The Calcium Conundrum. Both Versatile Nutrient and Specific Signal[1]

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Versatility and specificity are usually mutually exclusive terms. However, as we discuss calcium's role in plant nutrition, we are obliged to contrast the plethora of general housekeeping functions of this element against the ability of calcium (Ca^{2+}) to impart signaling specificity during biological responses (Marschner, 1995). Plants have evolved to rely on the unique properties of Ca^{2+} for a range of structural, enzymatic, and signaling functions. In this brief update, I will attempt to touch upon the various roles of Ca^{2+} in plant nutrition, growth, and development.

Ca^{2+} is a relatively large divalent cation, and in contrast to other macronutrients, a high proportion of total Ca^{2+} is found in the cell walls (apoplast). Some of this Ca^{2+} is associated to the cell wall, while another portion is exchangeable at the plasma membrane. Additionally, Ca^{2+} can be found at high concentrations within the vacuole of plant cells. In some tissues of particular plants, Ca^{2+} can be more than 10% of the dry weight and cause no deleterious effects to plant growth (Marschner, 1995). Juxtaposed against these concentrations of total calcium, the cytosolic-free Ca^{2+} activity within these cells remains around the 0.1 to 0.2 μM level. In this update, the first portion will detail the general role of Ca^{2+} in plant growth and development. In the middle section, we will focus on how almost imperceptible fluctuations in Ca^{2+} within the cytosol may be translated into plant growth and adaptation. This then leads to the final part of this broad review, to a discussion regarding how biologists are attempting to manipulate Ca^{2+} levels to improve plant productivity and human nutrition.

"EN GARDE": CALCIUM PROVIDES MEMBRANE STABILITY AND STRESS TOLERANCE

While we need Ca^{2+} to build strong bones, plants need Ca^{2+} to strengthen cell walls and provide stress protection. When human diets are low in Ca^{2+}, this leads to fragile bones or osteoporosis. With plants, soils or media deficient in Ca^{2+} can cause the disintegration of cell walls and the collapse of the affected tissues (Kirby and Pilbeam, 1984). Ca^{2+} deficiencies have been associated with bacterial diseases, fruit rotting, and other postharvest problems (Marschner, 1995). Thus, Ca^{2+} is cited for its beneficial effect on plant vigor and stiffness and also on grain and seed formation (Bennett, 1993). Increasing the Ca^{2+} content of fruits, for example, by spraying several times with Ca^{2+} salts during fruit development or by postharvest dipping in $CaCl_2$ solution, leads to an increase in firmness of the fruit and can delay fruit ripening (Bramlage, 1994; Bramlage and Weis, 1994).

Ca^{2+} stabilizes cell membranes by connecting various proteins and lipids at membrane surfaces. Additionally, Ca^{2+} can be exchanged with other cations (K^+, Na^+, or H^+) during stress responses. To protect the plasma membrane from various stresses, Ca^{2+} must always be present in the external solution, where it can regulate the selectivity of ion uptake. In fact, when plants are challenged with salinity stress, an increase in the concentration of Ca^{2+} often can ameliorate the inhibitory effect on growth (Epstein, 1972). In general, Ca^{2+} is involved in a plethora of plant functions (Marschner, 1995). Ca^{2+} is involved in cell elongation and cell division, influences the pH of cells, and also acts as a regulatory ion in the source-sink translocation of carbohydrates through its effect on cells and cell walls.

When Ca^{2+} is deficient in the media/soil, there will be problems with cell wall stability (see above; Marschner, 1995). Ca^{2+} in the xylem sap is translocated upward in the transpiration system, but once deposited, it is almost immobile. As a result of this immobility, deficiency symptoms are most pronounced in young tissues—where cell division is occurring. However, true Ca^{2+} deficiencies in soils are rather rare, and problems associated with low soil Ca^{2+} may be attributed to soil problems rather than a true Ca^{2+} deficiency. For example, Ca^{2+} deficiencies are favored by very low soil pH and on soils high in magnesium and potassium. Probably the most recognizable Ca^{2+} deficiency, especially to the weekend gardener, is blossom-end rot of tomato fruits, which is induced

by water stress (Bennett, 1993). At the time of fruit set, cells at the blossom end of fruits are injured when insufficient Ca^{2+} translocation to the flower results in a dry-rot area on the expanding fruit (Fig. 1).

