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## Phloem sap intricacy and interplay with aphid feeding

*Complexité de la sève phloémienne et impact sur l'alimentation des pucerons*Sylvie Dinant<sup>a,\*</sup>, Jean-Louis Bonnemain<sup>b</sup>, Christine Girousse<sup>c</sup>, Julia Kehr<sup>d</sup><sup>a</sup> UMR 1318 INRA-AgroParisTech, institut Jean-Pierre-Bourgin, bâtiment 2, route de Saint-Cyr, 78000 Versailles, France<sup>b</sup> CNRS, service de physiologie moléculaire du transport des sucres chez les végétaux (FRE 3091), université de Poitiers, 40, avenue du recteur Pineau, 86022 Poitiers cedex, France<sup>c</sup> INRA, UMR 1095, Genetics, Diversity and Ecophysiology of Cereals, 234, avenue du Brézat, 63100 Clermont-Ferrand, France<sup>d</sup> Centro de Biotecnología y Genómica de Plantas (CBGP), Campus de Montegancedo, 28223 Pozuelo de Alarcón, Madrid, Spain

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## ABSTRACT

*Aphididae* feed upon the plant sieve elements (SE), where they ingest sugars, nitrogen compounds and other nutrients. For ingestion, aphid stylets penetrate SE, and because of the high hydrostatic pressure in SE, phloem sap exudes out into the stylets. Severing stylets to sample phloem exudates (i.e. stylectomy) has been used extensively for the study of phloem contents. Alternative sampling techniques are spontaneous exudation upon wounding that only works in a few plant species, and the popular EDTA-facilitated exudation technique. These approaches have allowed fundamental advances on the understanding of phloem sap composition and sieve tube physiology, which are surveyed in this review. A more complete picture of metabolites, ions, proteins and RNAs present in phloem sap is now available, which has provided large evidence for the phloem role as a signalling network in addition to its primary role in partitioning of photo-assimilates. Thus, phloem sap sampling methods can have remarkable applications to analyse plant nutrition, physiology and defence responses. Since aphid behaviour is suspected to be affected by phloem sap quality, attempts to manipulate phloem sap content were recently undertaken based on deregulation in mutant plants of genes controlling amino acid or sugar content of phloem sap. This opens up new strategies to control aphid settlement on a plant host.

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## R É S U M É

Les *Aphididae* s'alimentent directement dans les éléments criblés du phloème, dans lesquels ils prélèvent la sève phloémienne riche en sucres, composés azotés et autres nutriments essentiels à leur développement et reproduction. Pendant la phase d'ingestion, leurs pièces buccales (*stylets*) pénètrent dans les éléments criblés, et en réponse à la pression hydrostatique élevée dans les tubes criblés, la sève phloémienne remonte dans les stylets. Le prélèvement de sève phloémienne à partir des stylets sectionnés (stylectomie) a été largement utilisé pour l'étude de sa composition. D'autres techniques de prélèvements, telles que l'exsudation spontanée par blessure, qui ne fonctionne que pour quelques espèces de végétaux, et la technique très populaire d'exsudation facilitée à l'EDTA, ont également été employées. Toutes ces méthodes ont permis des avancées

\* Corresponding author.

E-mail addresses: sylvie.dinant@versailles.inra.fr (S. Dinant), jl.bonnemain@voila.fr (J.-L. Bonnemain), girousse@clermont.inra.fr (C. Girousse), julia.kehr@upm.es (J. Kehr).

importantes pour l'analyse de la composition de la sève et la physiologie des tubes criblés. Un tableau assez complet des métabolites, des ions, des hormones, des protéines et des ARN présents dans la sève du phloème est désormais disponible. Ces avancées ont largement étayé le rôle du phloème comme réseau de signalisation, en plus de son rôle central dans la répartition des photo-assimilats. Ces méthodes d'échantillonnage de la sève élaborée ont aussi des applications potentielles remarquables pour l'analyse de la nutrition, la physiologie et les réactions de défense des plantes. Plusieurs études ayant suggéré que la qualité de la sève phloémienne pouvait affecter le comportement alimentaire des pucerons, des tentatives de manipulation de la composition de la sève phloémienne ont été engagées, basées sur la dérégulation des gènes contrôlant la teneur en acides aminés ou en sucres de la sève, dans les plantes transgéniques. De telles approches ouvrent des stratégies nouvelles pour limiter le développement des pucerons sur leur plante hôte et ainsi leur nuisibilité.

