What is the role of sporadic phloem sap nitrate?

Jing Cui[1], Andreas D. Peuke[2], Anis Limami[3], and Guillaume Tcherkez[4]

[1]ANU
[2]ADP International Plant Science Consulting
[3]University of Angers, (IRHS 1345) Institute of Research of Horticulture and Seeds
[4]Affiliation not available

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Abstract

Since the first description of phloem sap composition nearly 60 years ago, it is generally assumed that phloem sap does not contain nitrate and that there is little or no backflow of nitrate from shoots to roots. While it is true that nitrate can occasionally be absent from phloem sap, there is now substantial evidence that phloem can carry nitrate and furthermore, transporters involved in nitrate redistribution to shoot sink organs and roots have been found. This raises the question of why nitrate may or may not be present in phloem sap, why its concentration is generally kept low, and whether plant shoot-root nutrient cycling also involves nitrate. We propose here that phloem sap nitrate is not only an essential component of plant nutritional signaling but also contributes to physical properties of phloem sap and as such, its concentration is controlled to ensure proper coordination of plant development and nutrient transport.

Introduction

It has now been 61 years since phloem sap composition has first been described, using sap extracted from willow (Peel & Weatherley, 1959). In that study, using colorimetric methods, nitrate was found to be undetectable. Several subsequent studies also reported the absence of nitrate in other species such as castor bean (*Ricinus communis*) (Hall & Baker, 1972) and overviews of phloem sap composition established the generally very low, or undetectable, concentration in phloem sap nitrate (Ziegler, 1975). Furthermore, experiments with ^{15}N-nitrate labelling in legumes have shown that nitrate could not be transported from xylem to phloem at detectable levels (Pate *et al.* , 1975). There are more recent reports where nitrate was found to be undetectable, for example in maize (Lohaus*et al.* , 2000). It is thus widely accepted that phloem sap nitrate is of negligible importance and in particular, that nitrate circulation from shoots to roots does not occur. For example, in a recent review, the backflow of nitrate via the phloem to regulate root development is not mentioned (Fig. 3 in (Tegeder & Masclaux-Daubresse, 2018)). Also, in textbooks and university lectures, it is often reported that nitrate is absent from phloem sap and thus does not flow back from shoots to roots, see for example (Taiz *et al.* , 2015). However, many pieces of recent evidence suggest the contrary and provide possible reasons explaining why phloem nitrate concentration is usually low.

Phloem sap may contain nitrate

In fact, there are noticeable exceptions where nitrate is not absent from phloem sap composition (Fig. 1). It is the case of cereals (wheat, rice), with phloem sap nitrate of up to 8 mM (Hayashi & Chino, 1985; Hayashi & Chino, 1986). Also, in palm trees, nitrate has been found in phloem exudates for either trunk and inflorescence peduncles (van Die & Tammes, 1975). Extensive analysis of castor bean phloem sap has shown that average nitrate phloem concentration is 0.59 mM, ranging from no detectability to 2.4 mM (Peuke, 2010). This is of course much less than in xylem, which contains up to 25 mM nitrate. Interestingly, nitrate has been found to be much more abundant in phloem (0.29 mM) than xylem sap (3 µM) in Western candle

tree (*Banksia prionotes*) (Jeschke & Pate, 1995). These findings cannot be explained by the technique of sap collection (i.e. potential adulteration by other tissues upon sampling) since the above-cited studies used very different techniques (aphid-stylet and pure phloem sap bleeding) and therefore a systematic contamination is not a plausible explanation.

