Viewpoints

Are calcium oxalate crystals a dynamic calcium store in plants?

Summary

Calcium oxalate (CaOx) crystals occur as intravacuolar deposits in most angiosperm species. Different functions have been attributed to these crystals, some of which are very speculative, until now. Calcium regulation and homeostasis seem to be the most widespread function of CaOx crystals. Being rich in calcium, these crystals constitute a reserve of calcium for plants. However, despite being bioavailable, this reserve is functional in just a few situations due to the low mobility of calcium for phloem translocation. Therefore, CaOx crystals as a calcium reserve is a paradox because in most cases the reserve cannot be used. However, in most plants, these crystals occur in organs or tissues that will be discarded, which allows the elimination of excess calcium. This suggests that CaOx crystals have a functional role in excess calcium excretion. There is some evidence that, for calcium, this excretory function is relevant for plants since they lack an excretory system dedicated to discarding solid wastes, such as calcium salts.

Introduction

Calcium biomineralization, both as carbonate and oxalate salts, is a widespread phenomenon in the plant kingdom (Franceschi & Nakata, 2005; Bauer *et al.*, 2011). Calcium oxalate (CaOx) crystals occur in all vegetative and reproductive organs and represent a remarkable proportion of calcium in plants, reaching as high as 90% of total calcium content (Franceschi & Horner, 1980; He *et al.*, 2014).

Different functions have been attributed to CaOx crystals, some of which have been very speculative (see Nakata, 2003, 2012). Among these possible functions, calcium regulation and homeostasis, defense against herbivores and calcium reserve seem to stand out as the most studied, but they still remain controversial, with several lacking critical analysis and reliable supporting experimental evidence. Some other functions, which seem to be restricted to specific plant species or situations, include interesting roles for CaOx crystals, such as in heavy metal detoxification (Franceschi & Nakata, 2005; Pongrac *et al.*, 2018) and as a source of CO_2 for photosynthesis (Tooulakou *et al.*, 2016a,b).

Calcium oxalate crystals ($CaC_2O_4{\cdot}H_2O$) have a molecular weight of 128.096 g mol^{-1} in anhydrous form, of which calcium represents *c.* 30%. However, plant crystals have varying degrees of hydration, which reduces slightly the proportion of calcium. Despite this variation in calcium content, these crystals are obviously a way that plant cells store calcium. Thus, the question cannot be summed up by the simple and unquestionable fact that such crystalline forms constitute a reserve of calcium. The real question must be: is this calcium reserve functional? To address this question, I have combined evidence from the literature with some new arguments to shed light on this controversial matter.

Calcium transport

After calcium ion (Ca^{2+}) is taken up by roots it is transported to the aerial portions of the plant primarily through xylem, which is in turn controlled in most plants by the transpiration stream (Gilliham *et al.*, 2011). Several authors have shown a positive correlation between calcium levels in substrate and calcium in plant tissues (Smith *et al.*, 2009, and references cited therein). Similarly, there is a positive relationship between calcium in plant organs and transpiration (Paiva *et al.*, 1998; Gilliham *et al.*, 2011; Lee *et al.*, 2013; Hocking *et al.*, 2016). The effect of transpiration on calcium levels was observed in different organs of *Medicago truncatula* (Leguminosae); Nakata (2012) evaluated calcium levels in dry mass and found that leaves show 5× more calcium than roots or stems and 20× more than seeds. It should be noted that these values are consistent with the expected transpiration rates of these plant organs, and thus reinforces the hypothesis that calcium flows through xylem and that this process is affected by transpiration.

However, calcium has low mobility in phloem (Volk & Franceschi, 2000; Tang & Luan, 2017). While transport in xylem occurs through the apoplast, inside dead tracheary cells, the transport of sugar and other substances in phloem is inside the symplast, and thus the substances must be dissolved in the cytosol. This is a key point: Ca^{2+} levels are low in cytosol, while the vacuole and apoplast have higher levels of this ion (Gilroy *et al.*, 1993; Bush, 1995). Since sieve elements are not provided with vacuoles, and they possess poorly developed intracellular membrane systems with few if any organelles associated with Ca^{2+} storage (Bush, 1995), thus Ca^{2+} is actively pumped out of the cytosol (Higinbotham *et al.*, 1967; Raven, 1977; Sjolund & Shih, 1983; Poovaiah, 1988; Randall, 1992). Therefore, calcium in functional sieve elements must be in the micromolar range (10–3000 μM), and thus Ca^{2+} transport by phloem is not able to meet the demand of most tissues due to its low concentration in the cytosol. It is important to emphasize that the low concentration of calcium in the cytosol is not exclusive to sieve elements, but is a key trait of plant cells that remain alive at maturity. However, what makes sieve elements distinct from other cells with regard to calcium balance is that in the

