Table 2
Distribution of identified proteins as a function of the calculated grand average of hydropathy (GRAVY) values and the transmembrane α-helices (TMHs), using 6 TMHs as cut-off. The data were separately analyzed for different tissues of juvenile *C. populi*. Proteins with at least 6 TMHs that exhibit a positive GRAVY value were defined as integral membrane proteins (grey shaded).

GRAVY values	Number of proteins per tissue with either <6 or ≥6 TMHs							
	Gut		Malpighian tubules		Fat body		Defensive glands	
	<6	≥6	<6	≥6	<6	≥6	<6	≥6
< -0.51	122	0	60	0	69	0	50	0
-0.5 to 0	1012	85	661	52	775	71	529	27
0 to 0.5	373	237	303	185	271	195	232	90
> 0.51	18	10	18	12	3	7	2	6
Positive	391	247	321	197	274	202	234	96
Negative	1134	85	721	52	844	71	579	27

for the Malpighian tubules, 11.5 for the fat body and 9.3 for the defensive glands indicate that the measurement resulted in a well assessment of the transmembrane proteins.

3.2. Functional predictions for C. populi's IMPs

The *in silico* characterization of the IMPs was performed by BLAST analysis with subsequent classification in accordance to the biological function (Fig. 1, Table S–2). With the exception of the defensive glands we found in all tissues that transport proteins build the largest group of the identified IMPs (49% in the gut, 53% in the Malpighian tubules and 47% in the fat body). The second largest group of IMPs in the gut, the Malpighian tubules and the fat body was made up by proteins with enzymatic activities (22% in the gut, 19% in the Malpighian tubules and 26% in the fat body). In the defensive glands, the proportion of enzymes and transport proteins was about the same (32% enzymes vs. 28% transport proteins). Taken together, the functional prediction approach led to the identification of 122 transport proteins in the gut, 105 in the Malpighian tubules, 94 in the fat body and 27 in the defensive glands.

Besides the number of peptide matches to estimate the quality of the identification of IMPs mentioned above we have analyzed the localization of the matched peptides within the secondary structure of the transport proteins. From this analysis we found that ~5% of the identified peptides in all analyzed tissues are localized within the transmembrane-spanning regions; in detail most of these peptides matched a predicted TM helix partly (Fig. S-3). This could be due to the bias of tryptic digestion during sample preparation: trypsin rarely cleaves in these hydrophobic protein regions, which can lead to large peptides whose recovery from in-gel digested samples could be limited (Jonsson et al., 2001; Luque-Garcia and Neubert, 2009; Vermachova et al., 2014). Considering however the peptide-to-protein ratios stated above, IMPs can certainly be identified by using tryptic digestion. Our strategy to sort identified proteins represents an easy tool for the evaluation of sample preparation strategies for the identification of integral membrane proteins.

3.3. Transport proteins within the proteomic data

In order to further characterize the transport proteins that are possibly involved in the sequestration process of plant glucosides, we performed a classification of the transporters according to the approved system for membrane transport proteins known as the Transporter Classification (TC) system comprising pro- and eukaryotic taxa (Saier et al. 2014, 2016). From this analysis we found that ~80% of the proteins within the transporters from all tissues fall into two groups: the primary active transporters and the electrochemical potential-driven transporters (Fig. 2).

Primary active transporters couple the energy-releasing hydrolysis of ATP with the energy-requiring transport of an array of substances against their concentration gradient (Hollenstein et al., 2007). Electrochemical potential-driven transporters represent a diverse class of

