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Cell-Mediated Crystallization of Calcium Oxalate in Plants

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INTRODUCTION

Plants make crystals of calcium oxalate in an intriguing variety of defined shapes. Figure 1 illustrates a commercial preparation of calcium oxalate, which consists of a mix of crystals with variable sizes and irregular shapes. In contrast, crystals synthesized by plants typically exhibit quite specific morphologies. This is demonstrated in Figure 2, which shows characteristics of calcium oxalate isolated from bean (Figure 2A), velvet leaf (Figure 2B), and grape (Figure 2C). In higher plants, the distribution of crystals, like their morphology, follows species-specific patterns, indicating regulation over the sites and modes of calcium oxalate accumulation (Arnott and Pautard, 1970). Why and how do different plants make such diverse crystals from the same chemical substance, and what determines where they form? Very little is known about how plants control these patterns.

In higher plants, calcium oxalate typically develops within intravacuolar membrane chambers of specialized cells. The complex cellular features associated with calcium oxalate crystallization indicate that it constitutes a biologically controlled process, analogous to calcification processes that shape bones, teeth, and shells in animals (Arnott, 1966). This review addresses key questions about cell-mediated crystallization of calcium oxalate in plants, including how and why plant cells make crystals, and discusses important cellular, developmental, and physiological aspects of this phenomenon. The broader study of biomineralization provides a context for understanding calcium oxalate crystallization in plants.

What is biomineralization and why is it important? A vast array of organisms produce biological minerals, or "biominerals." Unique structural or crystallographic features typically distinguish biominerals from their counterparts that precipitate abiotically (Berman et al., 1993). Biominerals encompass a wide spectrum of scale and composition, from macroscopic structures such as vertebrate bone, composed of apatite (Anderson, 1995), to microscopic silicon frustules of diatoms (Li and Volcani, 1984) and subcellular ferrimagnetic particles in bacteria (Bazylinski et al., 1994). Biomineralization fulfills a variety of crucial functions, including important skeletal and protective roles.

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Anyone who has broken a bone has experienced firsthand the effects of biomineralization. Bone repair and healing, like the initial synthesis of bone during development, are conducted by cells with specialized roles in mineralization and demineralization that fashion and model bone structure according to genetically programmed patterns (Lowenstam and Weiner, 1989; Simkiss and Wilbur, 1989). One might naturally question what relevance bone repair and other biomineralization processes have to plant cell biology. In fact, as introduced above, the plant kingdom exhibits a varied assortment of patterned mineralized structures formed by cells, including deposits of silica, calcium oxalate, and calcium carbonate (Arnott and Pautard, 1970). Calcium oxalate crystals are by far the most prevalent and widely distributed mineral deposits in higher plants.

CALCIUM OXALATE CRYSTALLIZATION AND CALCIUM REGULATION

Why do plants sequester calcium oxalate? Calcium is very abundant in the natural environment in which most plants grow. A required element for plant growth and development, calcium plays many important roles, for example, as a structural component of cell walls (Demarty et al., 1984), a signal in various physiological and developmental pathways (Bush, 1995), and an osmoticum (Kinzel, 1989). Nonetheless, cytosolic free calcium must be restricted to levels of $\sim 10^{-7}$ M or less (Kretsinger, 1977), because higher concentrations interfere with a variety of crucial cell processes, including calcium-dependent signaling (Bush, 1995), phosphate-based energy metabolism (Kretsinger, 1977), and microskeletal dynamics (Hepler, 1994).

Although the mechanisms controlling calcium absorption at the root are controversial, plants accumulate calcium in excess of cytosolic requirements and limits (Loneragan and Snowball, 1969; Clarkson, 1984; Kirkby and Pilbeam, 1984; Kinzel, 1989). In addition, most plants, unlike animals, do not have well-developed excretory systems to dispose of excess calcium. Instead, higher plants appear to modulate differences between the natural abundance of environmental calcium and the very low levels required for cytosolic free calcium by controlling the distribution of calcium and its compartmentation within the cell (Clarkson, 1984; Kinzel,

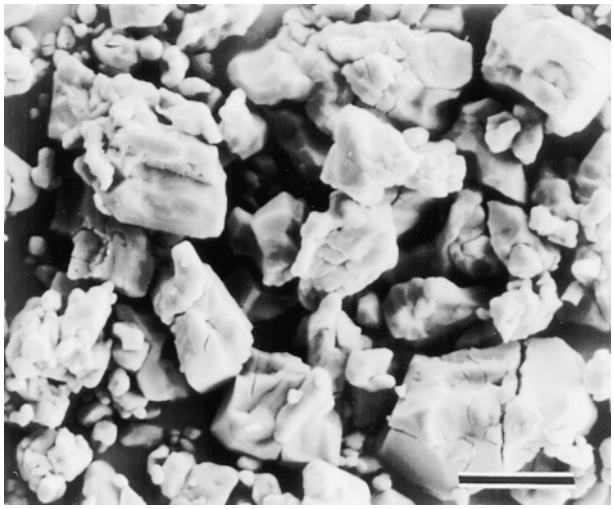

Figure 1. Commercial Preparation of Calcium Oxalate (Aldrich).