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## 1. Introduction

The phloem is a central actor in plant nutrition and development, allocating nutrients, water, energy and signals from source to sink organs. The conducting tubes, the sieve elements (SE), transport sugars, amino-acids, as well as a range of macromolecules, such as proteins or RNAs, acting potentially in long distance signalling in response to developmental or stress triggers [1–3]. SE are enucleated cells that mostly rely for the delivery of metabolites and macromolecules present in SE on the biosynthesis machinery of the adjacent companion cells (CC) or transfer cells. SE have developed sealing mechanisms, using both callose deposition and P-proteins. While callose deposition can remain for some time, in legumes, changes in P-protein conformation can reversibly and transiently interrupt mass flow and prevent sap leakage following injury [4]. The collection, storage and delivery of metabolites, such as sugars, is achieved through symplasmic and apoplasmic steps taking place in the whole phloem tissue, including CC and phloem parenchyma cells. Additionally, specialised storage cells, such as M-cells (that store myrosinases in crucifers [5]), or in some species laticifers or secretory ducts, often associated with vascular bundles [6], can also participate in the delivery of the secondary metabolites found in phloem sap. The sap is transported from source (i.e. photosynthetically active) to sink organs by mass flow, which is driven by a difference in hydrostatic pressure between source and sink [7]. Depending on plant species, there are three recognized strategies of sugar loading: apoplasmic and symplasmic with or without polymer trapping [8]. Apoplasmic loading is associated to the activity of membrane transporters whereas symplasmic loading relies on transport across plasmodesmata.

A basic characteristic of long distance translocation via the phloem is that not all sinks are supplied by the same source leaves. Source leaves can be connected in an independent succession known as orthostichy. The architecture of orthostichy is based on the phyllotaxy of the plant. For example, *Arabidopsis* has a 3/5-spiral leaf phyllotaxy, which means that every fifth leaf is vertically aligned after three spirals around the plant and leaves in an orthostichy are arranged approximately on a vertical line [9]. The pathway of systemic signalling in response to

various stresses, such as wounding, herbivory, or pathogen attack, follows this pattern along orthostichies [1], as evidenced by the demonstration that the translocation of systemic signal molecules, such as salicylic acid, essentially moves with assimilate movement along an orthostichy [10]. Another well-established example of this phenomenon is the pattern of systemic colonization during viral infection, which also follows phloem connections and therefore orthostichy [11]. These observations have confirmed a major role of phloem in long distance signalling, although other signalling pathways acting over long distances have been discovered, especially in plant defense responses [1,12], which recruit airborne signals, such as volatile organic compounds (VOC), and signals transported between phloem and xylem [10,13].

## 2. Stylectomy and comparison with other sampling methods

Over the last decades, the composition of the phloem sap has been studied in detail. Three main methods are available for sampling this fluid. Simple incisions into the phloem of certain trees (e.g. *Fraxinus americana*, *Robinia pseudoacacia*) and certain herbaceous plants (e.g. *Yucca filamentosa*, *Cucurbita maxima* and other members of the *Cucurbita* genus, *Ricinus communis* or *Brassica napus*) provide relatively large volumes of phloem sap that can be collected for analysis [14,15]. For example, *Yucca* and palms can provide several liters of phloem sap [3] and *Cucurbita maxima* can provide milliliters of sap. However, the method based on spontaneous exudation does not work in most plant species, because of a rapid arrest of bleeding, probably due to callose deposits and P-protein accumulation in the sieve plate pores that block the flow. In such a case, EDTA-facilitated exudation, which was initially described by King and Zeevaart, can be employed [16]. This method is based on the use of the calcium-chelator ethylene diamine tetraacetic acid (EDTA) in the collecting solution where the distal tips of cut petioles or stems are plunged. The addition of EDTA to the collecting solution is thought to prevent callose synthesis and P-protein plugging, processes that are normally induced by  $\text{Ca}^{2+}$  decompartmentalization in response to phloem injuries. The method of EDTA-facilitated exudation is quick and easy but does not allow the measurement of

component concentrations. It may induce several artefacts in the composition of the phloem sap, as mentioned below, although it appeared as useful for some applications [17].

Stylectomy is arguably the most elegant method, originally outlined by Kennedy and Mittler [18]. It requires the help of aphids or other phloem feeding insects, such as the brown plant hopper *Nilaparvata lugens*. It is based on cutting insect stylets that are inserted in a sieve tube while the herbivore is feeding. In the pioneer experiments, the stylets were severed by a razor-blade or microscissors. However, this technique is only feasible for big aphids settled on woody species such as the willow aphid [19]. Subsequently, laser microcautery and radiofrequency microcautery have been used to cut aphid stylets of various sizes, since the first experiments initiated by Barlow and McCully in 1972 and Downing and Unwin in 1977 [20,21].

The stylectomy method, which is less invasive than EDTA-facilitated exudation, allows collection of relatively pure phloem sap but this does not guarantee that the phloem sap content, especially the amino acid content, is not somewhat manipulated by aphid signals, which are produced in a process called facilitation, or by the initial wounding injury [22]. These potential secondary effects can be minimised or abolished by cutting stylets shortly after the aphids started feeding. The comparative analyses of phloem sap collected either by stylectomy or EDTA-facilitated exudation from the same plant species highlighted the artefacts induced by the latter method: changes in sugar and amino acids composition [23] and presence of large amounts of contaminative proteins [17]. However, changes in amino acid content may be low if the duration of EDTA-facilitated exudation does not exceed a few hours [24]. Reducing the duration of sap collection using EDTA also minimises the effect of ageing on amino acid metabolism and membrane activities. Moreover, spontaneous and abundant phloem sap exudation found in *Cucurbita* spp. and other species makes the problem of contamination from various cell types caused by cutting less critical. Stylectomy can be a time-consuming and exasperating technique. Stylet exudation is often unpredictable, both in terms of success rate and duration of sap collection, especially when aphids are settled on leguminous species [19,23,25]. The duration of stylet exudation from herbaceous species sieve tubes varies from a few minutes to a few hours and the volume of phloem sap from a few nanoliters to about 100 nl (with an exudation rate of 0.5–2 nl per minute).

