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REVIEW

Plant calcium oxalate crystal formation, function, and its impact on human health

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Abstract **Crystals of calcium oxalate have been observed among members from most taxonomic groups of photosynthetic organisms ranging from the smallest algae to the largest trees. The biological roles for calcium oxalate crystal formation in plant growth and development include high-capacity calcium regulation, protection against herbivory, and tolerance to heavy metals. Using a variety of experimental approaches researchers have begun to unravel the complex mechanisms controlling formation of this biomineral. Given the important roles for calcium oxalate formation in plant survival and the antinutrient and pathological impact on human health through its presence in plant foods, researchers are avidly seeking a more comprehensive understanding of how these crystals form. Such an understanding will be useful in efforts to design strategies aimed at improving the nutritional quality and production of plant foods.**

Keywords calcium, oxalate, crystals, biomineral, idioblast, nutrition

Introduction

Plant calcium oxalate crystals were among the first objects reported by Leeuwenhoek based on his observations using a simple light microscope (Leeuwenhoek, 1675). Since this initial report, crystals of calcium oxalate have been observed among members of most plant families. Many plants, including numerous crop plants, accumulate oxalate in the range of 3%–80% (w/w) of their dry weight with as much as 90% (w/w) of the total calcium of a plant occurring in the form of calcium oxalate crystals (McNair, 1932; Gallaher, 1975; Zindler-Frank, 1976; Libert and Franceschi, 1987; Horner and Wagner, 1995).

Based on their morphology, plant crystals are often classified into one of five categories: crystal sand, raphide, druse, styloid, or prismatic (Franceschi and Horner, 1980). The conservation of crystal morphology and spatial distribution within specific taxa are features useful in plant taxonomy and systematics (Horner and Wagner, 1995; Prychid and Rudall, 1999; Lersten and Horner, 2000; Monje and Baran, 2002; Hartl et al., 2007; Lersten and Horner, 2008a; Lersten and Horner, 2008b; Lersten and Horner, 2009; Lersten and Horner, 2011).

In this paper, I update our current understanding of selected aspects of calcium oxalate crystal formation and function, and highlight the potential for improving the nutritional value of plant foods through genetic manipulation of its calcium oxalate content. For additional insights into aspects of crystal formation and function not covered here the reader is encouraged to consult a number of earlier reviews (Arnott and Pautard, 1970; Hodgkinson, 1977; Franceschi and Horner, 1980; Libert and Franceschi, 1987; Zindler-Frank, 1987; Franceschi and Loewus, 1995; Horner and Wagner, 1995; Prychid and Rudall, 1999; Webb, 1999; Nakata, 2003; Franceschi and Nakata, 2005)

Oxalate biosynthesis

A number of oxalate biosynthetic pathways have been proposed to occur in plants. Isocitrate, glycollate, glyoxylate, oxaloacetate, and ascorbate have all been suggested as possible precursors in the biosynthesis of oxalate (Franceschi and Nakata, 2005; Kostman et al., 2007; Nakata and McConn, 2007b; Nakata and McConn, 2007c; Melino et al., 2009; Parsons and Fry, 2012). Of these precursors, ascorbic acid is most widely considered the primary substrate

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for the biosynthesis of the oxalate used in the formation of calcium oxalate crystals.

Radiolabel tracer studies, conducted with several plants, support ascorbate as the major precursor of oxalate production. The proposed mechanism involves a C2/C3 cleavage of ascorbate to produce oxalic and threonic acid (Wagner and Loewus, 1973; Loewus et al., 1975; Yang and Loewus, 1975; Nuss and Loewus, 1978; Li and Franceschi, 1990; Saito et al., 1997; Loewus, 1999). Microautoradiography studies, using C1-labeled ascorbate or precursors leading to C1-labeled ascorbate, allowed visual detection of the incorporation of oxalate into all 5 crystal morphological categories while the use of other proposed substrates did not result in labeled crystals (Horner et al., 2000; Keates et al., 2000; Kostman et al., 2001; Kostman et al., 2007).

The conversion of ascorbate to oxalate has been shown to occur both intracellularly (Kostman et al., 2001) and extracellularly (Green and Fry, 2005). Evidence supporting the intracellular production of oxalate showed that the biosynthesis of the ascorbic acid and its subsequent conversion to oxalate occurs directly within the calcium oxalate accumulating crystal idioblast (Kostman et al., 2001). The detection of the enzyme, L-galactono-γ-lactone dehydrogenase, responsible for the conversion of L-galatonolactone to ascorbic acid within the mitochondria of the crystal idioblast supports these findings (Kostman and Koscher, 2003). In addition, immunological (Kausch and Horner, 1985; Li and Franceschi, 1990) studies suggest that the crystal idioblast lacks glycolate oxidase, the enzyme proposed to catalyze the production of oxalate from glycolate or glyoxylate. Identification of an enzyme responsible for the conversion of ascorbate to oxalate; however, has remained elusive.