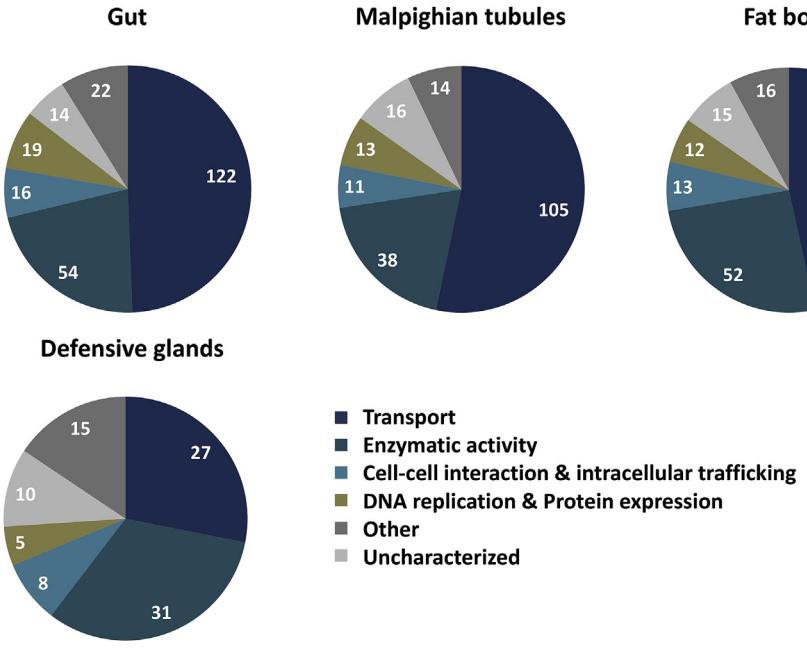

Fig. 1. Distribution of identified integral membrane proteins (IMPs) by the biological/cellular function. The data were separately analyzed for different tissues of juvenile *C. populi*. IMPs summarized in 'Other' function are involved in different cellular processes, e.g. apoptosis, autophagy, cell proliferation, protein folding, proteolysis, cell organelle organization, immune response, development or the maintenance of cell homeostasis. White numbers, number of predicted proteins.

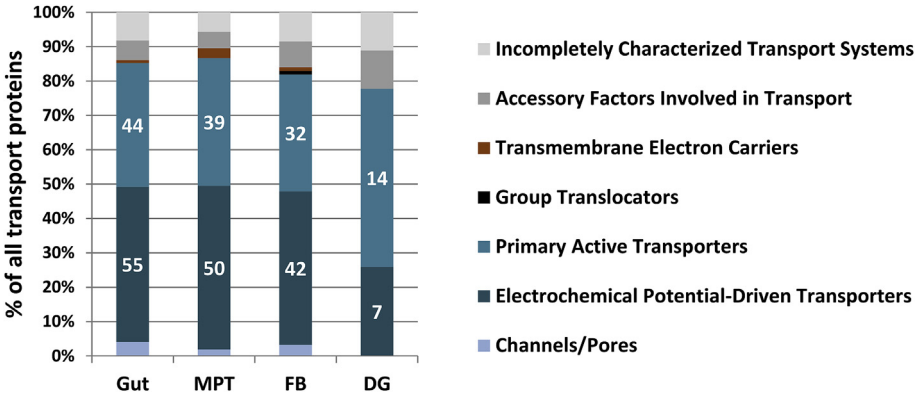

Fig. 2. Classification of transport proteins by the transporter classification database (TCDB) from the different tissues of juvenile *C. populi* analyzed in this study: gut, Malpighian tubules (MPT), fat body (FB), defensive glands (DG). White numbers, number of predicted transport proteins.

secondary transporters. As sequestration is used by herbivorous insects to overcome the chemical defense of their host plants it can be also interpreted as a kind of drug resistance mechanism (Erb and Robert, 2016; Petschenka and Agrawal, 2016). Several transporter superfamilies among these two groups have been identified to mediate translocation of xenobiotics and drugs. For example, among the secondary carriers the major facilitator superfamily (Pao et al., 1998; Saier, 2000) (MFS; 2.A.1), the resistance/nodulation/division (RND) superfamily (Tseng et al., 1999) (2.A.6), the drug/metabolite transporter (DMT) superfamily (2.A.7) (Jack et al., 2001), and the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) superfamily (Hvorup et al., 2003) (2.A.66) contain candidates known for drug resistance in pro- and eukaryotes (Yen et al., 2010).

Among the primary active transporters in particular ATP-binding cassette (ABC) transporters are of interest. These pumps translocate a wide variety of small compounds as well as ions across membranes, and are indispensable for the normal physiological and developmental processes in both pro- and eukaryotic organisms (Higgins, 1992; Holland, 2011; Rees et al., 2009). In insects they are also known to contribute to insecticide resistance (Gott et al., 2017; Sun et al., 2017) and to the distribution of plant allelochemicals across membranes (Dermauw and Van Leeuwen, 2014; Strauss et al., 2013). Therefore we decided to focus on secondary carriers as well as on primary active transporters for further classification and characterization (see Fig. 3).