Fortunately, recent advances in solute and macromolecule analyses have boosted the prospects of this technique. For instance, amino acid analysis by micellar electrokinetic chromatography with laser-induced fluorescence detection allowed the determination of the profile of amino acids in nanolitre volume samples (except methionine for technical reasons) [26]. Nano-flow liquid chromatography linked to a mass spectrometry enabled the detection of sap polypeptides that were undetectable by gel staining [27]. These methods allow the analysis of phloem sap exudates obtained by stylectomy for plant species yielding limited volumes (nanolitre range) of phloem sap. Finally, sophistication of the microcautery

technique itself and “employment” of aphid “battalions” led to the collection of phloem sap at an “industrial” scale (10  $\mu$ l within a few hours) and this contributed to improve transcript and protein analyses [28], although the increase in aphid workforce might cause potential problems with both facilitation and saliva injection.

### 3. Physicochemical properties of phloem sap

Analyses of phloem sap obtained either by incisions into the phloem of bleeding plants such as *Curcubita* spp. or *R. communis* or collected from severed aphid stylets (Table 1) indicate that sugars, potassium and amino acids are the main osmotic components [29,30]. The values of osmotic pressure of sieve tube sap vary from about 0.8 MPa in *Opuntia ficus-indica* or *Arabidopsis* [31–33] to about 2.0 or 2.5 MPa in species such as *R. communis*, *Hordeum vulgare*, *Zea mays* and *Sonchus oleraceus* [34–37]. For comparison, turgor pressure values measured in individual sieve tubes using severed aphid stylets [20] range from about 0.8 MPa in *Salix babylonica* [38] to around 1.2 MPa in *S. oleraceus* [37,39]. Provided that comparative measurements of osmotic pressure and hydrostatic pressure in both shoot and root are available [40,41], they support the Münch pressure flow theory establishing that the basic mechanism for phloem transport is thought to be bulk solution flow driven by an osmotically generated pressure gradient [42,43].

Another characteristic of sieve-tube sap is its pH value that is moderately alkaline, usually between 7.3 and 8.5, whatever method used to collect the sap, stylectomy or incisions into the phloem [20,44–46]. Acidic pH values sometimes reported in the past using the incision methods were probably due to phloem sap contamination with exudates from xylem vessels, damaged parenchyma cells, or laticifers [45]. The pH gradient between the symplast of the sieve cell-CC complex and the phloem apoplast ( $\Delta$ pH around 2.5 units) was, three decades ago, the energetic basis of a new hypothesis to explain phloem loading in apoplastic loaders, a  $H^+$ -sucrose symport energised by a proton pump ATPase [47]. This hypothesis was further supported by additional data. Particularly, measurements of the sieve tube transmembrane potential difference by inserting a microelectrode into sap exuding from severed stylets of the willow aphid gave values of around –155 mV. Addition of sucrose to the medium in which the *Salix* stem

**Table 1**  
Chemical composition of wheat phloem sap collected using stylectomy (from reference [29]).

Compounds	Concentration (mM)
Sucrose	251.0
Total amino acid	261.7
K <sup>+</sup>	299.0
Cl <sup>-</sup>	25.1
Na <sup>+</sup>	5.2
Mg <sup>2+</sup>	4.9
Ca <sup>2+</sup>	0.2
NH <sub>4</sub> <sup>+</sup>	2.5
PO <sub>4</sub> <sup>3-</sup>	8.2
NO <sub>3</sub> <sup>-</sup>	8.1
SO <sub>4</sub> <sup>2-</sup>	1.0

was immersed induced a rapid depolarization of the membrane potential [48]. These data are consistent with the concomitant influx of protons and sucrose molecules in the phloem described by other authors [49,50]. In apoplastic loaders such as *Vicia faba* and *Arabidopsis*, the plasma membrane H<sup>+</sup>ATPase generating the two components of the proton motive force ( $\Delta pH$  and potential difference) is highly expressed in CC [51,52].

#### 4. Nutrients, micronutrients and other small molecules in phloem sap

##### 4.1. Sugars and amino acids

The main metabolites found in phloem sap are organic compounds, mostly sugars and amino acids (Table 1). Three main types of sugars are known to be translocated in the phloem: sucrose, raffinose-family oligosaccharides (RFO: raffinose, stachyose, verbascose, ajugose), and polyols (mannitol, sorbitol). RFO are typically found in polymer trap loaders, whereas polyols are found either in symplasmic or apoplasmic loaders [8]. In many species, sucrose is the most abundant sugar, with concentrations ranging from 340 mM in the sap of *Arabidopsis* [53] to 1.8 M in the sap of potato [54]. In cucurbits, RFOs are most abundant, and the additive concentration of sugars in the SE-CCs of minor veins is about 600 mM, with stachyose and raffinose concentrations of 330 and 70 mM, respectively [55]. In peach and celery, sorbitol or mannitol, respectively, are the main sugars present in phloem sap [56]. Other sugars, such as galactinol and maltose, can also be found at lower concentration. For example, in *Arabidopsis*, galactinol, raffinose and maltose are detected [57,58]. Reducing sugars, such as glucose and fructose, are usually present as traces in phloem sap and a high ratio of sucrose to hexoses is generally considered consistent with limited contamination of phloem sap exudates with the content of other cell types. Nevertheless, this paradigm is challenged by the recent discovery, using the EDTA facilitated-exudation technique, that members of two plant families, *Ranunculaceae* and *Papaveraceae*, translocate more than 80% of carbohydrates in the form of hexoses [59].