latter, calcium can be sequestered by the endoplasmic reticulum, mitochondria, plastids, and vacuoles, or pumped out of the protoplast, to keep cytosolic levels of this ion low (see Sze *et al.*, 2000). Furthermore, because sieve elements are devoid of most membrane-bearing organelles, active pumping of Ca^{2+} out of the cytosol remains the only alternative to control cytosolic calcium levels. Such calcium pumping, however, has a high energetic cost since the calcium released outward would tend to return due to the concentration gradient, unless it is inactivated in the apoplast or directed to other cells. Calcium acts as a secondary messenger for several metabolic processes (McAinsh & Pittman, 2009; Tang & Luan, 2017) and its cytosolic levels must be precisely controlled since variation can trigger diverse metabolic responses (Webb, 1999; Bauer *et al.*, 2011; Tang & Luan, 2017).

Calcium controls cellular processes in sieve elements of phloem, such as callose synthesis (Kauss *et al.*, 1991; Volk & Franceschi, 2000) and reversible conformational changes to phloem proteins (Knoblauch *et al.*, 2001), both of which are related to sieve plate pore occlusion. The presence of Ca^{2+} channels along sieve elements permits Ca^{2+} influx from the specialized wall region immediately adjacent to the plasma membrane (Volk & Franceschi, 2000; van Bel *et al.*, 2014). This increase in cytoplasmatic Ca^{2+}, for example, induces callose formation during wounding (Kauss *et al.*, 1991; Volk & Franceschi, 2000) and controls hydrotropism of roots (Shkolnik *et al.*, 2018). Therefore, if there is an influx of calcium into the sieve tube elements, transport will be interrupted since the pores of the sieve plate will be immediately clogged, disturbing the transport of photoassimilates.

Calcium reserve for plant metabolism

Since immobilization of excess cytosolic calcium is one of the most important functions of CaOx crystals, it is natural that the amount of these crystals would be highly correlated with the amount of calcium available to the individual, as observed by several authors with different species of plants (Zindler-Frank, 1975, 1995; Franceschi & Horner, 1979; Zindler-Frank *et al.*, 1988, 2001; Mazen *et al.*, 2003; Franceschi & Nakata, 2005; Smith *et al.*, 2009), algae (Pueschel & West, 2007) and fungi (Tuason & Arocena, 2009, and references cited therein). Using the leguminous *Albizia julibrissin* and *Gleditsia triacanthos*, Borchert (1985) showed how inducible (and Ca^{2+} dependent) CaOx formation is in plants; at higher calcium concentrations crystals developed in up to 90% of mesophyll cells, whereas at low calcium concentrations crystals were restricted to the bundle sheath cells surrounding the veins. According to Borchert (1986), while calcium uptake, oxalate synthesis and the precipitation of CaOx are operational in the crystal cells of *G. triacanthos*, these processes must be induced in mesophyll cells by exposure to calcium.

The functionality of CaOx crystals as a calcium reserve has been questioned, mainly due to restrictions regarding calcium transport in phloem, which almost prohibits translocation. In fact, calcium transport in phloem can only occur at low to negligible rates as a result of its low concentration in the cytosol. This has led some authors to be quite restrictive, such as White & Broadley (2003), who stated 'Ca cannot be mobilized from older tissues and redistributed via the phloem', or admitting a certain rate of mobilization, such as Gilliham *et al.* (2011), who stated that 'once Ca is deposited in vacuoles it is rarely redistributed'. In fact, transport via xylem is essentially towards the transpiratory stream; so, calcium stored in plant cells can only be mobilized and transported a long distance following the transpiratory stream, which precludes, for example, directing calcium stored in a basal leaf towards an upper leaf. An interesting and ingenious experimental assay published by Malone *et al.* (2002) deserves mentioning with regard to this point: using leaf heating they were able to promote a reversal of the flow in the xylem, causing, in this artificial condition, calcium to be mobilized from an old leaf and transported by xylem.