3.4. Primary active transporters

The more detailed classification of primary active transporters according to the TCDB revealed two subclasses within our proteomic data: the P-P-bond-hydrolysis-driven transporters (3.A) and the oxidoreduction-driven transporters (3.D). The latter subclass is just represented by one member of the H^+ or Na^+-translocating NADH Dehydrogenase (NDH; 3.D.1) family in the gut and few members of the Proton-translocating Cytochrome Oxidase (COX; 3.D.4) superfamily in the gut, the Malpighian tubules and in the fat body. More interesting regarding sequestration is the subclass of P-P-bond-hydrolysis-driven transporters and within this subclass especially the ATP-binding Cassette (ABC) superfamily (3.A.1), which was found in a high percentage (~60%) in all tested tissues. Beside this superfamily, we identified members of ion or proton pumps (F-ATPase, 3.A.2; P-ATPase, 3.A.3) or protein translocation systems (3.A.5, 3.A.8, 3.A.16, 3.A.20) functioning in different cellular compartments and organelles.

From our further analysis we found that ABC transporters are often expressed simultaneously in several of the tested tissues (Fig. 4) and just few putative ABC pumps were specific only for one tissue. ABC transporters could therefore fulfill a universal rather than a tissue-specific function in the organs of juvenile *C. populi*.

ABC transporters from insects can be divided into 8 subfamilies (Dassa, 2011; Strauss et al., 2014). Based on BLAST results we have predicted candidates of five ABC subfamilies: one candidate of ABCA, four of ABCB, 24 of ABCC, five of ABCG and one of ABCH (Fig. S-4).

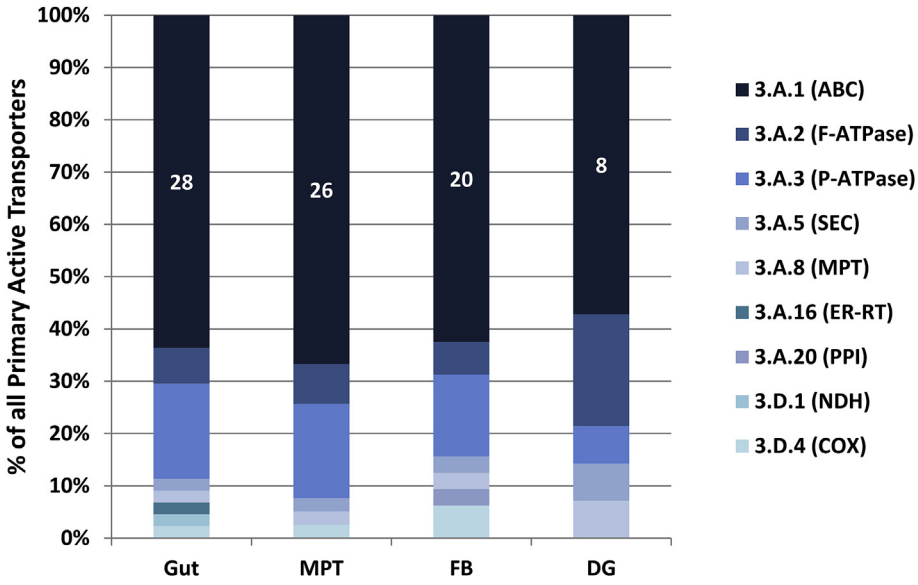

Fig. 3. Distribution of primary active transporters in different tissues of juvenile *C. populi* according to the TCDB classification: ATP-binding Cassette (ABC) Superfamily; H^+- or Na^+-translocating F-type, V-type and A-type ATPase (F-ATPase) Superfamily; P-type ATPase (P-ATPase) Superfamily; General Secretory Pathway (Sec) Family; Mitochondrial Protein Translocase (MPT) Family; Endoplasmic Reticular Retrotranslocon (ER-RT) Family; Peroxisomal Protein Importer (PPI) Family; H^+ or Na^+-translocating NADH Dehydrogenase (NDH) Family; Proton-translocating Cytochrome Oxidase (COX) Superfamily. Malpighian tubules (MPT), fat body (FB), defensive glands (DG). White numbers, number of predicted transport proteins.