Evidence supporting the extracellular production of oxalate was gathered using Rosa suspension cells (Green and Fry, 2005). The proposed extracellular pathway of oxalate production is thought to proceed non-enzymatically through novel metabolic intermediates *in vitro* and by extracellular enzymatic steps in the presence of cultured Rosa cells (Green and Fry, 2005; Parsons et al., 2011). Pathway investigations using this culture system led to the identification of three oxalyl-threonate intermediates in the extracellular growth media (Green and Fry, 2005; Parsons et al., 2011). Whether this pathway of oxalate production is utilized in calcium oxalate crystal formation remains to be determined, but it is an intriguing possibility.

Although ascorbate is likely to be the major substrate in the production of the oxalate used in calcium oxalate formation there are some recent studies that indicate that other pathways may exist. In contrast to an earlier study (Guo et al., 2005), Yu and his colleagues favor glyoxylate as the precursor for oxalate biosynthesis in rice (Yu et al., 2010). This proposed conversion of glyoxylate to oxalate is thought to be independent of glycolate oxidase (Xu et al., 2006) but the specific enzyme involved remains unknown. A genetic investigation using the *Medicago truncatula* calcium oxalate defective (*cod*) mutants indicated that independent pathways of oxalate biosynthesis and calcium oxalate formation for the two crystal types, druse and prismatic, exist (Nakata and McConn, 2007b). Whether the pathways leading to druse and prismatic oxalate crystals are actually "different" in that they use different precursors (e.g., ascorbate and some other precursor) and different enzymes or are "parallel" in that they use the same precursor (e.g., ascorbate), but have cell-specific transcription factors and/or isozymes (encoded by different nuclear genes) responsible for the conversion of ascorbate into oxalate remains to be determined. The presence of different or parallel pathways of calcium oxalate formation for the druse and prismatic crystals is plausible especially if the two crystal types serve specialized functional roles (Volk et al., 2002; Nakata and McConn, 2007b).

Calcium oxalate crystal idioblast

Crystals of calcium oxalate most commonly form within the vacuoles of specialized cells called crystal idioblasts (Fig. 1). The crystal idioblast ultrastructure has been studied in a number of plants (Arnott and Pautard, 1970; Franceschi and Horner, 1980; Horner and Wagner, 1995; Prychid and Rudall, 1999; Webb, 1999; Nakata, 2003; Franceschi and Nakata, 2005; Prychid et al., 2008). Such examinations have revealed that these specialized cells often exhibit characteristic features which include an enlarged nucleus, specialized plastids, increased ER, abundant golgi, elevated levels of rRNA, and unique vacuolar components (Arnott and Pautard, 1970; Franceschi and Horner, 1980; Li and Franceschi, 1990; Horner and Wagner, 1995; Webb, 1999; Kostman and Franceschi, 2000; Kostman et al., 2003; Nakata, 2003; Franceschi and Nakata, 2005).

Plastids

Many crystal idioblasts contain unique plastids (Fig. 1) called crystalloplastids (Arnott and Pautard, 1970). These crystalloplastids often lack thylakoids, rubisco, and photosynthetic capacity (Li and Franceschi, 1990). It has been speculated that the crystalloplastids might be the biosynthetic site of lipids required in the formation of the abundant membrane systems or as the biosynthetic site of oxalate used in the formation of the calcium oxalate crystal (Franceschi and Nakata, 2005). Interestingly, the calcium oxalate defective (*cod*) 4 mutant which has an increase in the number of cells accumulating crystals in its leaves, shows a corresponding decrease in chlorophyll content and presumed photosynthetic capacity (Nakata and McConn, 2003b). An increase in the number of chloroplasts differentiating into crystalloplastids is one possible explanation for this observation.

Endoplasmic reticulum

Crystal idioblasts have been observed to contain prolific networks of ER (Fig. 1) that extend throughout the cytoplasm (Lazzaro and Thomson, 1989; Horner and Wagner, 1995; Pennisi et al., 2001; Kostman et al., 2003; Nakata, 2003; Nakata et al., 2003; Franceschi and Nakata, 2005). Although this ER abundance may be partly associated with the high metabolic activity of the idioblast, as indicated by its rRNA content and rapid idioblast growth (Kostman and Franceschi, 2000), abundant ER is also needed for regulating the high influxes of calcium that can occur during calcium oxalate crystal formation (Franceschi et al., 1993; Kostman et al., 2003; Nakata et al., 2003; Mazen et al., 2004). For the ER to function in the regulation of calcium flux, it must possess a mechanism(s) to reduce its luminal calcium concentration to prevent the precipitation of calcium with phosphate and other anions. An initial report indicated that a calsequestrin-like calcium binding protein might function in this capacity (Franceschi et al., 1993). Since this first report, the high-capacity calcium binding protein calreticulin has been shown to be enriched in the crystal idioblast (Nakata et al., 2003). Protein localization studies showed that calreticulin was concentrated within distended regions of the idioblast ER (Nakata et al., 2003). Such an observation has been reported in other cell types where calreticulin was overexpressed to 100-fold that of wild-type levels (Crofts et al., 1999). Interestingly, these distended regions of idioblast ER appear to be the same regions found to be enriched in calcium (Kostman et al., 2003). Therefore, the high-capacity calcium binding protein, calreticulin, likely functions in sequestering the calcium within these distended regions which reduce the luminal calcium concentration in other areas of the ER. Such a mechanism would be crucial in controlling high calcium flux that has been shown to occur, in some plants, during rapid crystal formation (Franceschi, 1989; Mazen, 2004).