The distribution of Ca^{2+} at the cell wall and plasma membrane is mainly the result of a plethora of binding sites for Ca^{2+} in the cell walls as well as the carefully regulated transport of Ca^{2+} into the cytoplasm (Han et al., 2003; see below). Pectins and pectates bind a large portion of Ca^{2+}. As Ca^{2+} supplies increase within certain cells, the proportion of Ca^{2+} oxalate increases. In certain situations, oxalate-bound Ca^{2+} may represent the dominant binding form of Ca^{2+} (Fig. 1; Nakata, 2003). Intracellular Ca^{2+} is also found in the endoplasmic reticulum (ER) and chloroplast, and most of the water-soluble calcium is in the vacuole. By virtue of their size and capacity for calcium accumulation, the vacuole is the most prominent sink for calcium storage.

Much of our knowledge regarding the role of Ca^{2+} in cell wall stability and expansion has been obtained from classic physiology experiments. Since it is difficult (impossible?) to generate plant Ca^{2+} auxotrophs, the onus is now on plant biologists to generate a set of molecular tools to unravel the mechanisms behind these actions (Braam, 1999).

"HUSHHHHH": KEEPING FREE CYTOSOLIC CALCIUM LEVELS LOW

Numerous cytosolic proteins bind Ca^{2+} to dampen free cytosolic Ca^{2+} concentrations (Sanders et al., 1999; Luan et al., 2002; Sanders et al., 2002). Some of the most prominent Ca^{2+}-binding proteins are the molecular chaperone binding proteins, calnexin, calsequestrin, and calreticulin (CRT; Pittman and Hirschi, 2003). Of these, CRT is responsible for the main Ca^{2+}-retaining pool in plants. During signal transduction events (see below), proteins like calmodulin and Ca^{2+}-dependent protein kinases (CDPKs) also bind Ca^{2+}. Calmodulin and CDPKs contains four Ca^{2+}-binding EF-hand motifs. Arabidopsis appears to have around 250 putative proteins that contain at least one EF-hand motif (Day et al., 2002). Future work will need to be directed at the function of these various proteins in Ca^{2+} homeostasis, signaling, and plant architecture.
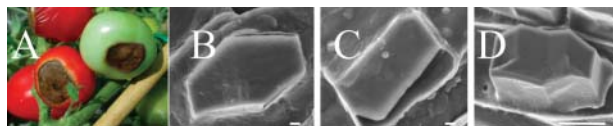
Figure 1. A, Blossom-end rot of tomato is caused in part by calcium deficiencies. Scanning electron micrographs of calcium oxalate crystals from *Trifolium pratense* (B), *Vigna unguiculata* (C), and *Vicia faba* (D). Note the different shapes among the various plants. Scale bar = 1 μm (from Nakata, 2003, with permission).

Another means of reducing cytosolic calcium levels is by transporting the Ca^{2+} into endomembranes such as the ER, chloroplast, and vacuole (Sze et al., 2000). Homeostasis of Ca^{2+} must be achieved by moving Ca^{2+} out of the cytosol across the plasma membrane and various endomembranes. Efflux across the plasma membrane may be the ultimate fate of excess cytosolic Ca^{2+} because both biochemical buffering and sequestration have finite capacities. At the plasma membrane, the Ca^{2+} concentration ratio (inside/outside) is typically of the order of 10^{-4}. Efflux of Ca^{2+} from the cytosol is mediated by pumps energized by either ATP hydrolysis or the proton motive force. Passive entry of Ca^{2+} into the cytosol is mediated by ion channels (Sanders et al., 2002).

## "STOP AND GO": CHANGES IN CYTOSOLIC CALCIUM LEVELS MEDIATE PLANT RESPONSES

Ca^{2+} is a fundamental component of eukaryotic signaling. Ca^{2+}-triggered events are critical for both normal cellular activity and for adapted responses (Sanders et al., 2002). At one level, these Ca^{2+} signaling events appear simple: cells at rest have a low level of cytosolic Ca^{2+} that rises during a signal transduction event. However, this signaling is quite complex when one contemplates how a ubiquitous nutrient becomes translated into a myriad of unique stimulus dependent responses.

Ca^{2+} signal transduction can be divided into four components (Fig. 2; Berridge et al., 2000): (1) signaling elicits various Ca^{2+} mobilizing signals; (2) the mobilizing signals feed Ca^{2+} into the cytoplasm generating the ON events; (3) Ca^{2+} functions as a messenger to activate Ca^{2+} sensitive processes, which are mediated by proteins like calmodulin and CDPKs; and (4) the OFF mechanisms, composed of transporters and binding proteins, remove the Ca^{2+} from the cytoplasm to restore the resting state.