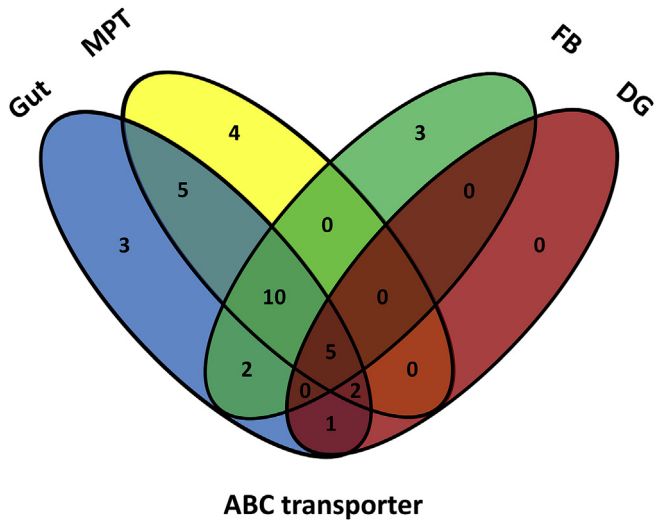

Fig. 4. Venn diagram depicting the distribution of ABC transporters among tissues of third-instar *C. populi* larvae. Malpighian tubules (MPT), fat body (FB), defensive glands (DG).

ABCD1-3 and ABCD4 are localized to peroxisomes and lysosomes (Kawaguchi and Morita, 2016), respectively, organelles not enriched in our sample preparation procedure. ABCE and F proteins lacking TMHs are localized to the cytoplasm and were therefore not found (Dean et al., 2001).

ABCA members are implicated in the transport processes of phospholipids, sterols, sphingolipids, bile salts, retinal derivatives (restricted to ABCA4) and other lipid conjugates indispensable for many biological processes (Albrecht and Viturro, 2007; Quazi and Molday, 2011). The only candidate was found in the gut tissue. The ABC candidates are dominated by putative proteins of the subfamilies B, C and G. These subfamilies gained sad fame through the mediation of multi-drug resistance described from bacteria to human cancer cells (Chen and Tiwari, 2011; Cole, 2014; Gottesman et al., 2002; Higgins, 2007; Lage, 2003; Moitra et al., 2011; Sarkadi et al., 2006). For the sequestration of plant glycosides, these subfamilies might be however the most relevant. They could contribute to the non-selective translocation from gut into the larval hemocoel; or, depending on the localization of the proteins in the intestinal cells, they may also take part in the detoxification of plant metabolites or xenobiotics by back-exporting them into the gut lumen. In the fat body ABCC, B and G candidates may govern the exchange of plant compounds for metabolic conversion, e.g. of aglycons into their glycosylated forms. In the Malpighian tubules they may play a role in the previously observed non-selective phytochemical extrusion by the excretion system (Discher et al., 2009). In the defensive glands, only *Cp*ABC35 (*Cp*MRP) has been characterized to date and was shown to mediate the transport of salicin within the defensive glands for the production of defensive secretions (Strauss et al., 2013). The remaining identified ABC pumps in the glands could contribute to the selectivity in the membrane of the hemolymph side of the glandular cells by extruding unused plant-derived compounds from these cells. ABCH has been suggested to contribute to the assembly of a lipid-based barrier of the cuticle to protect the insects against the uncontrolled loss of water (desiccation) and possibly also the penetration of xenobiotics (Yu et al., 2017).

In addition we complemented the results of the proteomic analysis with transcriptomic data available for the predicted ABC transporters of *C. populi* according to Strauss et al. (2014) (Fig. S-4). In 26 of 35 identified ABC transporters, we found no exact match of the proteome to the RNA seq data. In most cases, protein could be detected, which, however, correlated with only very low levels of mRNA (>0–25 normalized read counts). The reason might be that the proteins have such a half-life that they accumulate in the membranes independently of transcriptional activity. In contrast, if the mRNA levels were ≥25 normalized read counts, we also found corresponding hits in the protein data sets, with the exception of *Cp*ABC27, 50, 55 and 71. These exceptions may be explained by differences in the proteins' half-lives or by the efficiency of the protein extraction procedure, which may be tissue/cell type-dependent. Since we do not have quantitative proteomic records, we are however not able to correlate to the mRNA level directly. Nonetheless, our results demonstrate that proteomics and transcriptomics complement each other and an approach that combines both techniques can provide a solid base to build on further experiments. From the transcriptomic data we found two ABC transporters (*Cp*ABC35 and *Cp*ABC64) that show specific expression within the defensive glands. Whereas the function of *Cp*ABC35 (*Cp*MRP) has already been demonstrated (Strauss et al., 2013), there was no information about *Cp*ABC64 (ABCH subfamily). Our silencing experiments of this transporter by RNAi resulted in a down regulation of its expression accompanied by a significant higher larval mortality particularly during molting compared to a control group (Fig. S-5). Similar results were found in other insect species, suggesting that ABCH transporters might be crucial for the molting process by regulating lipid composition (Yu et al., 2017; Broehan et al., 2013; Guo et al., 2015). The observed phenotype is hence not due to a specific function of the putative ABCH in the defensive glands, but presumably to a general function in the larval integument.