Intravacuolar matrix

A striking feature of the crystal idioblast is the intravacuolar matrices that are associated with the calcium oxalate crystals (Fig. 1). These intravacuolar matrices (e.g., crystal chambers) are macromolecular structures that appear to play a role in defining the shape and growth of the crystal (Arnott and Pautard, 1970; Webb and Arnott, 1983; Barnabas and Arnott, 1990; Horner and Wagner, 1995). The crystal chamber, often composed of lipids and polysaccharides separates the vacuolar compartment from the space in which the crystal develops. These chambers are formed *de novo* within the vacuole and do not appear to be simply elaborations of the tonoplast membrane (Mazen et al., 2004). Based on the shape of these membrane chambers the morphology or shape of the crystal can often be predicted before it actually forms (Arnott and Pautard, 1970; Webb and Arnott, 1983; Barnabas and Arnott, 1990; Horner and Wagner, 1995). In some cases, the crystals have been observed to be encased in cell wall material (Frank and Jensen, 1970; Webb and Arnott, 1981), or surrounded by lamellated sheaths (Kausch and Horner, 1984;

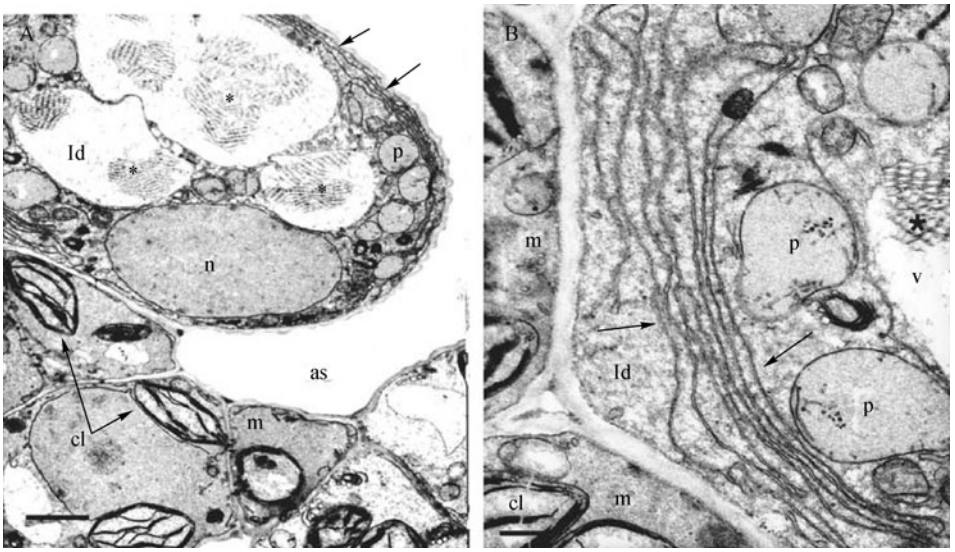

Figure 1 Ultrastructure of the calcium oxalate raphide crystal idioblast. (A) Low magnification TEM of a cross-section through a developing raphide crystal idioblast and adjacent mesophyll cells (for comparison) from a *Pistia stratiotes* leaf. Note some of the unique characteristic features of the crystal idioblast which include an enlarged nucleus, specialized plastids, increased ER, and unique vacuolar components. Bar = 2 μm. (B) Higher magnification view of crystal idioblast ER stacks, modified plastids, and adjacent mesophyll cells. The raphide calcium oxalate crystals appear as rectangular white profiles (*) within the vacuole. The ER stacks are denoted by arrows. Id, idioblast; m, mesophyll cell; n, nucleus; cl, chloroplast; p, plastid; v, vacuole; as, airspace. Bar = 1 μm. (Images used with permission from Elsevier, Kostman et al., 2003).

Katayama et al., 2007). These surrounding structures are hypothesized to regulate crystal shape possibly by controlling the relative delivery rate of calcium and oxalate to the crystallization space. Varying the molar concentrations of calcium and oxalate has been shown to affect crystal shape, *in vitro* (Frey-Wyssling, 1981; Thongboonkerd et al., 2006). Although the genes which encode the proteins that control this delivery process have not been reported, transcripts encoding proteins putatively involved in lipid, polysaccharide, and cell wall metabolism have been identified that are expressed at higher levels during calcium oxalate crystal formation (Nakata and McConn, 2002).

Crystal-associated proteins

A fundamental process in plant calcium oxalate crystal formation is the regulation of the crystallization process within the vacuole. One level of control may be through the use of proteins that are able to direct the nucleation and growth of the forming crystal. In animals, various endogenous proteins have been shown to promote or inhibit urinary stone (calcium oxalate) formation (Ryall and Stapleton, 1995). In plants, researchers have also been able to identify proteins associated with the crystal or crystal matrix (Webb et al., 1995; Bouropoulos et al., 2001; Li et al., 2003). Some of these crystal associated proteins from tobacco (Bouropoulos et al., 2001), tomato (Bouropoulos et al., 2001), bougainvillea (Bouropoulos et al., 2001), and water lettuce (Li et al., 2003) are enriched in acidic amino acid residues. Such acidic proteins have been shown to be important in regulating the formation of other biominerals (Weiner and Addadi, 1991; De Yoreo et al., 2006; Olszta et al., 2007; Kröger and Poulsen, 2008; Furuhashi et al., 2009). *In vitro* nucleation assays revealed that the assemblage of crystal-associated macromolecules from tobacco promoted nucleation of calcium oxalate crystals, while various control polypeptides did not (Bouropoulos et al., 2001). Li et al. (2003) identified and characterized a crystal matrix protein from the aquatic plant, *Pistia stratiotes* (Li et al., 2003) that possessed features consistent with a calcium binding protein. ^{45}Ca binding assays confirmed the calcium binding capacity of this protein. Further characterization of such proteins will be important in advancing our understanding of crystal nucleation and growth.