Some authors observed a reduction in the quantity and/or size of CaOx crystals when plants were submitted to calcium starvation, concluding that the calcium had been mobilized from them (Calmés & Carles, 1970; Franceschi, 1989; Volk *et al.*, 2002; Wu *et al.*, 2006). However, a reduction in crystal size does not necessarily mean the translocation of the eventually released calcium. In seeds of *Lotus miyakojimae* (Leguminosae), Yamauchi *et al.* (2013) observed that CaOx crystals appeared at the seed filling stage, being associated to the procambial strands. However, even with a high number of crystals in cotyledons, they were not degraded during germination, suggesting that CaOx crystals are not useful for calcium supply, thereby weakening the hypothesis that these crystals act in the regulation of calcium during germination. However, Ilarslam *et al.* (2001) observed that CaOx crystals are formed in soybean seeds during the initial stage of development and are subsequently removed as embryonic development proceeds, suggesting that these crystals are a source of calcium for embryonic development. Notice that there is no contradiction between the results of Ilarslam *et al.* (2001) and those of Yamauchi *et al.* (2013): in soybean CaOx acts as a source of calcium to neighboring cells inside the embryo, whereas in *L. miyakojimae* these crystals accumulate so late during seed formation and are not mobilized during germination since calcium would need to enter phloem cells to be directed to the new developing plant organs, and there are restrictions for calcium transport, as previously discussed.

The bioavailability of calcium in crystals was demonstrated in an elegant and convincing manner by Volk *et al.* (2002) using *Pistia stratiotes* (Araceae) subjected to calcium starvation. Oxalate oxidase, the enzyme that degrades oxalate, was located on the surfaces of crystals, showing that calcium can be remobilized from CaOx crystals.

Therefore, the question is not about the bioavailability of calcium from these crystals, which is readily available not only in plants but also in fungi (Tuason & Arocena, 2009). The dissolution, as well as the process of crystallization of CaOx, are reversible processes and depend on physico-chemical factors such as concentration, pH and the presence of nucleators (in the case of crystallization), and so they likely occur inside vacuoles.

The central point, which is neglected by most authors, is answering this question: is this known stock of bioavailable calcium functional?

The answer is simple: it depends!

In most cases, the calcium reserve is not functional, since the calcium from the dissolution of the crystals cannot reach the plant organs/cells that need calcium. The reason for this paradox is that the calcium present in leaves or in basal portions of the stem cannot reach, for example, the root system or the apical meristems, which demand a supply of calcium. In these cases, calcium translocation is prevented by the low ability of phloem to transport calcium. The low mobility of calcium in phloem was well documented by Tang & Luan (2017), who stated that 'calcium deficiency symptoms are usually observed in the young tissues partly because calcium is an immobile nutrient that can hardly be remobilized from old tissues and redistributed via the phloem'. Therefore, the calcium reserve contained in crystals is not functional, in most cases, for long-distance translocation, which explains the occurrence of symptoms of calcium deficiency in plants even under adequate calcium levels. According to Dayod *et al.* (2010), limitations on calcium transport can lead to local deficiencies despite abundance in supply. As pointed out by Malone *et al.* (2002), 'the situation can arise in which older leaves contain very high levels of calcium, whilst young developing fruit nearby on the same plant are severely deficient'.

When does the calcium reserve work?

Calcium oxalate crystals accumulated in roots, especially in cells associated with xylem or near this tissue, are liable to have the calcium reserve mobilized and translocated, over a short distance, via the apoplast until reaching tracheary cells. Once in the xylem this calcium can be directed, through the transpiratory stream, to any portion of the aerial part, meeting the calcium demand in the same way as the calcium just absorbed from the substrate. In fact, using *Lemna minor* (Araceae) roots, Franceschi (1989) showed how fast and reversible CaOx crystal formation is, allowing it to be recognized how efficient the CaOx crystals are as calcium reserves in roots.