Crystals were viewed in a scanning electron microsope. Bar = 5 μm.

1989; Leigh and Tomos, 1993). The cell wall and the vacuole provide major sinks for calcium in plants (Kinzel, 1989).

Many plants accumulate crystalline calcium oxalate in response to surplus calcium (Frank, 1972; Zindler-Frank, 1975, 1991, 1995; Franceschi and Horner, 1979; Borchert, 1985, 1986; Franceschi, 1989; Fink, 1991). With a solubility product of 1.3×10^{-9} in water, calcium oxalate provides a relatively insoluble, metabolically inactive salt for calcium sequestration (Kinzel, 1989). Calcium oxalate thus provides a high-capacity repository for calcium, and plants may accumulate this salt in substantial amounts, up to 80% of their dry weight (Zindler-Frank, 1976) or 90% of total calcium (Fink, 1991). The extent of calcium partitioning into calcium oxalate varies among different taxonomic groups of plants. In >100 species examined, calcium oxalate content averaged 6.3% of plant dry weight (Zindler-Frank, 1976). Numerous studies (Kohl, 1889; Arnott and Pautard, 1970; Franceschi, 1989) also indicate that crystals do not form an inert, nonretrievable pool but that they can be redissolved. However, despite the significance of calcium oxalate in sequestering and storing calcium, little is known about factors that direct calcium to this pool.

CRYSTAL STRUCTURE AND DISTRIBUTION

Variations of Crystal Distribution and Morphology among Plants

Calcium oxalate crystals may form in any organ or tissue within plants. For example, crystals occur in roots, stems, leaves, flowers, fruits, and seeds (Franceschi and Horner, 1980) and within epidermal (Zindler-Frank, 1975), ground (Horner and Whitmoyer, 1972), and vascular (Wang et al., 1994) tissues. Calcium oxalate often forms in idioblasts, cells that develop in isolation with structure or content distinct from surrounding cells (Foster, 1956). In other instances, crystals may develop in defined groups of cells, as in files of bundle sheath cells (Borchert, 1984), for example, or in a single layer of the seed coat (Webb and Arnott, 1982). Less often, entire tissues such as endosperm (Spitzer and Lott, 1982) or leaf epidermis (Brubaker and Horner, 1989) accumulate calcium oxalate in every cell or in a majority of cells.

Plant crystals display an astonishing variety of morphologies, most of which conform to one of the following categories defined by botanists (Franceschi and Horner, 1980): (1) prisms, consisting of simple regular prismatic shapes; (2) druses, which are spherical aggregates of crystals; (3) styloids, acicular crystals that form singly; (4) raphides, acicular crystals that form in bundles; and (5) crystal sand, small tetrahedral crystals that form in clusters. Calcium oxalate exists in two chemical forms, monohydrate and dihydrate, and both of these occur in plants (Arnott et al., 1965; Frey-Wyssling, 1981). The observed morphologies represent elaborations and modifications of basic crystal structure for either the monohydrate or dihydrate form. The monohydrate is more stable and is more commonly found in plants than is the dihydrate.

Defined Patterns of Calcium Oxalate Accumulation

Given the myriad possibilities, individual plant species typically display quite specific anatomical, morphological, and developmental patterns of crystal accumulation, reflecting genetic regulation of crystal formation. Crystal morphology and distribution are usually similar within specific taxa and differ among divergent taxa to the extent that they provide key characters for systematics. Cultured plant tissues typically produce crystals morphologically identical to those characteristic of the intact plant (Kausch and Horner, 1982).

The precise patterns exhibited by plants that produce calcium oxalate reflect multiple levels of organismal and cellular control over the crystallization process (reviewed in Arnott and Pautard, 1970; Franceschi and Horner, 1980; Horner and Wagner, 1995). Extensive observations indicate that calcium oxalate does not result from random precipitations wherever appropriate levels of calcium and oxalate happen to meet but that certain cells within the plant become specialized to accumulate calcium and crystallize calcium oxalate in a controlled and defined manner. The features of calcium oxalate crystals, their functions, and the plant cells that produce them have interested plant biologists for more than a century (Kohl, 1889; Haberlandt, 1914).