Microtubules

Most crystal idioblasts show a considerable increase in size during calcium oxalate crystal deposition. Careful coordination of cell and crystal growth is essential, especially under circumstances of rapid crystal formation. Since crystal idioblasts (e.g., raphide and styloid) become elongated during crystal growth, researchers proposed that cytoskeletal elements such as microtubules may be playing a role in coordinating crystal and cell growth (Horner and Wagner, 1995). Researchers showed in the aquatic plant model, *Pistia stratiotes*, that raphide crystal idioblasts possess a cortical microtubule network (Kostman and Franceschi, 2000). This microtubule network appears to limit an increase in cell diameter, but not elongation. Thus, the raphide idioblast is able to elongate at its ends, generating an American football-shaped cell. Using colchicine to prevent microtubule polymerization, researchers observed that the raphide idioblasts still formed but have a compacted cell shape. Even though the shape of the idioblasts are irregular the cell still accumulate raphide needles of calcium oxalate that are shorter in length (Kostman and Franceschi, 2000). Overall, these studies support a role for microtubules in coordinating the elongation of the idioblast and crystals within the cell.

Biological function

A number of functional roles for plant calcium oxalate crystal formation have been proposed. Such functions include roles in calcium regulation, sodium and potassium balance, plant protection, tissue support (plant rigidity), detoxification (e.g., heavy metals or oxalic acid), and light gathering and reflection (Horner and Wagner, 1980; Tillman-Sutela and Kauppi, 1999; Webb, 1999; Molano-Flores, 2001; Franceschi and Tarlyn, 2002; Hudgins et al., 2003; Nakata, 2003; Franceschi and Nakata, 2005; Jou et al., 2007; Kuo-Huang et al., 2007; Coté, 2009). Although there is a lack of experimental evidence in support of some of these proposed functions, evidence has accumulated supporting roles for crystal formation in bulk calcium regulation, defense against herbivory, and tolerance to heavy metals.

Bulk calcium regulation

Both physiological and biochemical studies have demonstrated a role for calcium oxalate formation in the bulk regulation of tissue calcium concentrations (Zindler-Frank, 1975; Franceschi and Horner, 1979; Borchert, 1985; Borchert, 1986; Franceschi, 1989; Kuo-Huang and Zindler-Frank, 1998; Ilarslan et al., 2001; Pennisi and McConnell, 2001; Zindler-Frank et al., 2001; Monje and Baran, 2002; Morrow and Dute, 2002; Volk et al., 2002; Prychid et al., 2008; Nakata, 2012). The crystal idioblast appears to serve as a localized calcium sink aimed at reducing the apoplastic calcium concentration around adjacent cells (Fig. 2). Studies in support of this role have shown, using a variety of plants, that the size and number of calcium oxalate crystals are responsive to changes in the concentration of calcium in the plant environment (Zindler-Frank, 1975; Franceschi and Horner, 1979; Borchert, 1985; Borchert, 1986; Franceschi, 1989; Kuo-Huang and Zindler-Frank, 1998; Ilarslan et al., 2001; Pennisi and McConnell, 2001; Zindler-Frank et al., 2001; Monje and Baran, 2002; Morrow and Dute, 2002; Volk et al., 2002). Under conditions of calcium excess new bundles

of raphide needles have been shown to accumulation (Fig. 2), in *Lemna minor*, in as little as 30 min (Franceschi, 1989). The spacing between the idioblasts also decreases suggesting that each idioblast services a certain area of tissue and when the calcium sequestration capacity is reached new idioblasts are induced to form. When environmental conditions change and calcium becomes limited, the crystals dissolve (Fig. 2), in some cases, presumably remobilizing the bound calcium for use in growth and development (Assailly, 1954; Calmes, 1969; Calmes and Carles, 1970; Franceschi, 1989; Volk et al., 2002).

Defense against herbivory

Temporal, spatial and morphological parameters of calcium oxalate crystal formation first led to the hypothesis that crystals can play a role in plant defense. Since then, evidence has accumulated supporting a role for sharp needle-shaped crystals in protecting plants against herbivory. The plant *Tragia ramosa,* for example, is covered with stinging hairs. Each stinging hair consists of an elongated cell which encompasses a large needle-shaped styloid crystal(s) made of calcium oxalate (Thurston, 1976). When an animal comes into contact with this plant, the tip of the elongated cell ruptures exposing the needle-shaped crystal which can puncture the dermis of the animal. A toxin is then channeled from the base of the cell along a groove that runs along one edge of the needle-shaped crystal. It is this toxin which then causes the stinging sensation (Thurston, 1976).