3.5. Electrochemical potential-driven transporters

As mentioned above, there are also superfamilies and families of electrochemical potential-driven transporters that are known to be involved in drug resistance processes. For this reason we performed a more detailed classification of this transporter class according to the TCDB (Perland and Fredriksson, 2017; Saier et al., 2016). Within the electrochemical potential-driven transporters all identified transport proteins belong to the subclass of porters (2.A), which are known to mediate uniport, symport and antiport. Currently this subclass contains 127 different superfamilies and families of transport proteins, of which 26 could be identified in our proteomic analysis (Table 3).

In particular candidates of the Major Facilitator Superfamily (MFS, 2.A.1) were most frequently represented among all tested tissues (23 identified in the gut (42%), 34 in the Malpighian tubules (68%), 21 in the fat body (50%) and 5 in the defensive glands (71%)) and belong to different MF families. The MFS is an ancient and omnipresent superfamily, found usually in high copy numbers in genomes (Quistgaard et al., 2016; Reddy et al., 2012). The MFS comprises im- and exporters that play a crucial role in a multitude of physiological processes by moving an extraordinarily broad spectrum of substrates across plasma and intracellular membranes (Goswitz and Brooker, 1995; Quistgaard et al., 2016; Yan, 2015).

The Resistance-Nodulation-Cell Division (RND) Superfamily (2.A.6) is represented by five members and all of these RND transport proteins belong to the Eukaryotic (Putative) Sterol Transporter (EST) Family (Prinz, 2007). In humans the EST members, Niemann-Pick C1 (NPC1) and C2, form a sterol transport system that localizes at the late endosome or lysosome, where it binds free cholesterol and mediates its export to diverse cellular compartments (Castellano et al., 2017; Davies et al., 2000; Kwon et al., 2009). NPC1 is also known to translocate small apolar molecules including acriflavine and oleic acid (Davies et al., 2000). In insects, however, a function has never been shown and to what extent they are involved in the translocation of sterols and other apolar compounds remains to be elucidated. Two members of the Drug/Metabolite Transporter (DMT) Superfamily (2.A.7) were detected only in the fat body tissue. These two candidates possess similarity to carriers involved in the translocation of nucleotide sugars and may not be relevant for the sequestration of phytochemicals (Jack et al., 2001). Besides the typical multi-drug resistance transporter families, it can be

Table 3
Distribution of putative electrochemical-driven transporters (subclass porters) in the different tissues of juvenile *C. populi*. MPT, Malpighian tubules; FB, fat body; DG, defensive glands; minus, not detectable.

TCDB classification		Number of proteins per tissue			
		Gut	MPT	FB	DG
2.A.1	The Major Facilitator Superfamily (MFS)	23	34	21	5
2.A.3	The Amino Acid-Polyamine-Organocation (APC) Superfamily	4	–	–	–
2.A.4	The Transient Receptor Potential Ca^{2+} Channel (TRP-CC) Family	–	–	1	–
2.A.6	The Resistance-Nodulation-Cell Division (RND) Superfamily	1	4	4	–
2.A.7	The Drug/Metabolite Transporter (DMT) Superfamily	–	–	2	–
2.A.17	The Proton-dependent Oligopeptide Transporter (POT/PTR) Family	2	1	–	–
2.A.18	The Amino Acid/Auxin Permease (AAAP) Family	2	–	3	–
2.A.21	The Solute:Sodium Symporter (SSS) Family	–	2	3	–
2.A.22	The Neurotransmitter:Sodium Symporter (NSS) Family	–	1	–	–
2.A.23	The Dicarboxylate/Amino Acid:Cation (Na^+ or H^+) Symporter (DAACS) Family	–	–	1	–
2.A.29	The Mitochondrial Carrier (MC) Family	3	–	3	1
2.A.30	The Cation-Chloride Cotransporter (CCC) Family	2	1	–	–
2.A.31	The Anion Exchanger (AE) Family	3	–	–	–
2.A.36	The Monovalent Cation:Proton Antiporter-1 (CPA1) Family	3	–	–	–
2.A.41	The Concentrative Nucleoside Transporter (CNT) Family	1	–	–	–
2.A.43	The Lysosomal Cystine Transporter (LCT) Family	1	–	–	–
2.A.49	The Chloride Carrier/Channel (ClC) Family	1	1	–	–
2.A.53	The Sulfate Permease (SulP) Family	–	1	–	–
2.A.55	The Metal Ion (Mn^{2+}-iron) Transporter (Nramp) Family	–	–	2	–
2.A.57	The Equilibrative Nucleoside Transporter (ENT) Family	5	–	–	–
2.A.60	The Organo Anion Transporter (OAT) Family	–	1	–	–
2.A.82	The Organic Solute Transporter (OST) Family	–	–	1	–
2.A.92	The Choline Transporter-like (CTL) Family	3	1	1	–
2.A.94	The Phosphate Permease (Pho1) Family	1	1	–	–
2.A.106	Ca^{2+}:H^+Antiporter-2 (CaCA2) Family	–	2	–	–
2.A.123	The Sweet; PQ-loop; Saliva; MtN3 (Sweet) Family	–	–	–	1