Nitrate redistribution via phloem sap circulation

Nitrate redistribution via phloem circulation has been recently suggested to occur in three physiological contexts (Fig. 1): remobilization from old leaves, development of reproductive structures and nitrate backflow from shoots to roots. In *Arabidopsis* , the nitrate transporter NPF5.5 is involved in controlling embryo N content: in knock-out mutants, embryos at the bent cotyledon stage have a 7-8% reduction in the N content suggesting that the import of nitrate to the developing seed has been impacted (Léran *et al.* , 2015). There is presently no direct evidence that NPF5.5 only participates in the import of nitrate into seeds but its implication in nitrate transfer from the phloem to developing tissues is likely. NPF2.12/NRT1.6 appear to be expressed intensively in funiculus vascular bundles, which are mostly made of phloem tissue in *Arabidopsis* (Almagro *et al.* , 2008), and mutants affected in this transporter have altered isotopic (^{13}N) nitrogen transfer to reproductive structures (Babst *et al.* , 2019). Three nitrate transporters have been shown to be involved in nitrate transfer and/or redistribution to the phloem from xylem or source nitrate-containing tissues: NPF2.13/NRT1.7, NPF1.1/NRT1.12, and NRT1.2/NRT1.11 (reviewed in (Iqbal *et al.* , 2020)). In particular, it has been recently shown that NRT1.7 is essential for remobilization of nitrate from old leaves to deliver nitrate into the phloem, making it available for new, developing leaves and enhancing nitrogen use efficiency (Chen *et al.* , 2020). Also, in mutants affected in NRT1.9 (transporter expressed in root phloem), the nitrate content in root phloem exudates is lower (by 20 to 30%) (Wang & Tsay, 2011); furthermore, downwards nitrate transport to root tips, when assayed with a compartmentalized root system, is reduced, suggesting that NRT1.9 plays a role in the backflow of nitrate to roots (Wang & Tsay, 2011). Double mutants affected in both NRT1.11 and NRT1.12 have a strong growth phenotype suggesting that xylem-to-phloem nitrate transfer and/or phloem-mediated nitrate is essential for plant growth (Hsu & Tsay, 2013). Taken as a whole, recent data on nitrate transporters clearly show that phloem can carry nitrate upwards (developing sink organs in shoots) and downwards (back to roots).

Phloem sap nitrate is a signaling molecule

There is now considerable evidence that nitrate can play a signaling role to control plant development (Kant, 2018). Historically, an important step forward was the demonstration that nitrate shoot content acts as a signal to regulate the shoot:root ratio. In fact, manipulating nitrate reductase activity showed a correlation between leaf nitrate concentration and shoot:root ratio, across different levels of nitrate supply (Scheible *et al.* , 1997). Furthermore, in split-root experiments, root growth is inhibited by the accumulation of nitrate in the shoot, regardless of the fact that one part of the root system was supplied with high nitrate and the other part with low nitrate (Scheible*et al.* , 1997). Molecular data have now demonstrated a link between nitrate and phytohormones, and this aspect has been reviewed recently elsewhere (Vega *et al.* , 2019). The signaling cascade associated with nitrate has also been dissected recently and shown to involve Ca^{2+}-sensor protein kinases and NIN-like proteins (NLP) transcription factors to control gene expression, including gene encoding enzymes and transporters involved in nitrogen assimilation (Liu *et al.* , 2017; Chu *et al.* , 2020). The role played by nitrate as a signal from shoots to roots implies that its concentration in phloem must be controlled to avoid both fluctuations and high concentrations. That is, signal-carrying molecules have to be at low concentration under steady-state conditions and their transient increase can play the role of a signal. Unfortunately, relatively little is known on possible fluctuations of nitrate concentration in phloem sap and whether its appearance in sap is linked to specific circumstances (but see below, *Perspectives for N cycling in plants*). It is worth noting that in root tips, nitrate absorption is small, compared to other root parts (Lazof *et al.* , 1992). In addition, root tips are the sites of longitudinal root growth (increase in root length), where protophloem develops first (i.e. before protoxylem) (Mahonen *et al.* , 2000; Bauby *et al.* , 2007). It is plausible that downwards transport of nitrate via (proto)phloem plays a role in controlling root tip nitrate concentration and thus root growth.