The placement and number of crystals within the tissues of a plant have also been shown to be an important factor in deterring herbivory. While observing the feeding pattern of gazelles, researchers noticed that only the leaf tips of the desert lilies were being eaten (Ward et al., 1997; Saltz and Ward, 2000). Microscopic examination of these leaves revealed that the leaf tips were the only portion of the leaves devoid of the needle-shaped raphide crystals (Ward et al., 1997). A correlation was also noted between the amount of herbivory at a particular location and the amount of needle shaped crystals accumulated in the desert lilies at each location (Ardon valley, Neqarot canyon, and Machmal valley). The authors found that the lilies grown at the locations of high grazing stress contained the largest amount of crystals while lilies found in locations where grazing was

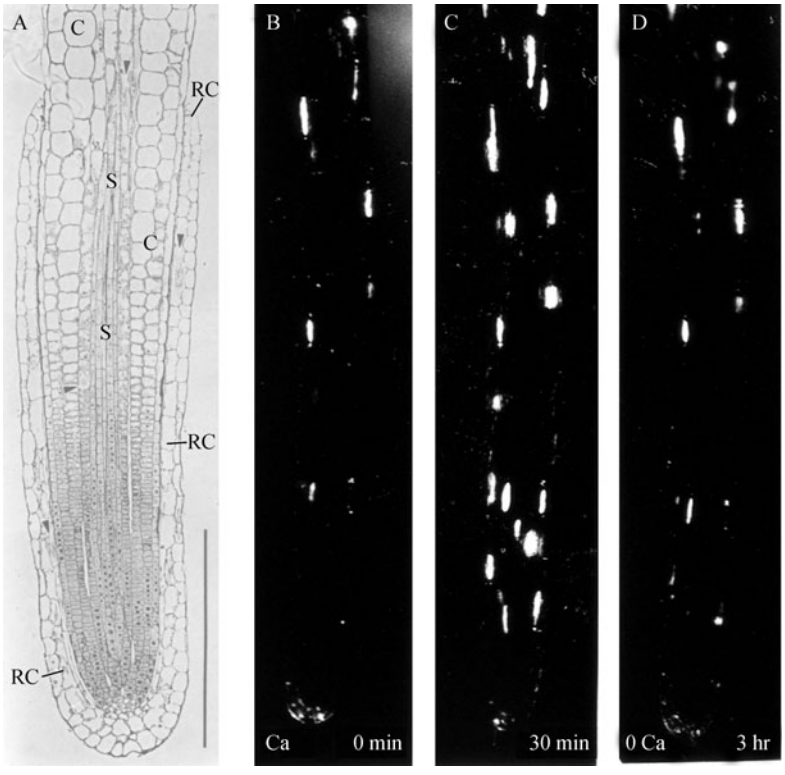

Figure 2 Calcium oxalate crystal formation in high-capacity calcium regulation. High calcium flux can result in rapid formation of calcium oxalate crystals as exhibited in this sequential series of calcium treatments using a single live root of *Lemna minor*. Under calcium deficient conditions, the calcium bound in the crystal is remobilized for use in plant growth and development. Raphide bundles of calcium oxalate crystals appear as white blotches. (A) Longitudinal section through a *Lemna* root tip. RC, root cap; S, root stele; C, root cortex. Bar, 250 μm. (B) Whole-mount of live root tip after pretreated with 0 mM calcium solution as viewed between crossed-polarizers. (C) Whole-mount of the same live root tip after a 30 minute exposure to a 7 mM calcium solution as viewed between crossed-polarizers. (D) Whole-mount of the same live root tip viewed in (B) after returned to a 0 mM calcium solution for 3 hours as viewed between crossed-polarizers. (Images used with permission from Springer-Verlag, Franceschi, 1989).

minimal accumulated few, if any, crystals. Thus, it appears that the formation of the needle-shaped crystal may have been under the selective pressure of herbivory (Ruiz et al., 2002a; Ruiz et al., 2002b).

One can easily envision that needle shaped crystals can act as a deterrent against herbivory. But what about situations where the crystals are not needle shaped, can these crystals also play a role in plant protection? This question was recently answered in studies utilizing the model legume, *Medicago truncatula*. In *M. truncatula*, as well as other legumes, prismatic crystals of calcium oxalate accumulate in cells surrounding the secondary vascular strands of leaves (Nakata and McConn, 2000). Using calcium oxalate defective (*cod*) mutants and wild type *M. truncatula*, researchers show a role for these crystals in protecting plants against chewing insects such as the caterpillar larvae of the beet armyworm, *Spodoptera exigua* (Korth et al., 2006; Park et al., 2009). Feeding experiments revealed that the caterpillar exhibit a clear feeding preference for the plants lacking the prismatic calcium oxalate crystals. Caterpillars reared on the prismatic crystal containing plants showed reduce growth rates and increased mortality compared to those fed a diet of plant material lacking crystals. Upon completion of an extensive microscopic study researchers determined that the crystals exerted their deterring effect by acting as a physical abrasive on the caterpillar mandibles (teeth) during feeding (Korth et al., 2006; Park et al., 2009).