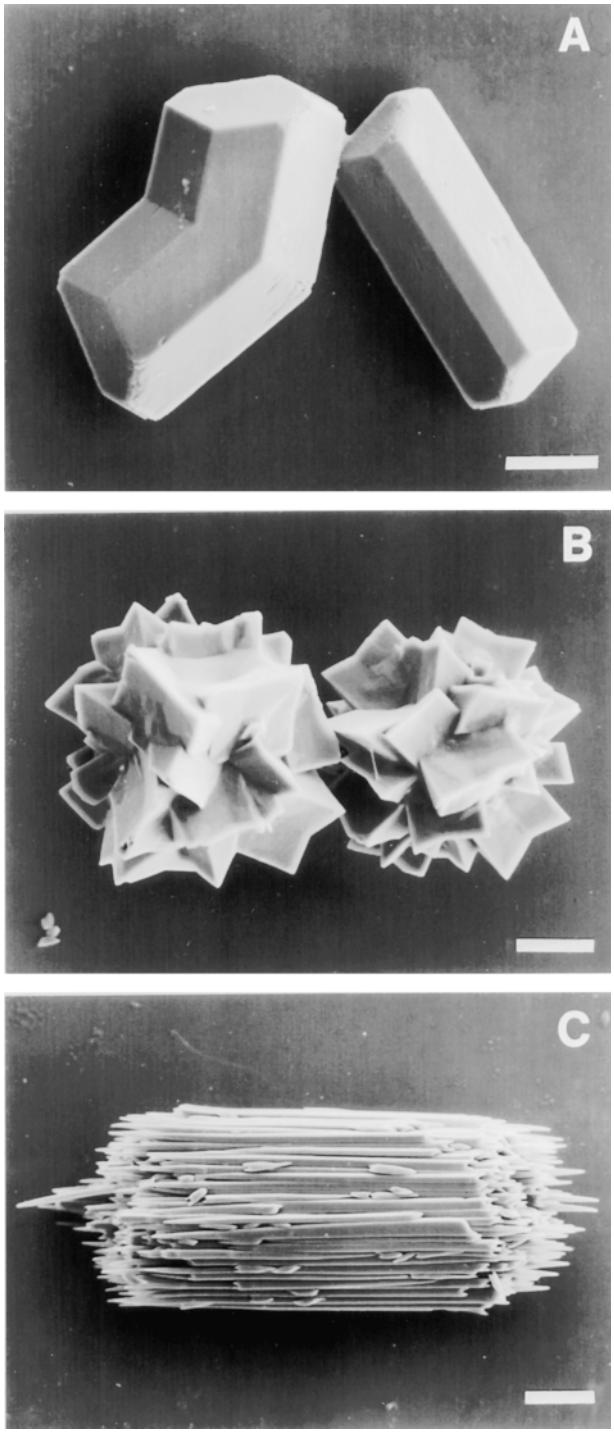

Figure 2. Characteristic Morphologies of Calcium Oxalate Crystals Isolated from Selected Plants.

Crystals were viewed in a scanning electron microscope (SEM). Bean and velvet leaf crystals were prepared by homogenizing leaves in distilled water in a blender and filtering the extract through cheesecloth. Crystals were isolated by swirling filtrate in a spot plate well until crystals accumulated at the center of the well. Crystals were then washed with several changes of distilled water, mounted on a glass cover slip on an aluminum stub, dried, and sputter-coated with gold-palladium for SEM observation. Raphide bundles were isolated in ethanol, as described by Webb et al. (1995). Bars = 5 μm.

(A) Twinned prismatic crystals from the seed coat of bean (*Phaseolus vulgaris*). Both mirror-image (left) and rotational (right) twins form in hypodermal cells of the seed coat (Arnott and Webb, 1983).

(B) Druse crystals isolated from velvet leaf (*Abutilon theophrasti*), a common weed. The druses are spherical aggregates of calcium oxalate that form in mesophyll cells.

(C) Isolated bundle of raphides, needle-shaped crystals, from leaves of grape (*Vitis labrusca*). Several hundred raphides form per cell in idioblasts that are distributed throughout the plant. The micrograph is from Webb et al. (1995).

SPECIALIZED CRYSTAL-FORMING CELLS AND CRYSTAL CHAMBERS

Calcium Oxalate Crystallization in Specialized Plant Cells

In higher plants, calcium oxalate crystals typically form inside the vacuoles of specialized cells. Some studies have suggested that excess apoplastic calcium induces differentiation of these cells (Borchert, 1985, 1986; Franceschi, 1989), but other research has shown that they differentiate even when calcium supply is limited (Frank, 1972). A large body of ultrastructural research documents the unusual development and unique features of crystal-forming cells in a variety of plants, reviewed previously by Arnott and Pautard (1970), Franceschi and Horner (1980), and Horner and Wagner (1995). However, detailed correlative information about the physiological and biochemical features that characterize these cells is generally lacking. Because structural and developmental features of crystal-forming cells in plants have been extensively reviewed, as noted above, they are not covered exhaustively in this review. Rather, selected examples are used to examine key questions.

In most biomineralization processes, specialized cells and/or organic macromolecules in or around specialized cells govern and mediate crystal formation. These cells and their associated molecules, collectively termed the organic matrix, function in a variety of ways to compartmentalize the crystallization process, to nucleate crystals, and to modify crystal growth and morphology (Lowenstam and Weiner, 1989; Simkiss and Wilbur, 1989).

Crystal Formation within Intravacuolar Membrane Chambers

Although structural features of crystal-forming cells vary in relation to crystal morphologies in diverse plant species

(reviewed in Arnott and Pautard, 1970; Franceschi and Horner, 1980; Horner and Wagner, 1995), certain characteristics are common to most systems. For example, crystals typically develop inside the vacuole. They form within intravacuolar membrane chambers, termed crystal chambers, that differentiate and proliferate exclusively in the vacuoles of crystal cells (Arnott, 1966; Arnott and Pautard, 1970). Additional substances commonly accumulate specifically within the vacuoles of these cells, including paracrystalline bodies, tubular or fibrillar structures, and various densely staining substances that associate with the crystal chambers (Arnott and Pautard, 1970; Horner and Wagner, 1995). Mucilage, consisting mainly of complex polysaccharides, also may accumulate within such vacuoles, particularly in association with raphides (Kausch and Horner, 1984; Wang et al., 1994; Webb et al., 1995). In certain plants, paracrystalline bodies (Horner and Wagner, 1980) or tubules (Franceschi, 1984) appear to give rise to crystal chambers. In many other plants, the origin of the crystal chambers has not been established.