These distinctions are somewhat arbitrary given that the mechanisms are tightly coupled and there is often a link between Ca^{2+} efflux or depletion and Ca^{2+} entry. A central facet of these four components of signaling is that local spatial and temporal patterns of Ca^{2+} signals are important in encoding the specificity of cellular responses (Putney, 1998). In other words, Ca^{2+} signaling, like real-estate prices, depends on location.

These localized alterations in Ca^{2+} must be judiciously regulated. A marked and prolonged increase in Ca^{2+} is harmful to cells because it leads to activation of particular Ca^{2+}-dependent enzymes, having a potentially adverse effect. In some mammalian cells, Ca^{2+} overload can also cause mitochondrial failure; if this becomes irreversible it leads to cell death (Duchen, 2000).

There are several fundamental aspects of plant Ca^{2+} signaling that should be noted. While animals predominately utilize Na^+ as the coupling ion to circulate
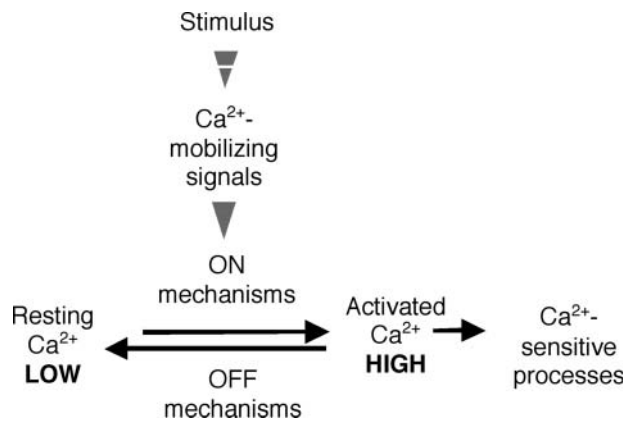
Figure 2. The four units of the Ca^{2+}-signaling network (Berridge et al., 2000). Stimuli act by generating Ca^{2+}-mobilizing signals that act on various ON mechanisms to trigger an increase in the intracellular concentration of Ca^{2+}. The response is terminated by OFF mechanisms that restore Ca^{2+} to resting levels.

Ca^{2+} across biological membranes, plants almost exclusively use protons as the coupling ion (Sze et al., 1999). Also, the design and architecture of the plant cell mediate spatial features to the Ca^{2+} spike not seen in mammalian systems—in particular, the Ca^{2+} spikes around the vacuole. Did you know the plant vacuole can occupy up to 99% of a plant cell's volume (Marty, 1999)? Aside from the Ca^{2+} fluctuations that occur around the ER and plasma membrane, various findings now suggest that localized Ca^{2+} spikes around the plant vacuole play a pivotal role in determining a plant's growth, development, and adaptation to environmental responses (Sanders et al., 2002). However, Ca^{2+} spikes in and around the mitochondria and other endomembranes are also likely to be important in particular cellular responses (Logan and Knight, 2003).

The technology to visualize fluctuations in cytosolic Ca^{2+} levels and combine these approaches with molecular genetics is an exciting new vista in plant biology (Allen et al., 1999; Kiegle et al., 2000). For example, this technology has been used to visualize the perturbations in cytosolic calcium oscillations associated with reduced vacuolar H^+-ATPase (V-ATPase) activity (Allen et al., 2000, 2001). These studies have gone on to demonstrate that specific cytosolic Ca^{2+} oscillations are essential to elicit processes like stomatal closure.

DESIGNING FOR YIELD: ALTERING CALCIUM LEVELS FOR INCREASED PLANT PRODUCTIVITY

For more than 2,000 years, it has been standard agricultural practice to add mineral elements to soils to improve plant growth. As we mentioned previously, increased Ca^{2+} levels in the soils can improve membrane stability, and Ca^{2+} is applied to soils to ameliorate salinity effects and decrease pathogen infection. It is also added exogenously to ripe fruits to improve durability. The genetic manipulation of the processes that govern the passage of Ca^{2+} through the cytoplasm may also have a substantial impact not only on improving growth but also on manipulating particular cellular responses (Pittman and Hirschi, 2003). For example, rice plants have been generated with increased levels of a particular CDPK (Saijo et al., 2000, 2001). The extent of tolerance to cold and salt/drought stresses of these plants correlated well with the level of CDPK proteins. Plants have also been engineered to express higher levels of the Ca^{2+}-binding protein CRT (Persson et al., 2001; Wyatt et al., 2002). These plants are more vigorous than the controls and contain slightly more total Ca^{2+} than wild-type plants. Therefore, it appears that the manipulation of CDPK, CRT, and other Ca^{2+}-binding proteins may be one way to engineer more robust plant varieties.