Tolerance to heavy metals

Micromolar concentrations of aluminum can inhibit root growth and negatively affect the acquisition of water and other nutrients. This reduction in root function is a major issue in the production of many agriculturally important crop species (Kochian 1995; Ma et al., 2001; Guo et al., 2005). The growth and development of some plant species; however, are less affected by the presence of elevated levels of aluminum. Investigations into how these tolerant plants are able to grow and develop in the presence of aluminum revealed their use of organic acids such as malate, citrate, and/or oxalate to decrease the toxic effect of the metal (Ryan et al., 2001). Two mechanisms of oxalate utilization, exclusion and internal tolerance, have evolved that enable plants to resist aluminum toxicity (Taylor, 1991; Kochian, 1995). The exclusion mechanism involves the excretion of oxalate into the environment by the roots and occurs in response to external aluminum stress (Ma et al., 1997a). The internal tolerance mechanism involves the sequestration of aluminum, in the non-toxic form of aluminum-oxalate, within the aerial portion of the plant (Ma et al., 1997a). Plants such as buckwheat use both mechanisms of aluminum detoxification. Some plants appear to produce oxalate as a mechanism to detoxify other hazardous metals such as lead (Yang et al., 2000), strontium (Franceschi and Schueren, 1986; Zindler-Frank, 1991), and cadmium (Choi et al., 2001), when present in the environment. Studies have noted that the exogenous application of such metals to the plant environment often results in the incorporation of the heavy metal into oxalate crystals within the plant tissues (Franceschi and Schueren, 1986; Zindler-Frank, 1991; Ma et al., 1997b; Choi et al., 2001; Mazen, 2004).

Impact of plant oxalates on human health

Forms of oxalate

Oxalate in plant foods exists in two general forms, soluble and insoluble, and each form can have a negative impact on the health of the person consuming the plant food. In the soluble form, oxalate often occurs as the sodium salt. In this form it can be absorbed directly from the diet and contribute to the pathological condition of renal stone disease (Holmes et al., 1995; Holmes et al., 2001) where over 75% of all kidney stones contain calcium oxalate as their primary component (Nordin et al., 1979). Studies have shown that high dietary oxalate consumption often results in an increase in urinary oxalate excretion (Holmes et al., 1995; Holmes et al., 2001) and it is during this excretion process that the oxalate can precipitate with calcium and sometimes other substances leading to the formation of calcium oxalate kidney stones. Since plant foods are the main source of dietary oxalate, reducing the oxalate content in these foods should aid those prone to kidney stone formation.

In the insoluble form, oxalate is usually found in the calcium oxalate crystal. In this form oxalate is acting as antinutrient rendering the bound calcium unavailable for nutritional absorption. Plants with high calcium oxalate content such as spinach were found to have poor calcium bioavailability compared to plants with low calcium oxalate content such as kale (Weaver et al., 1987; Heaney et al., 1988; Heaney and Weaver, 1989). Thus, effort has been put forth to identify germplasms (Libert and Franceschi, 1987; Massey et al., 2001; Siener et al., 2006a; Siener et al., 2006b; Catherwood et al., 2007; Gélinas and Seguin, 2007; Ritter and Savage, 2007), growth conditions (Sugiyama and Okutani, 1996; Ahmed and Johnson, 2000; Rinallo and Modi, 2002; Ji and Peng, 2005; Proietti et al., 2009; Smith et al., 2009; Rahman et al., 2010), breeding practices (Libert, 1987), and food preparation practices (Savage et al., 2000; Oscarsson and Savage, 2007; Moreau and Savage, 2009; Savage et al., 2009) that result in plant foods with lower oxalate content. Alternatively, it may be possible to reduce the oxalate content in plant foods through genetic modification as described below.

Genetic manipulation of plant calcium oxalate content

Through the use of genetic screens researcher have been able to show that genetic manipulations to alter calcium oxalate

content is possible (Nakata and McConn, 2000, 2007c; McConn and Nakata, 2002). Such mutant screens have led to the isolation of several classes of calcium oxalate defective (*cod*) mutants from the model legume, *Medicago truncatula*, that have alterations in various aspects of calcium oxalate formation including crystal nucleation, morphology, distribution, and/or amount (Nakata and McConn, 2000, 2007c; McConn and Nakata, 2002). The sheer number of complementation groups indicates the complexity in forming this biomineral. One mutant, *cod5*, lacks the ability to accumulate prismatic crystals (Fig. 3A) and proves the feasibility of generating modified plants with reduced oxalate content. The *cod5* along with the original parent wild-type line also provide a near isogenic plant system to more accurately determine the impact of oxalate on calcium bioavailability (Nakata and McConn, 2000).