Characterizing the nature and ontogeny of vacuolar constituents associated with crystal formation and understanding how these specialized vacuoles differ from normal vacuoles are essential for clarifying the mechanisms of cellular control over the crystallization process. This review emphasizes, in particular, questions about the structure, composition, and function of crystal chambers in calcium oxalate crystallization.

The configuration of crystal chambers, before mineralization and at very early stages in the process, may predict the morphology of crystals, as illustrated in Figure 3. For example, cells in the bean seed coat that form prismatic crystals (Arnott and Webb, 1983) exhibit relatively simple arrays of intravacuolar membranes (Figure 3A; Barnabas and Arnott, 1990). In cells that form druses in *Capsicum* anthers (Horner and Wagner, 1980), membrane chambers radiate around a central core to form a spherical aggregate (Figure 3B). A similar phenomenon is also found in druses in grape endosperm, where crystals form around a central core of organic material (Webb and Arnott, 1983). In raphide-forming cells, hundreds to thousands of individual crystals may form inside a single vacuole, and each of these crystals is enclosed by a membrane (Arnott and Pautard, 1970; Webb et al., 1995). In the grape seed coat, raphides initiate from linear membranous structures (Figure 3C, arrows) within complexes of whorled membranes. Membrane chambers surround and enclose raphides throughout their development (Webb et al., 1995).

As crystal-forming cells mature, further elaborations of crystal chambers or matrix may occur. In *Yucca* roots, for example, lamellated sheaths develop around older raphides (Kausch and Horner, 1984). In certain plant groups, crystals eventually become enclosed by a cell wall sheath that may be suberized (Frank and Jensen, 1970; Webb and Arnott, 1981). Although it is not clear how these wall sheaths develop around vacuolar crystals (Horner and Zindler-Frank, 1982), they effectively exclude the crystal from the protoplast and thus may provide more permanent sequestration.

How Do Intravacuolar Crystal Chambers Define the Crystallization Space?

The crystal chambers clearly constitute key components of specialized cells that store calcium oxalate. Cell-specific synthesis and accumulation of these chambers signify the differentiation of a crystal-forming cell early in its development (Arnott and Pautard, 1970; Frank and Jensen, 1970; Horner and Whitmoyer, 1972; Franceschi, 1984; Kausch and Horner, 1984). Because they function to compartmentalize developing crystals, an accurate understanding of their fine structure is relevant to their function. In most cases, crystal chambers exhibit unit membrane structure characteristic of phospholipid bilayers (Arnott and Pautard, 1970). When unit membrane structure is not apparent, it is not clear whether the unit membrane is supplanted by an alternative structure, such as a phospholipid monolayer, or is lost due to inadequate structural preservation. Fixation for microscopy may be problematic in crystal cells that also accumulate large amounts of mucilage or other materials within their vacuoles such that preservation and subsequent visualization of the chambers may be impaired. The clearest images of crystal chamber structure come from materials fixed in potassium permanganate, which preserves the membranes that bound the chambers but dissolves calcium oxalate within them before embedding. Such images have consistently shown clear unit membrane structure (see Arnott and Pautard, 1970). However, the possibility that crystal chambers in some plants have an alternative structure cannot be ruled out.

Another question sometimes raised is whether the membrane chambers completely enclose crystals or have gaps or holes continuous with the vacuolar solution (Franceschi, 1984). This point is important in understanding what defines the crystallization space. Are crystal chambers closed compartments separated from the vacuole proper, or does the vacuole as a whole constitute the crystallization medium?

The continuity of crystal chambers around developing or mature crystals can often be difficult to assess in ultrathin sections. Visualization of these membranes at the ultrastructural level is complicated by damage resulting from mechanical impedance caused by crystals during ultrathin sectioning. However, images of thick sections with high-voltage electron microscopy or of isolated whole chambers provide evidence that crystals are isolated in closed compartments (Arnott and Pautard, 1970; Wagner, 1983; Webb et al., 1995). This closed structure separates the crystallization space from the vacuolar sap and thus provides control over the environment in which crystals form.

Do Crystal Chambers Nucleate Crystals?