The major transport form of reduced nitrogen in the phloem is amino acids [60]. Ammonium (NH<sub>4</sub><sup>+</sup>) is usually undetectable [61] and nitrate (NO<sub>3</sub><sup>-</sup>) is found either at low concentration or undetectable [29,61–63]. The uptake of sucrose and amino acids, and subsequently their concentration in phloem sap, is interdependent [64]. Amino acids can be abundant (Table 2), with concentrations ranging from 0.18 M in *Arabidopsis* [32], around 0.4 M in alfalfa, wheat, potato and *B. napus* [25,54,65–67] and over 1.23 M in maize [68]. The relative concentrations of amino acids, especially of the four essential amino acids threonine (Thr), histidine (His), tryptophane (Trp), and valine (Val) vary between plant species [69] (Table 2). One of the amino acids glutamine (Gln), asparagine (Asn), glutamate (Glu) or aspartate (Asp) is often predominant, depending on the plant species, although most essential and non-essential amino acids can be detected [25,32,70]. Cysteine (Cys) is present just as a trace in phloem sap, and glutathione was

**Table 2**

Amino acid content (mM) of phloem sap collected from wheat (*Triticum aestivum* L.) leaf [29] and alfalfa (*Medicago sativa* L.) stem [23] using stylectomy.

Amino acid	Wheat (mM)	Alfalfa (mM)
Ala	6.9	8.2
Arg	6.1	5.4
Asn	10.2	273.0
Asp	51.4	18.3
Cys	na	1.3
Glu	79.3	7.5
Gln	9.7	8.9
Gly	1.7	2.6
His	4.9	2.6
Ile <sup>a</sup>	10.6	6.1
Leu <sup>a</sup>	12.4	5.8
Lys <sup>a</sup>	12.4	3.3
Met <sup>a</sup>	1.8	n.d.
Phe <sup>a</sup>	8.4	4.5
Pro	na	6.6
Ser	14.9	15.8
Thr <sup>a</sup>	12.9	10.3
Trp <sup>a</sup>	na	na
Tyr	5.9	1.7
Val <sup>a</sup>	12.2	9.0
γ-amino butyric acid	n.d.	1.7
Total essential amino acids	70.7	39.0
Total amino acids	261.7	392.6

Values are obtained from 1 sap sample in the case of wheat and are means of 9 samples in the case of alfalfa.

n.d.: non detected; na: not available. Note that in the alfalfa phloem sap, the total of essential amino acids represents only 10% of the total amino acid content.

<sup>a</sup> Essential amino acids.

proposed to be the main form of transport of sulfur amino acids [71], which are found in the sap [72]. In addition to glutathione, in wheat leaves, the non-protein amino acid S-methylmethionine is also a major form of reduced sulfur in phloem sap [73]. The non-protein amino acid γ-amino-butyric acid (GABA) is detectable in the sap of some species although a high level of GABA was suggested to be in some cases an artefact of the exudation technique [23]. Other organic components, such as polyamines, are also found in phloem sap [74], and organic acids, especially malic, succinic and citric acid, are normally present in a range of species [34,61,62].

##### 4.2. Inorganic ions and micronutrients

Inorganic ions, especially potassium, are present in the phloem sap (Table 1), with concentrations varying between plant species and environmental conditions [29,75]. In *Arabidopsis*, potassium, sulphur and phosphorus are found at concentrations of 125, 15, and 25 mM, respectively [53]. Potassium, the major cation, plays an important role in provision of turgor and membrane potential, favouring sugar loading in source leaves [53,76] and is also implicated in the transmission of electric signals via membrane depolarisation [77]. Phosphate and other anions, such as bicarbonate and malate, are involved in the charge balance and are thought to play a role in the control of phloem sap pH. Minerals, such as phosphorus can also fluctuate with both daytime and seasons [75,78]. Small

amounts of  $Mg^{2+}$ , sodium and chloride are found in the sap. In maize, they accumulate around 1.5, 0.7 and 8 mM, respectively, and sodium and chloride increase in response to salt treatment, indicating that phloem translocation of these ions contribute significantly to the leaf solute balance and prevents ion accumulation in apoplasm and symplasm [61]. Micronutrients such as iron, copper, manganese, zinc, boron and molybdenum, are also present [79], although their presence in phloem sap can be divergent in different plant species depending on the mechanisms involved in their translocation. For example, the reallocation of boron via the phloem is only found in species, such as celery, that transport sugar alcohols, which form a complex with boron in the sap [80]. Vitamins, such as thiamine and ascorbic acid (AsA), are also found in phloem sap [45,72,81]. In the potato, strong evidence suggests that AsA, which has a major role as antioxidant, is synthesised in source leaves and then translocated via the phloem to sinks organs, such as tubers, although the relative contribution of phloem-derived AsA to overall AsA accumulation in potato tubers is still unknown [82].