Generation of calcium oxalate deficient plant enhances calcium bioavailability

Both *in vitro* (Nakata and McConn, 2006, 2007a) and *in vivo* (Morris et al., 2007) nutritional models have been utilized in assessing the nutritional enhancement of the genetically modified oxalate plants. Initial investigations centered on the use of an *in vitro* dialysis method that simulated the processes of human digestion and absorption. This quick and economical procedure allowed nutritional screening of calcium availability in the various *cod* plants. This investigation showed that calcium availability generally correlated inversely with the amount of calcium bound in the form of the oxalate crystal (McConn and Nakata, 2004; Nakata and McConn, 2006, 2007a). Thus, the plants with more calcium bound in the oxalate crystal had reduced calcium availability while the plants with less calcium in the form of the crystal showed enhanced calcium availability, compared to controls. Based on these encouraging results, a more definitive *in vivo* study was undertaken to prove the nutritional improvement in an animal model (Morris et al., 2007). Mice were fed either extrinsically or intrinsically ^{45}Ca-labeled diets derived from labeled wild-type or *cod5* plants (Fig. 3B). By measuring the amount of ^{45}Ca incorporated into bone it was determined that the mice fed the *cod5* diet were able to absorb and utilize more calcium from this modified plant food than mice fed a wild-type control diet containing an equal amount of total calcium (Morris et al., 2007). The increase in calcium absorption was found to be proportional to the amount of calcium oxalate that was genetically removed from the *cod5* plant (Fig. 3C). Overall, these studies prove that reducing the oxalate content in edible plants is a viable strategy to improve the nutritional value of plant foods. The modification, in this case, was also accomplished using a non-transgenic approach. Such an approach should alleviate the problems associated with the public acceptance of transgenic foods. In addition to the nutritional improvement, the ability to genetically reduce the oxalate content in plant foods also has another potential benefit. Studies have shown that the absorption of oxalate from plant foods contributes more than previously thought to kidney/urinary stone formation (Holmes et al., 1995; Holmes et al., 2001). Therefore, removing the oxalate content in plant foods will benefit those prone to renal stones.

Growth and development of calcium oxalate deficient plant

A nutritional improved crop plant would only be beneficial to the consumer if the farmer was able to grow the crop with suitable yields and if the phenotypic appearance of the plant food was similar to wild-type. To assess these two characteristics, researchers grew *cod5* and wild-type *M. truncatula* plants, over a 90 day period, under controlled greenhouse conditions (Nakata and McConn, 2003a). Under these conditions no difference was observed in phenotype or biomass production (Nakata and McConn, 2003a). However, when challenged with adverse environmental conditions such as exposure to chewing insects, the *cod5* plant showed an increase in its susceptibility compared to controls (Korth et al., 2006; Park et al., 2009). Thus, strategies aimed at improving calcium bioavailability through a reduction in calcium oxalate content will have to take this finding into consideration. One could envision designing a targeted strategy to reduce the accumulation of calcium oxalate crystals, for example, in edible legume seeds (e.g., dry beans) while maintaining crystals production in the protecting pod walls as well as the other non-edible parts of the plant for protection against such chewing insects.

Potential nutritional improvement of plant foods through the generation of calcium oxalate deficient plants

The primary difference governing calcium bioavailability between vegetables such as spinach and kale is thought to be the high levels of oxalate found in spinach and the low levels of oxalate present in kale (Weaver et al., 1987; Heaney et al., 1988; Heaney and Weaver, 1990). As described above, recent research shows that genetic manipulation to create oxalate-free plant foods is now possible (Nakata and McConn, 2000, 2003a) and results in the desired nutritional improvement (Nakata and McConn, 2006, 2007a; Morris et al., 2007). Based on this knowledge, one would predict that the generation of a “no oxalate spinach” would have a calcium bioavailability similar to that of kale. If this proves true, the “no oxalate spinach” plant would have a substantial nutritional improvement delivering as much calcium as glass of milk (Table 1).

Such a nutritional improvement is significant considering the important link between calcium and health-related conditions such as osteoporosis, rickets, cancer, and kidney stone formation as well as the reliance of different populations around the world on plant foods as their main source of