DESIGNING FOR FEED: INCREASING BIOAVAILABLE CALCIUM LEVELS IN FOODS

Some portion of Ca^{2+} in foods is bioavailable, meaning it is digested, absorbed, and metabolized. This bioavailable Ca^{2+} affects various developmental processes, including bone formation and calcification. An estimated \$13.8 billion in health-care costs each year is used on osteoporosis-related care (Bachrach, 1999, 2001). Unfortunately, the majority of people do not consume enough Ca^{2+}. The low Ca^{2+} content of the most widely consumed vegetables make them a minor contributor to the Ca^{2+} intake for most Americans (Weaver et al., 1999). Many plant foods are enriched in Ca^{2+}, but the Ca^{2+} is often found sequestered as an oxalate salt (Fig. 1). Oxalate is an antinutrient that sequesters Ca^{2+} in a state that renders it unavailable for nutritional absorption (Weaver et al., 1987). In general, Ca^{2+} absorption is inversely proportional to the oxalic acid content of the food. The ability to genetically alter the Ca^{2+} content of agriculturally important crops is just emerging.

Recently, scientists have manipulated Ca^{2+} oxalate crystal formation in *Medicago truncatula* (a forage legume; Nakata, 2003). Medicago is not consumed by humans; however, the plant contains Ca^{2+} oxalate crystals in the leaf tissue that are very similar to those found in other plant foods such as spinach (Fig. 1). Several allelic mutants (*cod 5*) devoid of crystal formation have been isolated. In the greenhouse, plant phenotype and growth studies indicated little difference between the *cod 5* and unmutagenized control plants. Oxalate measurements show that *cod 5* has oxalate levels at the limit of detection. Ca^{2+} levels, on the other hand, are comparable to unmutagenized control plants. Overall, the isolation of *cod 5* shows the feasibility of manipulating Ca^{2+} oxalate formation in

plants, including many crop plants (e.g. leafy green vegetables), via a mutation at a single loci. These findings suggest that in the future, it may be possible to alter the function of a single gene in spinach to remove all Ca^{2+} oxalate crystals.

Another approach to alter the content of bioavailable Ca^{2+} content in plants is to engineer high expression of Ca^{2+} transporters in the edible portion of the plant. Simplistically, this strategy can be thought of as nutrient mining, where the nutrient is transported from the soil into the edible portions of the plant. Specifically, one potential model for increasing the Ca^{2+} content in edible foods would be to manipulate plant endomembrane transporters to transport more Ca^{2+}. In plants, we have characterized a vacuolar Ca^{2+} antiporter termed cation exchanger 1 (CAX1). In both tobacco and carrots, high-level expression of CAX1 displays dramatic increases in calcium content when compared to vector control plants (Hirschi, 1999; Park et al., 2004). In a similar fashion, ectopic expression in tobacco of a wheat cation transporter LCT1 increases shoot Ca^{2+} levels (Antosiewicz and Hennig, 2004). In the future, using mouse and human feeding studies, biologists will test if these genetically modified plants have altered calcium bioavailability.

CONUNDRUM REDOX

In this general overview, I have attempted to illustrate that while Ca^{2+} is required for basic plant nutrition, it is also the most common signal transduction element in all eukaryotic cells. To paraphrase the paradox, Ca^{2+} levels can climb to a huge percentage of the plant dry mass; however, minute fluctuations in cytosolic Ca^{2+} levels determine how plants respond to developmental and external cues. While Ca^{2+} is required for life, prolonged high intracellular Ca^{2+} levels lead to cell death. Ca^{2+} cannot be metabolized like other second messenger molecules, so cells tightly regulate cytosolic levels through numerous binding proteins and transporters.

Remember, next time you bite into an apple or squeeze a tomato, you are, in part, assessing the Ca^{2+} status of the fruit. Using the tools of modern molecular genetics and in vivo Ca^{2+} imaging, plant scientists are trying to assess the cytosolic Ca^{2+} fluctuations in plants. The outcome of these studies should aid in conceptualizing and harnessing this useful signal/nutrient.

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