One crucial role suggested for crystal chambers is that they nucleate crystals (Simkiss and Wilbur, 1989). Indeed, developmental studies show that chambers arise in the vacuole before crystal initiation, and the first signs of crystallization

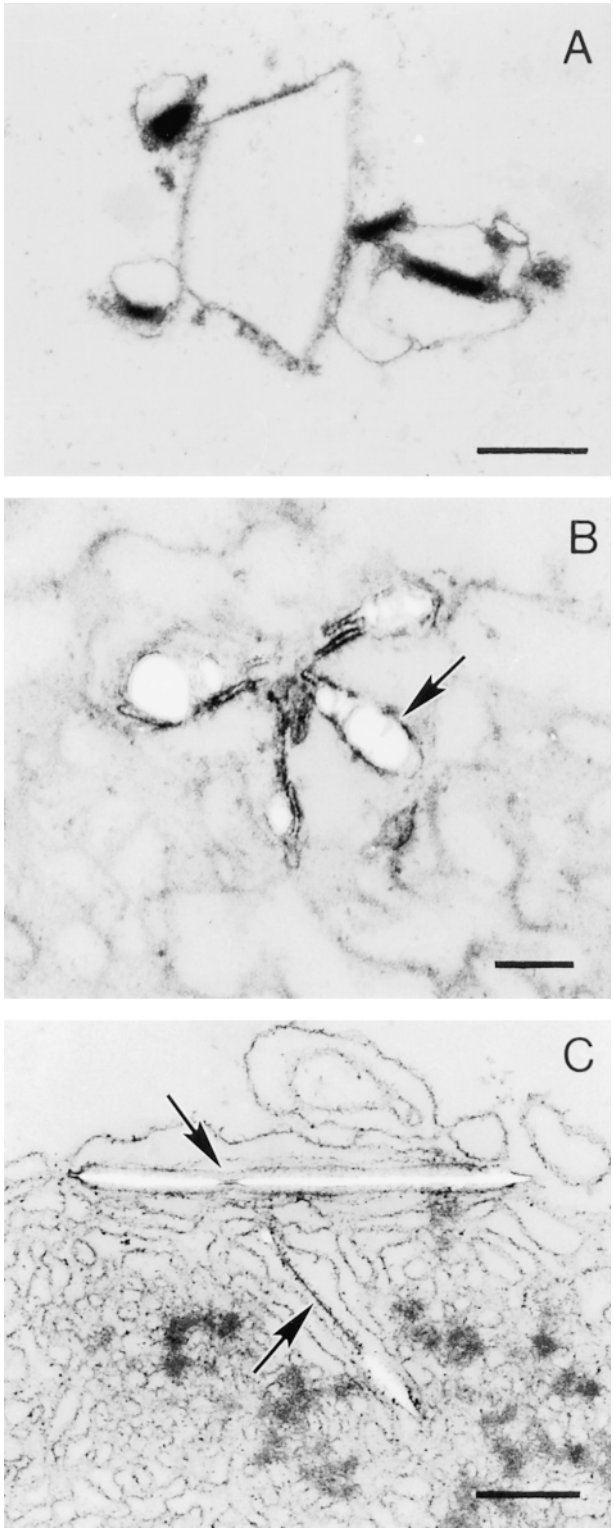

Figure 3. Intravacuolar Crystal Chambers in Crystal-Forming Cells.

In all examples, tissue was fixed in glutaraldehyde and osmium tetroxide and then embedded in resin. Ultra-thin sections were stained with lead citrate and uranyl acetate and viewed in a transmission electron microscope. Crystals are not penetrated by the embedding resin and appear as holes in the micrographs.

(A) Crystal chambers in hypodermal cells of the bean seed coat produce prismatic crystals, like those seen in Figure 2A. Vesicles coalesce within the vacuoles to produce the chamber complex, and electron-dense bodies develop at vesicle contact sites (Barnabas and Arnott, 1990). The micrograph is courtesy of Barnabas and Arnott (1990). Bar = 0.5 μm.

(B) An aggregate of crystal chambers (arrow) radiates from a central core in cells of sweet pepper (*Capsicum annuum*) anthers. A reticulate complex of membranes surrounds crystal chambers in the vacuole. Crystal aggregates, or druses, produced in these cells are morphologically similar to druses from velvet leaf, seen in Figure 2B. The micrograph is courtesy of Horner and Wagner (1980). Bar = 0.1 μm.

(C) Two stages of raphide initiation (arrows) observed in a grape vacuole in association with raphide bundles, like that seen in Figure 2C. At the earlier stage (lower arrow), mineral formation has just begun at opposite ends of a linear membrane structure that will form the crystal chamber (Webb et al., 1995). Bar = 0.5 μm.

appear within them (Arnott and Pautard, 1970; Horner and Whitmoyer, 1972). A role for the chambers in crystal nucleation gains support from observations that they have a regular repeating or ordered structure (Frank and Jensen, 1970; Franceschi, 1984; Webb et al., 1995), an important characteristic of substrates that promote nucleation (Lowenstam and Weiner, 1989). Additional support comes from demineralization experiments in which crystal chambers isolated from grape leaves and demineralized with sulfuric acid serve as nucleation sites for secondary crystallization of calcium sulfate (Webb et al., 1995). In this role, the number of nucleating centers (chambers) that develop, and their configuration within the vacuole, would provide control over the number and morphology of crystals that form, as illustrated for selected examples in Figure 3. The crystal chamber membranes in this regard may have a direct effect on calcium oxalate nucleation, or they may anchor or enclose other molecules or complexes that promote nucleation.

Interestingly, the first step in calcification of cartilage, bones, and teeth in vertebrates takes place within membrane-bound vesicles, termed matrix vesicles (Anderson, 1995). Needle-like crystals of calcium phosphate initiate within these vesicles and provide centers for further proliferation of apatite (crystalline calcium phosphate). In developing bone, matrix vesicles originate from the plasma membrane of specialized cells, which release them into the extracellular matrix, where the mineral apatite is deposited.