anticipated that also the other families contain candidates for the shuttling of phytochemicals. For example, a member of the Organo Anion Transporter (OAT) family (2.A.60) has recently been linked with the protection of insects against dietary cardenolides (Groen et al., 2017). For the human homologues from hepatocytes digitalis-derived compounds have been assigned as substrates (Gozalpour et al., 2014). We have found one putative OAT candidate in the Malpighian tubules, where it needs to be shown if this protein might play a role in the detoxification of xenobiotics or plant metabolites. Regarding sequestration members of the Solute:Sodium Symporters (SSS) family (2.A.21) and the Sweet; PQ-loop; Saliva; MtN3 (Sweet) family (2.A.123) are also of interest. Although in insects SSS have been associated so far with survival under salt stress (Stergiopoulos et al., 2009), the substrate range of human homologues encompasses various compounds including monosaccharides and flavonoid glycosides (Walgren et al., 2000; Wright et al., 2017). We have identified members in fat body and Malpighian tubules. The Sweet family is currently under extensive investigation (Deng and Yan, 2016; Szablewski, 2017a). In vascular plants transporters of this family are involved in a wide variety of physiological processes by mediating sucrose and hexose transport (Eom et al., 2015; Julius et al., 2017; Li et al., 2018; Zhang and Turgeon, 2018); in Metazoa, however, the functional characterization of these proteins is still in its infancies and it is unclear whether glycosides are among the substrates (Chen et al., 2010; Feng and Frommer, 2015). From *D. melanogaster* a Sweet gene has though been reported to be expressed in embryonic salivary glands, but it has not been functionally studied to date (Artero et al., 1998). We have found one Sweet member in the defensive glands. Hence, from the current state of knowledge, several secondary transporter families contain promising carriers that could come into consideration for the sequestration process of phytochemicals by juvenile *C. populi*. Based on the high numbers found in all larval tissues, the MFS candidates were subject for further analysis.

3.6. Candidates of the major facilitator superfamily

Within the MF superfamily, we found that members of the Sugar porter (SP) family (2.A.1.1, containing also human glucose transporters (GLUTs) of the Solute Carrier (SLC) 2 family (Mueckler and Thorens, 2013)) are present in large proportion especially in the Malpighian tubules (73%) and in the defensive glands (100%) (Fig. 5). They were also detected in the gut and in the fat body, but just in lower proportions, 35% and 24%., respectively. Members of the SP family act in pro- and eukaryotes as uni- or symporters in the translocation of among others hexoses, pentoses, disaccharides, amino sugars, sugar alcohols, dehydroascorbic acid, or uric acid (Chen et al., 2015; Deng and Yan, 2016; Mueckler and Thorens, 2013). Sugar porters differ in transport capacity, substrate affinity and specificity, and tissue distribution (Cura and Carruthers, 2012; Manolescu et al., 2007). In humans members of the SP family (GLUTs) have received wide attention due to their links with various metabolic disorders, including diabetes, obesity, and cancer (Barron et al., 2016; Koekkoek et al., 2017; Szablewski, 2017b; Tanasova et al., 2018; Tanasova and Fedie, 2017). In insects, recent studies of genomes and transcriptomes revealed that SP genes exist as large multigene families within the genomes (Ge et al., 2015; Govindaraj et al., 2016; Kikuta et al., 2015; Nicholson et al., 2015; Pimentel et al., 2018; Price and Gatehouse, 2014; Stock et al., 2013; Yang et al., 2017). In comparison with the large number of genes, only a few insect SP candidates have been functionally characterized. For example, the *Nilaparvata lugens* hexose transporters NlST1 (NlHT1) and NlST16 have been demonstrated to be glucose transporters with different affinities (Kikuta et al., 2015; Price et al., 2007). Another SP transporter from the brown planthopper, Nlst6, was characterized as a facilitative glucose/fructose transporter (Kikuta et al., 2010). The aphid, *Acyrthosiphon pisum*, expresses at least two sugar transporters, Ap-ST3 and Ap-ST4, both of which transport glucose and fructose (Price and Gatehouse, 2014; Price et al., 2010). The trehalose transporter TRET1 orthologs of *Anopheles gambiae* (Km: 45.74 ± 3.58 mM) and *Bombyx mori* (71.58 ± 6.45 mM) showed low trehalose affinity, whereas those of *Apis mellifera* (9.42 ± 2.37 mM) and *Drosophila*