Free  $Ca^{2+}$  is also present in phloem sap and appears as important for phloem physiology and signalling. Thus, it was shown to be important for sieve plate occlusion and forisome dispersion in *V. faba* in response to injury [83,84]. The estimation of the concentration of  $Ca^{2+}$  in the phloem sap was a matter of debate for a long time. It was determined using both  $Ca^{2+}$ -selective electrodes and fluorescent  $Ca^{2+}$ -sensitive dyes. In *V. faba*, it has been estimated to be around 50 nM, rising in response to wounding up to 200 nM, establishing that free  $Ca^{2+}$  is present in a concentration range similar to that found in other cell types [85], although much lower than previously estimated for phloem sap of *R. communis* [86].

#### 4.3. Secondary metabolites, hormones and lipids

A large range of secondary metabolites accumulate in phloem sap, but the content varies a lot and depends on the plant species. Phenolic glycosides, for example, are found in *Salicaceae* [87], glucosinolates in *Brassicaceae* [88], cardenolides [89] and iridoid glycosides [90–92] in *Scrophulariaceae* or *Bignoniaceae*, quinolizidine and pyrrolizidine alkaloids in *Fabaceae* [93–95] and glycosides of hydroxamic acid in *Poaceae* [96]. Only one comprehensive survey of the metabolome of the phloem sap was achieved in pumpkin by analysis of bleeding sap [97]. This study established a large range of compounds besides sucrose and amino acids, such as organic acids, a range of sugars, hexose phosphates, polyamines, aromatic amino compounds and small hydroxy acids [97].

In addition, many phytohormones have been identified in phloem sap [98], including auxin, cytokinins, gibberellins, abscisic acid, 1-aminocyclopropane-1-carboxylic acid (the precursor of ethylene), methyl jasmonate, and salicylic acid [13,99–112]. In contrast, brassinosteroids were not reported. Other signal molecules, such as nitric oxide, are detected in phloem sap of *V. faba* [113]. The dicarboxylic acid, azelaic acid, is found in Arabidopsis phloem sap and acts in systemic resistance in the priming of systemic immunity [114]. Only a few reports analysed

the amounts of lipids and sterols present in the sap. In canola, the phloem lipid composition is clearly distinguishable from that of membranes and previously characterised lipid particles [107]: it includes phospholipids, diacylglycerol, triacylglycerol, steryl wax esters and unesterified fatty acids. Some of these fatty acids might participate in signalling pathways. Some sterols, such as sitosterol, are phloem mobile as reported in barley sap samples [115].

#### 5. Effects of environmental factors and stresses on the nutrient content of phloem sap

As previously stated, sugars, amino acids and  $K^+$  are the dominant solutes and account for the majority of the osmotic potential [116] and their overall concentration maintains a relatively stable rate of transport into the SE independent of day-night transitions or climatic variations [75,117]. However, the precise composition of phloem sap is influenced by multiple timescales: from the diurnal cycle to the season [65,75,118–123]; by the developmental age of the plant [72,97,123–125].

It is also influenced by abiotic factors, such as temperature, nitrogen and water availability [67,70,126].

It was shown that the C/N ratio fluctuates along the diurnal cycle and plant development [118,124], consistent with the interdependence of sugar and amino acid uptake [64]. Thus, the levels of total amino acids, sucrose and  $K^+$  ions in phloem exudates present an overall diurnal periodicity [119,120,127]. Analyses of wheat phloem sap collected from aphid stylets also indicate complex diurnal variations of individual amino acids. The increase of amino acid content during the afternoon is correlated to a covariation of Arg, Tyr, Phe, His/Val, Leu/Ile, Pro and Asn concentrations, while the level of the other amino acids remains unchanged [65].

Furthermore, specific changes in amino acid composition can be induced by abiotic stresses. For instance, the technique of EDTA-facilitated exudation induces a variety of artefacts, notably a drop of Asn content and a strong increase of GABA [23]. These data are consistent with previous observations indicating that GABA dramatically accumulates in leaf tissues floating on an incubation medium [128]. On the other hand, a decrease of the predawn water potential of alfalfa leaves from –1 to –2 MPa induced an increase of the total amino acid concentration mostly due to certain amino acids, especially Pro (60-fold increase), Val, Leu, Ile, Glu, Asp and Thr [25]. These and other data, such as the observation of a synthesis of Pro and an induction of amino acid transporter in leaves in response to a water deficit [129,130] contribute to explain the dramatic accumulation of Pro observed in the growing region of the primary root in response to drought [131]. Specific changes in amino acid composition of phloem sap can also be noted in cases of biotic stress. For example, the infestation of wheat plants by aphids such as *Schizaphis graminum* and *Diuraphis noxia*, which induce chlorotic lesions, led to modification of phloem sap composition, especially to an increase of amino acid concentration, particularly Gln and the essential amino acids His, Ile, Leu, Met, Phe, Trp and Val [132]. These

changes can be in some cases nutritionally advantageous for the aphids and can even be induced by an aphid-mediated facilitation process during feeding, as mentioned earlier on.