Matrix vesicles competent to induce mineralization differ in specific ways from similar vesicles produced by other cells. Specifically, they are enriched in phosphatidylserine, a calcium-binding phospholipid, and annexin V, a phospholipid-dependent calcium binding protein that may form a

calcium channel. Matrix vesicles also have high levels of alkaline phosphatase activity and thus are able to release phosphate from organic phosphate and pyrophosphate (Kirsch et al., 1997). At the earliest stage of mineralization, these vesicles can thereby establish a membrane-associated "nucleation core" that consists of a complex of Ca^{2+}, Pi, phosphatidylserine, and annexin. This complex induces initial crystallization of calcium phosphate, which is then propagated by protein-bound Ca^{2+} and Pi present in the lumen of the vesicles (Wu et al., 1997).

High-resolution ultrastructural studies have shown that center-to-center distances between particles in the inner face of matrix vesicle membranes are consistent with calcium phosphate nucleation (Plate et al., 1996). Isolated matrix vesicles also promote mineralization in vitro (Boskey et al., 1997). In developing bone, the polarized release of matrix vesicles competent for mineralization by cells specialized for skeletal formation determines the initial region of calcification within the extracellular matrix. Although subsequent mineralization takes place in the extracellular matrix after initiation in matrix vesicles, specialized bone-forming cells mediate this process and model the resulting mineralized tissue into mature bone (Anderson, 1995).

Because crystal chambers may well function in an analogous way to nucleate calcium oxalate, knowledge about bone matrix vesicles points to specific directions for further investigations in plants. One would expect, given their unique role, that crystal chambers would differ in composition from other membrane systems in plant cells. However, at present there is virtually no information about their biochemical features. Cell-specific expression of membrane components, including crystal nucleators, could be an important element controlling distribution of crystals within the plant body. Relevant questions about the composition of crystal chambers might include the following: What are the protein and lipid compositions of these membranes? Is annexin V or an alternative calcium transporter present in the membranes where crystals initiate? Are calcium-binding proteins present inside crystal chambers? Does a membrane constituent catalyze release of oxalate from a precursor or derivative in the surrounding vacuolar solution? Such information about the high-resolution structure, ontogeny, and biochemical composition of these membranes is essential to clarify their potential role in nucleation.

We have isolated raphide bundles from grape under conditions in which proteins associated with raphides are preserved. Detergent-soluble extracts of raphide-associated proteins contain a complex assortment of polypeptides, as would be expected for a biological membrane (Webb et al., 1995). We have recently identified several cDNAs putatively encoding these proteins and are currently pursuing their characterization (J.M. Cavaletto, V.S. Eccleston-Goodwin, W.M. McDowell, and M.A. Webb, unpublished results). Similar studies examining crystal-associated proteins in other plant systems are needed to gain critical information about constituents of crystal chambers.

CALCIUM AND OXALATE ORIGIN AND TRANSPORT

How Are Calcium and Oxalate Ions Transported to Crystal Chambers?

Because crystal chambers compartmentalize the crystallization process within the vacuole, it is logical to conclude that they provide selectivity and control over entry of ions into the crystallization space. However, there is no information about transport proteins in these membranes. Whether or not accumulation of calcium and oxalate in the chambers requires energy depends on mechanisms of transport (Bush et al., 1995).

One consideration important to formulating hypotheses or models about how calcium and oxalate accumulate within crystal chambers is the physical relationship of the chambers to other cell constituents. Do crystal chambers "float free" within the vacuolar space, attach to the tonoplast or other membranes, or contact the ground cytoplasm? Different requisites apply to calcium import from the vacuole, where Ca^{2+} concentration is high, versus the cytosol, where Ca^{2+} concentration is low. Many ultrastructural studies have described "de novo" formation of crystal chambers within the vacuole. However, methods have generally not been used to determine whether chambers connect with the cytosol, tonoplast, or elements of the endomembrane system out of the plane of section. Although the vacuole is now recognized as a major intracellular sink for calcium, studies of calcium transport into the vacuole are limited (Blumwald and Gelli, 1997), and intravacuolar crystal chambers add another dimension to this process.

In addition, little is known about pathways for calcium and oxalate transport within crystal-forming cells. Because calcium oxalate has a very low solubility product, one would not expect to find free calcium in the presence of oxalate (Kinzel, 1989). Thus, an important aspect of chamber-mediated crystallization involves preventing precipitation of calcium and oxalate outside crystal chambers. This can be accomplished by separate pathways of transport for calcium and oxalate, by their sequestration in separate compartments, or by synthesis of oxalate in close proximity to crystal chambers. One study (Franceschi et al., 1993) identified a calsequestrin-like protein, localized to the endoplasmic reticulum in crystal-forming cells of *Pistia*, that may play a role in calcium sequestration. However, additional knowledge about ion transport and compartmentation within crystal-forming cells is sorely needed.