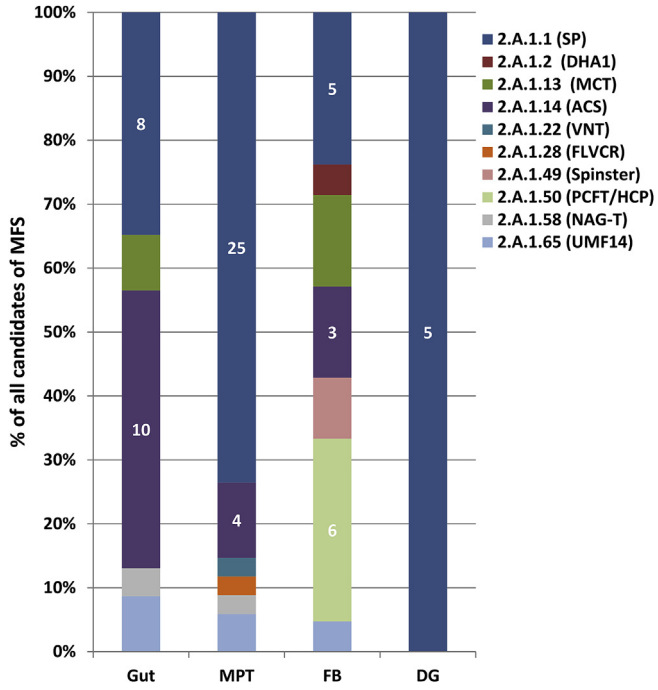

Fig. 5. Distribution of candidates of the MF superfamily in different tissues of juvenile *C. populi* according to the TCDB classification: The Sugar Porter (SP) Family; The Drug:H^+ Antiporter-1 (12 Spanner) (DHA1) Family; The Monocarboxylate Transporter (MCT) Family; The Anion:Cation Symporter (ACS) Family; The Vesicular Neurotransmitter Transporter (VNT) Family; The Feline Leukemia Virus Subgroup C Receptor (FLVCR) Family; The Endosomal Spinster (Spinster) Family; The Proton Coupled Folate Transporter/Heme Carrier Protein (PCFT/HCP) Family; The N-Acetylglucosamine Transporter (NAG-T) Family; The Unidentified Major Facilitator-14 (UMF14) Family. Malpighian tubules (MPT), fat body (FB), defensive glands (DG). White numbers, number of predicted transport proteins.

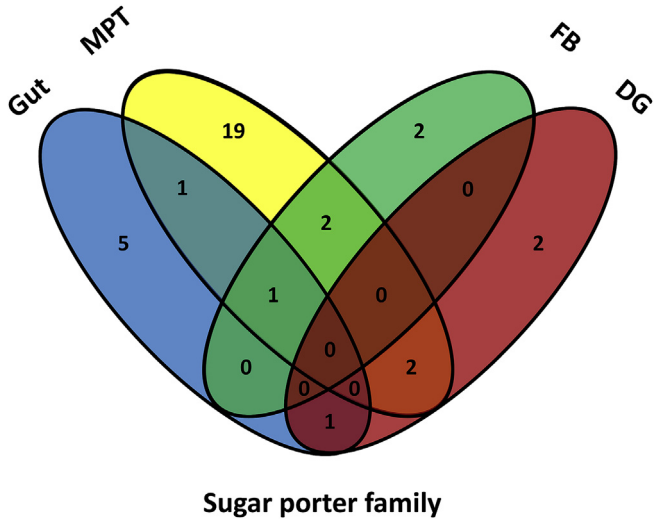

Fig. 6. Venn diagram depicting the distribution of sugar porters among tissues of third-instar *C. populi* larvae. Malpighian tubules (MPT), fat body (FB), defensive glands (DG).

melanogaster (10.94 ± 7.70 mM) showed high affinity (Kanamori et al., 2010).