In any portion of a plant, if calcium demand is adjacent to cells that accumulate CaOx crystals, the mobilization of calcium from them can be possible. Indeed, apoplastic transport (cell-to-cell) is effective for calcium, even on cells other than xylem tracheary elements, as shown by White (2001). Therefore, we can predict calcium movements from the crystals to the neighboring cells that demand this mineral regardless of xylem transport. In his assay regarding CaOx crystals in *L. minor* roots, Franceschi (1989) stated that 'each idioblast 'services' a finite tissue volume, thus allowing adjacent cells to perform their normal functions'. In the anther tapetum, for example, this situation seems to be quite common: CaOx crystals were observed in tapetum cells of species of Commelinaceae and other families, and seems to serve as a calcium source during the development of pollen grains (see Gebura & Winiarczyk, 2016, and references cited therein). In this case, to understand the relevance of crystals as a calcium source it is necessary to consider that the anthers are confined in the floral buds and, consequently, have a low transpiratory rate; thus, phloem is the main supplier of water, minerals and carbohydrates to these structures. Therefore, the transport of calcium to anthers is slow and gradual given the difficulty of translocation of this ion in phloem, a situation similar to that reported by Hocking *et al.* (2016) for fruits. This is, therefore, a case where the calcium reserve from CaOx crystals is functional because it will meet the demand for calcium in neighboring cells.

Reserve organs such as tubers or rhizomes could mobilize calcium from the CaOx crystals, which could reach the new organs via xylem. Sunell & Healey (1979) showed evidence that CaOx crystals in *Colocasia esculenta* (Araceae) serve as a calcium reserve that can be mobilized to new aerial organs during resprouting.

It is necessary to reemphasize that calcium reserve represented by CaOx crystals is functional in roots and/or in the basal portions of the stem, and can be mobilized to any part of the plant body; however, in most of the aerial part this reserve is limited to attend demand for calcium from neighboring cells and cannot be translocated over long distances.

Concluding remarks

If in most cases the reserve of calcium as CaOx crystals is poorly functional, what is the reason for the frequent accumulation of crystals in most plants?

Calcium oxalate crystals as calcium reserve is an obvious fact, but, in an apparent contradiction they are not an effective calcium reserve in most situations because it is not functional and plants die by calcium starvation even with a plentiful presence of such crystals.

Crystal idioblasts usually appear scattered in epidermal or parenchymatic tissue layers, especially in leaves and stems, and noticeably near veins and especially near phloem (see Paiva & Machado, 2005). Most eudicots, which present secondary growth, discard nonfunctional phloem by ritidome formation; most perennial plants lose their old leaves; and cortex and epidermis of stem and root are usually lost due to the secondary growth – all of these disposable parts and tissues have an abundance of CaOx crystals. This seems to be strong evidence that the main function of these crystals is not Ca^{2+} reserve, because if it were, they would not be stored in organs or tissues that are frequently discarded. Plants do not have an excretory system, and so have no way to eliminate excess calcium other than by eliminating the cells, tissues or organs in which it is stored. Therefore, the elimination of excess calcium emerges as a relevant function of CaOx crystals for plants. However, there is no indication that this pathway for the elimination of excess calcium is relevant for all plants, such as mutants of *M. truncatula*, for example, in which the formation of these crystals is not essential for normal growth and development (Nakata & McConn, 2000, 2003). In this sense, an interesting relation between the elimination of the excess of cytosolic calcium, through the formation of CaOx crystals, was presented by De Silva *et al.* (1996) in two calcicoles in which the formation of these crystals in trichomes or mesophyll parenchyma cells adjusts the level of calcium to allow the adequate functioning of the stomata.

Excess calcium as CaOx crystals eliminated in this manner is recycled when discarded organs decompose, thus returning a noticeable amount of calcium from CaOx crystals to the soil (Smith *et al.*, 2009; Dauer & Perakis, 2014; Borrelli *et al.*, 2016; Uren, 2018). However, some species of Cactaceae, which are devoid of leaves and incapable of discarding nonfunctional phloem,

accumulate CaOx crystals, which can reach up to 80% of their dry weight (see Franceschi & Nakata, 2005), reinforcing this hypothesis of excess calcium excretion as the function of CaOx crystals.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES, Finance Code 001). EASP thanks the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil, process 305638/2018-1) for a research grant and Dr Denise M. T. Oliveira for her helpful comments.

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Key words: calcium oxalate, calcium reserve, calcium transport, excretion, phloem, plant crystals.

Received, 2 April 2019; accepted, 3 May 2019.