Related questions center on the derivation of calcium and oxalate that go into crystal formation. Calcium clearly must be imported into the crystal cell from its surroundings. The pathway(s) for calcium transport may vary, depending on the location of crystal-forming cells and the source of calcium. For example, calcium may be transported directly from the soil solution, or it may result from internal reallocation via degradation of cell walls (Horner and Wagner, 1980)

or from reabsorption of crystals elsewhere in the plant (Franceschi, 1989). In contrast, oxalate may be generated within crystal-forming cells, or it may be imported from surrounding cells, as discussed below.

What Is the Source of Oxalate for Crystallization?

The source(s) of the oxalate that must be synthesized in the substantial amounts required for crystal formation is unknown. In certain plants, oxalate synthesis increases with increased calcium supply and uptake (Zindler-Frank, 1975; Kinzel, 1989). A number of pathways give rise to oxalate in plants (Raven et al., 1982; Libert and Franceschi, 1987; Franceschi and Loewus, 1995), and any of these could potentially provide oxalate for crystal formation. Previous research supports the idea that oxalate does not originate from the same source in all plants. For example, Raven et al. (1982) underscored the demonstration of four different pathways for oxalate synthesis in spinach (*Spinacea oleracea*). Two pathways that have been examined extensively in higher plants (Franceschi and Loewus, 1995) are (1) conversion of the photorespiratory products glycolate and glyoxylate to oxalate and (2) ascorbic acid catabolism, which yields oxalate and threonic or tartaric acid. Additional possibilities include cleavage of isocitrate (Millerd et al., 1963a) or oxaloacetate (Chang and Beevers, 1968), or other pathways that produce gylcolate (Millerd et al., 1963b). Many plants accumulate significant soluble salts of oxalate (Libert and Franceschi, 1987; Kinzel, 1989) in addition to crystalline calcium oxalate, and soluble and insoluble oxalates could originate from different pathways.

Another crucial consideration is localization of oxalate synthesis and accumulation in relation to crystal-forming cells and intravacuolar crystal chambers. Previous studies have not found enzymes for oxalate synthesis from glycolate inside crystal-forming cells (Kausch and Horner, 1985; Li and Franceschi, 1990). Although oxalate could be imported from surrounding cells or tissues, such import would necessitate its compartmentation or modification to avoid cytotoxic effects (Libert and Franceschi, 1987), including precipitation of calcium outside the crystal chambers. Additional studies are needed to establish the source(s) of oxalate for crystal formation and to determine the cellular and intracellular localization of oxalate synthesis.

CELL-MEDIATED CONTROL OF CRYSTAL MORPHOLOGY

Other Potential Roles for Controlled Crystallization

Why has sequestration of calcium oxalate evolved as a primary mechanism for controlling excess calcium in so many plants rather than exclusion or excretion of calcium? One answer may lie in examining potential roles for crystals other than calcium regulation. In the same way that bone provides both a reservoir for calcium and an important skeletal framework in animals (Lowenstam and Weiner, 1989), calcium oxalate deposits in plants may provide multiple benefits. It has already been noted that crystals may be reabsorbed and thus provide an internal reservoir for calcium. Another well-supported role proposed for crystals is defense against herbivory. Specifically, acicular crystals such as raphides and styloids often form in specialized cells that also produce toxins, and these crystals appear to facilitate passage of toxin through the skin of the herbivore (Sakai et al., 1972; Thurston, 1976). In other cases, crystals may provide structural reinforcement analogous to cell wall sclerification. For example, crystals often form in epidermal or subepidermal tissues (Brubaker and Horner, 1989), in cells ensheathing vascular bundles (Zindler-Frank, 1995), and in cell layers within the seed coat (Webb and Arnott, 1982). These distributional patterns suggest roles for crystals in physically reinforcing structural hardness in protective tissues (Haberlandt, 1914). In addition, a wide variety of other functions has been suggested for calcium oxalate crystals, including roles in anther dehiscence (Horner and Wagner, 1980), promotion of lignin polymerization (Lane, 1994), and stomatal functioning (Ruiz and Mansfield, 1994). Further clarification of these many potential functions is essential to understanding the rationale for controlled crystallization in plants.

Crystal morphology and distribution are crucial elements in the potential secondary functions detailed above. Additionally, they may be important factors mediating calcium sequestration. In this role, the number, size, and morphology of developing crystals within a cell would affect surface area available for crystal growth and thus the rate at which calcium and/or oxalate ions can be removed from the surroundings. Thus, different crystal morphologies and distributions could accommodate different rates of ion removal within and among tissues and may have evolved with respect to environmental niches occupied by particular plant species and in concert with physiological parameters affecting calcium uptake and oxalate synthesis.

How Do Plant Cells Control Crystallization and Crystal Morphology?

A key feature of calcium oxalate crystallization in plants is species-specific crystal morphology (Figure 2), yet very little is known about how plant cells produce these elegantly crafted morphologies. Knowledge about intravacuolar matrices, specifically crystal chambers (Figure 3), is undoubtedly crucial to understanding this aspect of cell-mediated crystallization. Within crystal-forming cells, crystal chambers provide compartments bounded by a biological membrane, separating the crystallization space from the vacuolar sap. In this way, they allow regulation of physical factors such as pH, water, and ion composition and concentration inside the

compartment. In addition, they may contain or enclose macromolecules that inhibit or modify crystal growth, or they may provide anchors for such molecules, as discussed below.