Finally, phloem sap composition is far from being uniform across SE, as illustrated by the variation in amino acid concentration among replicate samples of exudates from severed stylets [25,65,133].

## 6. Alteration of phloem sap composition and downstream effects on plant aphid interactions

Variation in the performance and abundance of aphids has been suggested to be correlated to variation in host plant quality [70,79,134–136] and more specifically to the sugar, amino acid and sucrose: amino acid ratio in phloem sap. In order to test for this hypothesis, aphid growth and fecundity were first evaluated using sap-copied holidic diets differing by their amino acid or sucrose concentrations [137]. More recently, several groups investigated aphid performance on transgenic plants, in which phloem sap sugar or nitrogen content was modified.

### 6.1. Modification of sugar content and aphid settlement and feeding

Sugars accumulate to high concentrations within phloem sap and are responsible for a high osmotic pressure in the aphid diet, which in turn requires a strong osmoregulation within the insect body [136]. Modification of sugar content in phloem sap has been achieved by different approaches, modifying either carbon metabolism in mesophyll cells or sucrose transport in the phloem. For example, the alteration of the accumulation of triose phosphate translocator or ADP-glucose pyrophosphorylase in potato in antisense transgenic lines [122] led to a modification of day/night sugar composition in the sap. Other approaches were based on the alteration of sugar transporters, which are required for apoplasmic loading. To date, only one report studied the aphid responses to alteration of phloem sap sugar content. The analysis of potato plants affected in sugar transporter *StSut1*, which were affected in the amount of sucrose present in phloem sap, showed no effect on the performance of *Myzus persicae* and *Aulacorthum solani*. Only *Macrosiphon euphorbiae* performed worse on these lines, suggesting that these aphids are dependent on a high sugar concentration in phloem sap to successfully colonise potato leaves [54]. Since sucrose has been suggested to act as a phagostimulant [79,138,139], the authors concluded that aphid feeding might be promoted by temporal variations in sucrose composition [54]. In this study, aphid behaviour was recorded using electric penetration graph (EPG), a technique based on electric recording of aphid behaviour during the successive steps of feeding [140].

### 6.2. Modification of amino acid content and aphid performance

It is well accepted that the supply of nitrogen in the form of free amino acids is a limiting factor in the aphid

diet for growth and fecundity [79]. Moreover, nine amino acids cannot be synthesised by the aphids and have to be present in the diet [136]. Uptake and transport of amino acids is controlled by a number of amino acid transporters expressed in various tissues, including phloem, and displaying a large range of affinity and substrate specificity [141], in close relationship with nitrogen remobilisation mechanisms. Attempts to manipulate amino acid sap composition were based on the deregulation of genes encoding amino acid permeases in *Arabidopsis* [32,33]. *ANT1* is a permease that displays moderate affinity for neutral and aromatic amino acids [142]. In the knockout mutant *ANT1*, the contribution of several amino acids was increased in phloem sap, consistent with the hypothesis that *ANT1* moves amino acids out of the SE [33]. However, no significant differences in the feeding behaviour and fecundity for *M. persicae* aphids feeding on the mutant could be detected [33]. *AAP6* is a neutral amino acid permease with high affinity [129]. It is mainly expressed in sink tissues and is thought to mediate the transfer of amino acids from the xylem to the phloem. Although knockout mutants presented a clear decrease in amino acids in the phloem sap, *M. persicae* feeding on the mutant showed only limited alteration of their metabolism, behaviour and reproduction, besides a mild effect on salivation time [32], as monitored by EPG.

It is becoming clear that prediction of aphid performance is more complex than a simple correlation with the nitrogen content of the diet, many other metabolite factors potentially contributing to output [32]. Moreover, the endosymbiotic bacteria *Buchnera* enables aphids to subsist on an unbalanced amino acid diet provided by phloem sap [136,143] by synthesising several essential lacking amino acids from relatively abundant non essential phloem amino acids [143,144] or possibly from other sources, such as the non-protein amino acid S-methylmethionine, which was suggested to be a potential precursor [145].

## 7. Phloem sap macromolecules in relation to defence against phloem feeding insects

### 7.1. Protein content and trafficking

An exciting field of recent investigations is the identification of a large range of proteins in phloem sap [17,146–150]. Protein content in phloem sap ranges from 0.1 to 1 mg/ml in most plants up to 30 mg/ml in cucurbit exudates [148], and protein sizes range from 1 to more than 100 kDa. Since the size exclusion limit of plasmodesmata between CC and SE is up to 67 kDa [151], it is believed that sorting mechanisms enable larger proteins to be unfolded to enter the sieve tubes via plasmodesmata [2]. In addition, some of these proteins traffic long distance within sieve tubes and act in long distance signalling [2,3]. For example, the protein FT was shown to act as the phloem long distance signal for flower induction in *Arabidopsis* [152], and the long distance transport of the transcript of *BEL1* through the phloem is correlated with tuber formation in potato [153].