A comprehensive phylogenetic analysis by using a transcriptome library from a leaf beetle species related to *C. populi*, the mustard leaf beetle *Phaedon cochleariae*, revealed 68 putative members of the SP family (Stock et al., 2013). RNA-seq experiments of sugar porter-silenced *P. cochleariae* larvae showed the down-regulation of the silenced porter but concurrently the up-regulation of other SP members suggesting an adaptive system to maintain sugar homeostasis in the defensive glands. However, the extent to which sugar transporters contribute to the regulation of sugar homeostasis in the insect's body and how their substrate spectrum looks in detail awaits further experimental tests. In view of the large number of genes, it is conceivable that glycosides could also be accepted as substrates by sugar porters, which represent hence candidates implicated in the sequestration process. In a pilot experiment, we have silenced, for example, *Cp*SP-like15 in juvenile beetles by using RNAi and found a phenotype that was not able to produce defensive secretions anymore (data not shown). Further investigations regarding function of this putative transporter are currently being carried out.

Additionally, we complemented the results of the proteomic analysis with RNA-seq data available for the predicted SP members of juvenile *C. populi* (Fig. S-6). Proteome and RNA-seq data of the SP family were in general more consistent than the ABC transporter data sets. In 23 out of 35 cases we found an exact match between the proteomic and transcriptome data (mRNA level ≥25 normalized read counts) of the analyzed sugar porters. For four SP candidates, we were able to determine an mRNA level, but no protein, and in eight cases we had a protein hit but found no corresponding level of transcripts. The striking difference in our analysis results between ABC transporters and SP members could be due to the different half-lives of these transporters in the membranes (Hou et al., 2009; Katayama et al., 2015).

From our proteomic analysis we found that sugar porters, in contrast to ABC transporters, are predominantly expressed in only one tissue: 5 in the gut, 19 in the Malpighian tubules, 2 in the fat body and 2 in the defensive glands (Fig. 6). Their organ-specific expression is already described by other studies, e.g. from humans (Mueckler and Thorens, 2013; Yan, 2017), indicating a distinct rather than a general function of sugar porters in an organism. At the time of our analysis, however, we cannot rule out that some of the identified transporters operate also as translocator-sensors or as sensors of glycosides (Diez-Sampedro et al., 2003; Leturque et al., 2009; Thorens, 2015).

In the midgut not only sugar porters were prominent, but also members of the Anion:Cation Symporter (ACS) family (2.A.1.14) found in pro- and eukaryotes. ACS members translocate phosphate and organic anions (Reimer, 2013). In insects these transporters have been investigated mainly in the context of neuronal activity (Rossano et al., 2017) and a biological significance in sequestration cannot be proposed currently. In the fat body tissue several members of the Proton Coupled Folate Transporter/Heme Carrier Protein (PCFT/HCP) family (2.A.1.50) have been identified. Although they most likely do not facilitate the shuttling of secondary metabolites in the beetle larvae, a crucial role in the innate immune response recently reported from *D. melanogaster* should be noted (Paik et al., 2017).

Other MFS transporters that could be taken into account for the sequestration of plant metabolites would be members of the Drug:H^+ Antiporter DHA1 (2.A.1.2) and DHA2 (2.A.1.3) family found in pro- and eukaryotes (Abdel-Motaal et al., 2018; Gbelska et al., 2006). DHA1 and 2 members mediate predominantly multidrug resistance in bacteria and fungi. From Metazoa (including insects) only DHA1 members are reported. In these organisms they contribute to the accumulation of cationic neurotransmitters in the neuronal system (Martin and Krantz, 2014; Omote et al., 2016). We have identified in our membrane protein analysis one DHA1 member in the fat body of *C. populi* larvae, but we yet cannot assign any function to it.

Taken together, we assume that ABC pumps and members of the MFS are promising candidates for governing the sequestration of plant-derived compounds in insects. With our proteomics approach, complemented by RNA-seq quantitative analyses, we provide an inventory of transporters to which further experimental validation of protein function can be built on.

Author contributions

LS, AB, WBo designed research; LS, NW performed research; LS, NW, DW analyzed data; LS, AB, WBo wrote the paper.

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Appendix A. Supplementary data

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