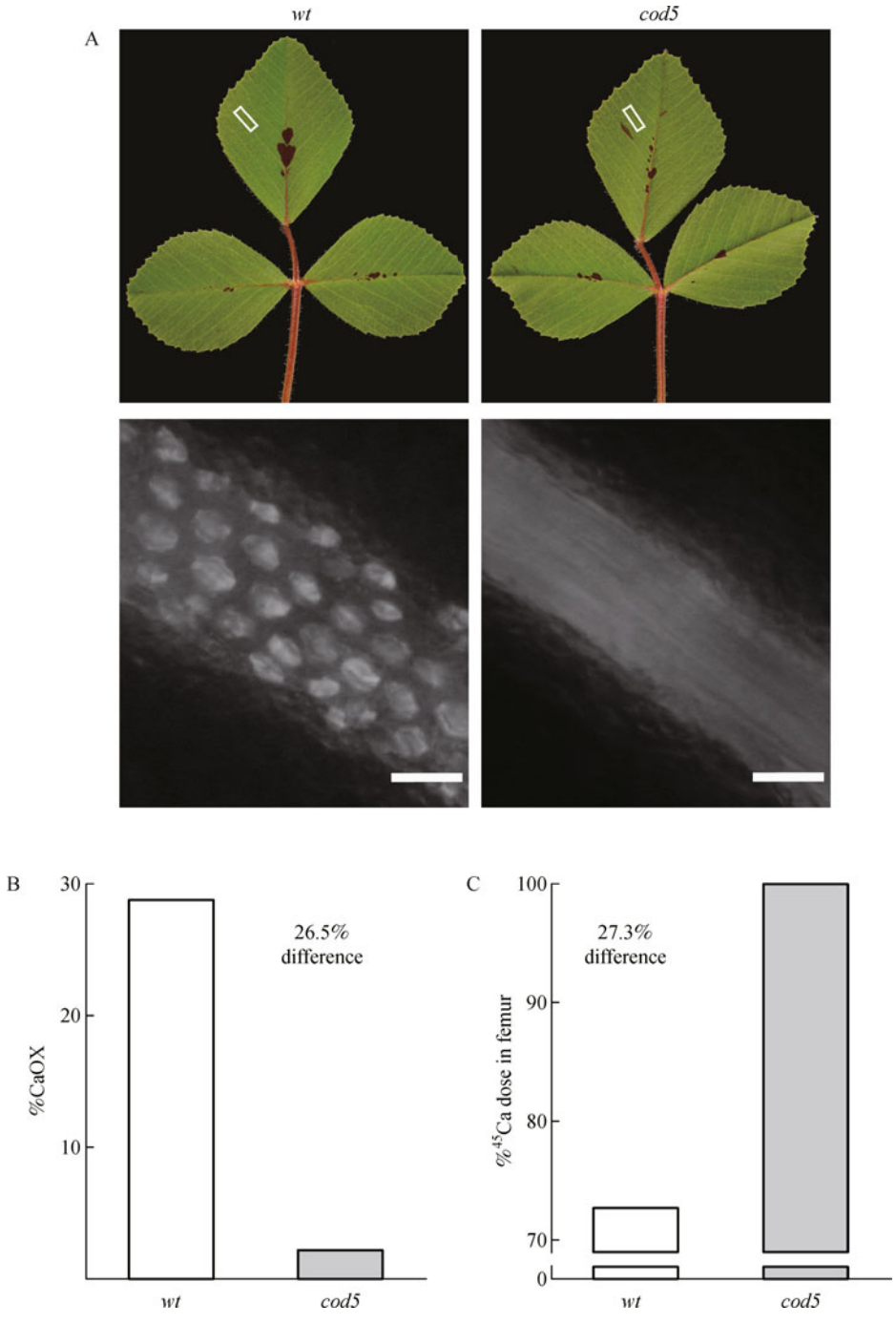

Figure 3 Generation of a calcium oxalate deficient plant with improved calcium bioavailability. Wild-type and *cod5* plants were grown hydroponically in the presence of ^{45}Ca. (A) A comparison of the leaves (top) and calcium oxalate crystal phenotypes (bottom) of wild-type and *cod5*. Note the crystals along the secondary vascular strand (boxed region on leaf image) in wild-type and the absence of crystals in the modified plant, *cod5*. Bar = 25 μm. (B) Percent difference of the calcium found in the form of the calcium oxalate crystal in wild-type and *cod5*. Both plants had similar calcium content but differed in the amount each sequestered as the calcium oxalate salt. (C) Percent difference in ^{45}Ca absorbed and incorporated into the bones from wild-type and *cod5* fed mice. Note, that the increase absorption and utilization of calcium from the modified *cod5* plant was directly proportional to the decrease in the percentage of calcium bound in the oxalate crystal (Morris et al. 2007).

calcium and other minerals. These studies also remind us of an important point that is often overlooked. It is not just how much of a nutrient you eat in your diet that is important but the form of the nutrient. It is the form of the nutrient that determines its bioavailability, meaning how much the human body can absorb and ultimately utilize.

Table 1 Potential nutritional impact

Food	Serving size	Calcium content (mg)	Fractional Absorption (%) *	Est. absorbable Ca/serving (mg)
Cow milk	1 cup	288	32	92
Kale, boiled	1 cup	94	41	39
Spinach, boiled	1 cup	244	5	12
"No CaOx" spinach	1 cup	244	41 ?	100 ?

*: Values from Heaney et al. (1988) and Heaney and Weaver (1990).

Conclusions

Plant calcium oxalate crystal formation serves diverse functional roles that protect the plant from a variety of environmental stresses. Such roles include the regulation of bulk tissue calcium, defense against herbivory and/or tolerance to heavy metals. Although there are large gaps in our understanding of the mechanisms controlling oxalate biosynthesis and calcium oxalate crystal formation, researchers are gaining insights into this remarkable biomineralization process. There is mounting evidence supporting ascorbic acid as the primary precursor in the biosynthesis of the oxalic acid utilized in crystal formation. Some of the protein components that compose the calcium oxalate crystal formation machinery have been identified and isolated. The addition of a genetic model and the isolation of calcium oxalate crystal mutants have aided efforts to elucidate the pathway of crystal formation. In light of the important roles of calcium oxalate crystal formation to a plant's survival as well as its impact on human health through its presence in plant foods, a better understanding of the crystal pathway is desired. Such an understanding could lead to new strategies for improving the production and nutritional value of plant foods. Recent studies have shown that creating oxalate-free plant foods is possible and that such a change results in the desired nutritional enhancement of calcium bioavailability. Recent work has also shown that such manipulations can have other consequences that need to be considered such as a negative impact on insect resistance. Thus, researchers will have to find a balance between nutritional improvement and insect resistance in the development of a cost-effective modified crop plant. The development of such modified crop plants appear to be within reach but further studies are necessary before the potential of such plants can be fully realized.

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