Phloem sap proteomes were analysed in rice, melon, cucumber, pumpkin, *B. napus* and *R. communis*

[17,27,146,148–150,154–157]. One striking feature is the abundance of enzymes involved in redox regulation and antioxidant defence systems [155,158]. They include the enzymes peroxidase, ferredoxin, monodehydroascorbate reductase, dehydroascorbate reductase, Cu/Zn superoxide dismutase, and thioredoxin H [150,155,159,160]. Also, other defence proteins were reported in several phloem sap analyses [157]. Since sieve tubes are the targets of aphids, such proteins were proposed to be involved in protection mechanisms against phloem feeding insects [158]. The set of putative defence proteins includes protease inhibitors, lectins, components of the myrosinase system (found in crucifers), some proteins known to be induced by wounding or insect feeding (CSF-2, SN-1, SLW-1, SLW-3), and enzymes involved in the biosynthesis of jasmonic acid or ethylene [158]. Many components of signal transduction pathways were found, including kinases, calcium binding proteins, annexins or calmodulin [158], which can act either in the activation of Ca<sup>2+</sup>-binding kinases or in Ca<sup>2+</sup> sequestration to prevent occlusion of sieve tubes [84,85]. P-proteins, especially phloem lectins, that are thought to act on the transient and reversible occlusion of SE following phloem injury, were also frequently found [158].

Some phloem sap proteins are also involved in RNA trafficking in the phloem [2] and RNA-binding proteins were consistently found in phloem sap. In pumpkin sap, at least 82 proteins annotated as RNA-binding were described [157]; they include CmPP16, CmPP1, CmPP2, glycine-rich RNA binding proteins and eIF-5A, a putative component of the protein synthesis machinery, and helicases. Some of these proteins were proposed to be involved in the unfolding and refolding of RNAs trafficking through plasmodesmata between CC and SE [2,157].

Enzymes involved in the metabolism of sugars appear enriched in pumpkin sap exudates, consistent with the possibility of a conversion of sugars into hexoses [157]. Components of the cytoskeleton, actin, actin associated proteins and profilin, were reported in several surveys [150,157]. Of particular interest is the discovery of many components of the ubiquitin proteasome complex found in pumpkin sap, rice and rape. Such components are thought to participate either in protein turnover or in defence or developmental signalling pathways [161,162]. To date, there is no report of proteome analysis of phloem sap in response to aphid feeding, although there are potentially many proteins in phloem sap that could be acting in response to aphids.

Eventually, when delivered into sieve tubes, proteins which are not anchored to membranes are translocated long distances by bulk flow, as demonstrated for the green fluorescent protein (GFP) that moved non-selectively in sieve tubes [163]. Nevertheless, whereas translocation towards the shoot appears to be passively carried by bulk flow, movement of some phloem RNA-binding proteins in the direction of the root was selectively regulated [164], suggesting that the delivery of macromolecules into sink tissues might be tightly controlled. However, our understanding of the control mechanisms for long-distance movement remains limited, particularly due to the difficulty to observe long distance trafficking of

macromolecules *in vivo*. Evidence of a destination-selective translocation of phloem proteins was obtained in rice, using the MUSI (micro-introduction using stylet of insect) method [165] and the brown planthopper *N. lugens*. The approach is based on application of fluorescent or biotinylated tagged tracer protein to the cut stylet of the insect, which allowed for protein diffusion into the sieve tube, then incorporation into the phloem translocation stream and observation of its long distance delivery into distant organs [164,165]. Using tagged CmPP16 proteins from *C. maxima*, the authors demonstrated that both CmPP16-1 and CmPP16-2 form a complex with rice phloem sap proteins, whereas CmPP16-1 is translocated towards the root in addition to the shoot, in contrast to CmPP16-2 that moves only towards the shoot. These results suggest that the selective movement is regulated by protein-protein interactions and the formation of complexes in phloem sap [164].

In addition to CmPP16 proteins, various proteins have been identified in such complexes, including, for example, a translationally controlled tumor-associated protein (TCTP), an eukaryotic translation initiation factor 5A (eIF5A), a polypyrimidine tract binding protein, (RBP50) and a molecular chaperone Hsc70 found in rice or pumpkin phloem sap [164,166]. Some of these proteins are RNA-binding proteins and are thought to form ribonucleoprotein complexes acting on the translocation of the RNA present in phloem sap.

## 7.2. RNAs species and trafficking

Various RNA molecules have thus been found in SE sap, and these phloem-mobile RNAs are thought to act in long distance signalling [2,167,168]. They include mRNAs, siRNAs, miRNAs, rRNAs and tRNAs. Hundreds of mRNAs have been identified in the phloem sap [28,167,169–171]. These mRNAs encode proteins involved in a range of functions, including transcription factors, stress response proteins, metabolic enzymes, transporters or other structural components. The subset of mRNAs present in the phloem sap appears distinct from those present in other tissues or organs [172]. For example, in melon a higher percentage of transcripts related to stress and stimulus responses, metal-ion binding, proteinase-inhibitor activity, and the ubiquitin–ligase complex was found in phloem sap compared to leaves and fruits [170]. In Arabidopsis, the transcript profile of phloem sap does not reflect the profile of CC, indicating that not all transcripts of the CC are transported into the SE [172]. Importantly, not all mRNAs are transported long distances, as demonstrated in the melon, in which only a small fraction of mRNAs were translocatable through graft junctions [170]. This suggests that besides long distance signalling, other functions may be undertaken by these RNAs.