Research on other systems has provided evidence that a diversity of macromolecules may affect crystal growth and morphology (see Lowenstam and Weiner, 1989; Simkiss and Wilbur, 1989). These include proteins, particularly acidic proteins and glycoproteins (Addadi and Weiner, 1985; Walters et al., 1997), polysaccharides (Kok et al., 1986), and lipids (Letellier et al., 1998). Proteins may also be incorporated into crystals (Berman et al., 1993; Aizenberg et al., 1995; McKee et al., 1995), thereby affecting morphological and mechanical properties. A variety of proteins that influence growth of kidney stones, pathological accumulations of calcium oxalate in humans and other animals, have been identified and studied (Nakagawa et al., 1987; Campbell et al., 1989; Shiraga et al., 1992; McKee et al., 1995; Grover et al., 1998). However, it is not known whether these proteins occur in plants.

Studies of calcium oxalate crystallization have identified other factors that influence morphology. For example, relative concentrations of calcium and oxalate affected hydration of crystals produced in vitro (Frey-Wyssling, 1981). When oxalate was introduced into a concentrated solution of calcium, crystals of calcium oxalate dihydrate predominated, whereas calcium introduced into a concentrated solution of oxalate resulted in the monohydrate form. The order in which calcium and oxalate ions are imported into crystal chambers during development and relative concentrations of each ion within the chambers could thus have significant effects on resulting crystal morphology. Other studies of calcium oxalate crystallization in vitro (Cody and Horner, 1984; Cody and Cody, 1987) have demonstrated the potential of a wide variety of additives to alter crystal morphology so as to produce crystals that mimic certain morphologies made by plants.

The composition of crystal chambers and associated materials in plants is clearly relevant to understanding how plant cells control crystal morphology. Previous research has provided limited information about substances associated with crystals or crystal chambers in situ. A number of histochemical studies have shown that periodic acid-Schiff–positive substances often coat the surfaces of calcium oxalate crystals in plants (see Horner and Wagner, 1995). Immunological studies (Trull et al., 1991) identified an antigen associated with calcium oxalate crystals in anthers of tobacco and other members of the Solanaceae; however, this has not been pursued further. Webb et al. (1995) determined that a complex assortment of polypeptides could be solubilized from raphides isolated from grape leaves. This study also showed that complex polysaccharides in the vacuoles of raphide-forming cells included novel polymers of glucuronic acid, which could potentially modify crystal growth (Webb et al., 1995). Further examination and characterization of these and other crystal-associated macromolecules are essential to understanding how plants mediate calcium oxalate crystallization. In addition, knowledge about how specific biological molecules affect crystallization has applications in other areas of biomineralization as well as in materials sciences and nanofabrication, in which "biomimetics" provides new possibilities for manipulating crystal size and shape (Heuer et al., 1992).

CHALLENGES FOR FUTURE RESEARCH

This review has highlighted numerous questions about cell-mediated crystallization of calcium oxalate in plants. Future research in this area will benefit from applying a variety of integrated approaches to these questions. There is a critical need for correlative biochemical and biophysical characterization, which may entail traditional approaches such as organelle and membrane isolation and characterization. Contemporary approaches that expand the existing spectrum of immunological and molecular probes can also provide new information about expression and localization of specific proteins within crystal-forming cells. Additionally, fluorescent probes may allow measurement of physical factors such as pH and calcium gradients in and around crystal cells (Pierson et al., 1996; Swanson et al., 1998).

Structural studies will also be indispensable for clarifying how crystal growth may be controlled and modified by plant cells. Crystallographic analyses (Berman et al., 1993) can reveal morphological characteristics and determinants in different types of plant-synthesized crystals. New applications of microscopy, such as atomic force microscopy, show tremendous potential for gaining detailed information about the effects of proteins on growing crystal surfaces (Walters et al., 1997).

Molecular genetic studies provide additional opportunities to identify cellular factors affecting calcium oxalate crystallization, including genes specifically expressed in crystal-forming cells. Because crystal-forming cells have unique characteristics, their differentiation may involve novel genes and regulatory mechanisms related to processes such as membrane ontogeny, oxalate synthesis, and calcium transport. In an analogous mineralization system, molecular studies of silicon deposition in diatoms have recently led to identification of a new class of silicon transporters (Hildebrand et al., 1993, 1997). Antisense technology could also prove useful in dissecting the role of specific genes in crystal cell differentiation and the crystallization process.

A model plant for genetic studies would be an extremely valuable tool in this field. Although Arabidopsis does not normally make calcium oxalate crystals, it may be possible to isolate crystal-forming mutants by random screening or targeted mutagenesis. Because a type of kidney stone disease in humans may result from mutation of a single gene (Purdue, 1990), this approach could be more feasible than it may seem.

These integrated approaches can begin to answer many

outstanding questions, including: How many genes are required for crystal formation in the plant cell? What do these genes encode, and how does their expression affect crystallization and crystal cell differentiation? How are they induced and regulated? What determines cell-specific expression/repression of genes related to crystallization? The pursuit of these problems may well contribute to understanding a variety of processes important to plant cell biology and development, including cell and membrane specialization, calcium transport and sequestration, oxalate biosynthesis, and vacuole function.

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