Low nitrate concentration may avoid protein aggregation

Phloem sap contains very high content of sucrose (about 400 mM in castor bean), amino acids (about 60 mM) and K^+ (about 60 mM). Mg^{2+} and Na^+ are present in lower abundance and Ca^{2+} is at low concentration (1 mM or less) (Ziegler, 1975). Anions that counterbalance K^+high content are mostly Cl^-, phosphate, sulphate and organic acids (malate). Nitrate is thus a minor participant in phloem electroneutrality. Also, phloem sap contains significant amounts of proteins, at about 1 g L^{-1}, and an important proportion is made of the so-called P-proteins (SEOR proteins in*Arabidopsis*) that are believed to play a role in phloem occlusion by Ca^{2+}-dependent aggregation (Anstead *et al.* , 2012; Jekat *et al.* , 2013; Knoblauch *et al.* , 2014; van Bel*et al.* , 2014). Of course, phloem sap contains many other proteins, and recent proteomics analyses have shown that this includes not only enzymes, but also translation initiation or elongation factors, proteins involved in redox homeostasis, chaperones, etc. (Rodriguez-Celma *et al.* , 2016). It is worth mentioning that with such high salt concentration (in particular K^+), there is a risk of uncontrolled protein aggregation. In fact, both experiments and theory have provided evidence that ion species have different propensity to trigger protein aggregation (chaotropism), via denaturation and/or instability (Kunz, 2010). Ca^{2+} is the most chaotropic cation while tertiary amines are the least chaotropic, K^+ being intermediate. This provides a physical justification for the role of Ca^{2+} in phloem occlusion. Similarly for anions, phosphate, sulphate and organic acids are the least chaotropic, but nitrate is much more chaotropic, just behind perchlorate and iodide (Kunz, 2010). Accordingly, experiments with lysozyme have demonstrated that the displacement of the solubilization-aggregation equilibrium towards aggregation is larger with nitrate than chloride forms of sodium salts (Kastelic *et al.* , 2015). As such, having high concentrations of nitrate is not desirable for phloem protein stability when K^+ (and/or Mg^{2+}) is present at high concentration. Conversely, in sucrose concentrated solutions (like phloem sap), ethylamine nitrate is beneficial to protein renaturation and decreases viscosity due to the rescuing property of ethylamine as a non-chaotropic cation (Byrne*et al.* , 2007).

Perspectives for N cycling in plants

While there are good reasons to explain why nitrate concentration must remain low in phloem sap (signaling and chaotropism), the occasional presence of nitrate raises the question of its potential role in nutrient cycling. Pioneering N mass balance in castor bean proposed that nearly 50% of nitrate translocated to the shoot cycled back to roots (Marschner *et al.* , 1997). While this number is certainly overestimated, recent isotopic data (^{15}N natural abundance, $\delta^{15}N$) also suggest that a small flux of a few percent of xylem translocation to shoots can cycle back to roots, in both sunflower and oil palm (Cui *et al.* , 2020). Although quantitatively minor, this flux is important to explain the natural^{15}N-enrichment in root nitrate. It is also possible that variations in nitrate concentration, in addition to organic N, contribute to the diel pattern of $\delta^{15}N$ of phloem in castor bean (Peuke *et al.* , 2013). The $\delta^{15}N$ value of aphids (feeding on phloem sap) has been shown to be lower (depleted) compared to host plants and related to nitrate reduction capacity, suggesting that the aphid-host isotopic difference is partly explained by the ^{15}N-enrichment in phloem nitrate – as opposed to the ^{15}N-depletion in phloem amino acids (Wilson *et al.* , 2011).

The backflow of nitrate from shoots to roots depends on growth conditions impacting on overall nutrition, since (Cui *et al.* , 2020) showed it depends on K nutrition and root hypoxia (waterlogging). Also, phloem sap nitrate increases when nitrate availability increases and declines with salinity (Peuke *et al.* , 1996). Surprisingly, meta-analyses have shown that phloem nitrate concentration does not correlate significantly to other cations and only correlates with xylem, leaf and root nitrate content (Peuke, 2010). However, the nitrate flow in the phloem (expressed in µmol nitrate g^{-1} FW d^{-1}) correlates reasonably well with phloem carbon and Ca^{2+} flows (Peuke, 2010). The nitrate backflow thus depends on other nutrients and salinity and is maybe linked to metabolites (organic acids and amino acids) present in phloem sap. The supply of amino acids to roots via the phloem participates in the control of root N acquisition (for a specific discussion, see (Tillard*et al.* , 1998)) and as discussed above, nitrate also plays a regulatory role. Thus, more than individual concentrations, the nitrate-to-amino acid ratio of phloem sap might be a crucial component of plant development, root growth and nitrogen assimilation. In the past years, there have been an increasing number

of publications on phloem (including proteomics data) but due to the difficulty of phloem sap collection, there is limited information on phloem composition under varying conditions, including metabolite profiling (metabolomics), nitrate content and $\delta^{15}N$ value. Future studies are warranted to provide more data and therefore to appreciate the generality and significance of the transport of nitrate in the phloem.

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Data availability

There is no data associated with this manuscript.

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Figure 1. Nitrate circulation via phloem sap : (a-f) Typical species where phloem nitrate has been directly measured or inferred from isotopic mass-balance: palm trees (a), cereals (wheat and rice) (b), Western candle tree *Banksia prionotes* (c), castor bean (d), sunflower (e), and Arabidopsis (f). (g) Summary of hypothetical roles of nitrate circulation via phloem sap (see text for further details).