Small RNAs, including siRNAs and miRNAs, were identified in *C. maxima*, *Cucumis melo*, *Lupinus albus* and *B. napus* [173–175]. The siRNAs are non-cell autonomous and are indeed very abundant in phloem sap where they act on systemic silencing [176]. The presence of several miRNAs, usually considered as cell autonomous [177] is perhaps more surprising. Additionally, specific miRNAs

found in phloem sap are regulated by abiotic stresses, as demonstrated for sulphate, phosphate or copper deficiency [174,175,178]. This suggests that stress-induced miRNAs could possibly act as systemic silencing signals between distant organs. Other classes of small RNAs, i.e. rRNAs and tRNAs, have also been detected in phloem sap exudates. The observation of truncated tRNAs, presenting inhibitory activity in *in vitro* translation assays is consistent with involvement in a regulatory mechanism to down-regulate protein synthesis [179].

Interestingly, some RNAs present in phloem sap can also be delivered to plant parasites. For example, in the host-parasite junction of *Cuscuta pentagona* with tomato, several mRNAs were found to move from host to the dodder [180,181]. Some of the genes with mobile transcripts have roles in mediating plant response to the environment [180], and were proposed to allow some coordination between the parasite and its host [181]. This observation provides the potential to use gene-silencing technologies to increase resistance of crop plants against parasites by exploiting the interspecific trafficking of small interfering RNAs to target vital functions in the parasite. The use of dsRNA or RNA interference expressed *in planta* has already been validated against the western corn rootworm and the cotton bollworm [182,183].

## 8. Conclusion and perspectives

Aphid stylectomy, spontaneous exudation and EDTA-facilitated exudation have been successfully used in many studies that have significantly increased our knowledge of phloem sap composition. Despite its technical difficulty, the use of aphid stylectomy is an useful tool for collecting SE sap so that its composition can be analysed, for studying phloem function and integrating with other aspects of plant biology. It minimises many sampling artefacts caused by wounding, not withstanding that aphids do manipulate their host and induce in the phloem tissue gene expression reprogramming [184]. Stylectomy was used for investigations on phloem sap main metabolites, such as sugars, amino acids or K<sup>+</sup>, that contributed to a better understanding of phloem physiology. Other potential applications are possible. Recently, it was used for analysing the unexpected secondary positive effects of transgenic maize lines expressing the *Bacillus thuringiensis* toxin on the performance of a non targeted insect feeding on these plants, the aphid *Rhopalosiphum maidis*. In these plants, the amino acid content in phloem sap was proposed to partially explain the observed increased aphid performance [68]. Nevertheless, application of stylectomy is mainly restricted to plant species on which phloem feeding insects can produce extended and abundant exudation, such as rice or maize [27,44,68], although several other plants, such as potato or *Arabidopsis*, were recently successfully used for analysis of abundant SE components, despite exudation volumes in the nanoliter range [32,33,54].

Many studies on phloem sap composition have been performed on the polymer trap loader cucurbits that allow the collection of large amounts of phloem exudates from simple incisions [147,149,155,157,170,173]. Other species,

such as *B. napus* or *R. communis*, that also spontaneously exude upon wounding, also emerged as additional useful models for phloem sap analysis [150,156,169,174].

Besides stylectomy, other techniques based on the use of aphids are currently being developed to extract and analyse phloem sap. Limited so far to rice, the MUSI technique [165] was shown to be an efficient technique for studying macromolecular trafficking through sieve tubes. EPG [140] can be useful for plant physiologists, since it can show alteration in the feeding behaviour of an insect on different plants, which might indicate variations in the content of phloem or other cell layers. For example, it was used to demonstrate the role of PAD4 (phytoalexin deficient 4), a lipase-like protein involved in the regulation of defence, in the control of the phloem phase of feeding of *M. persicae* in *Arabidopsis* [185]. EPG was also applied to demonstrate that the plugging of sieve plates by forisomes induced by burning affects the behaviour of the aphid *Megoura viciae* on *V. faba* [186].

These advances in phloem physiology have contributed to identifying new cues potentially controlling plant aphid interactions. So far, this has largely been achieved using mutant plants in which genes encoding for sucrose or amino acid transporters were knocked out [32,33,54]. The potential of such approaches in limiting aphid performance in the field still needs to be established and as reported here, it is clear that the interaction between plants and aphids is not as clear as earlier proposed. This indicates that plant-aphid interactions are probably subtly regulated and involve a range of nutrients, repellents and volatiles, and that a better understanding of metabolism, transport and signalling in the phloem will potentially provide new targets for strengthening plant defence against aphids.

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