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Calcium acetate induces calcium uptake and formation of calcium-oxalate crystals in isolated leaflets of *Gleditsia triacanthos* L.

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Abstract. During treatment of isolated, peeled leaflets of *Gleditsia triacanthos* with 0.5–2 mM [^{45}Ca]acetate, saturation of the cell-wall free space with Ca^{2+} occurred within 10 min and was followed by a period of 6–10 h during which there was no significant Ca-uptake into the protoplast, but apoplastic Ca^{2+} was periodically released into the medium. Later, Ca^{2+} was absorbed for 3–4 d at rates of up to 2.2 μmol $Ca^{2+} \cdot h^{-1} \cdot (g\ FW)^{-1}$ to final concentrations of 350 μmol $Ca^{2+} \cdot (g\ FW)^{-1}$. The distribution of absorbed Ca^{2+} between cell wall, vacuole and Ca-oxalate crystals was determined during Ca-uptake. Wheras intact, cut leaflets deposited absorbed Ca^{2+} as Ca-oxalate in the crystal cells, peeled leaflets lacking crystal cells accumulated at least 40–50 μmol·$(g\ FW)^{-1}$ soluble Ca^{2+} before the absorbed Ca^{2+} was precipitated as Ca-oxalate. These observations indicate that the mechanisms for the continuous uptake of Ca^{2+}, the synthesis of oxalate and the precipitation of Ca^{2+} as Ca-oxalate are operational in the crystal cells of intact leaflets, but not in the mesophyll cells of peeled leaflets where they must be induced by exposure to Ca^{2+}. The precipitation of absorbed Ca^{2+} as Ca-oxalate by the crystal cells of isolated *Gleditsia* leaflets illustrates the role of these cells in the excretion of surplus Ca^{2+} which enters normal, attached leaves with the transpiration stream.

In addition to acetate, only Ca-lactate and Ca-carbonate lead to Ca-uptake, but at rates well below those observed with Ca-acetate. Other small organic anions (citrate, glycolate, glyoxalate, malate) and inorganic anions (chloride, nitrate, sulfate) did not permit Ca-uptake. Acetate-^{14}C was rapidly absorbed during Ca-uptake, but less than 20% was incorporated into Ca-oxalate; the rest remained mostly in the soluble fraction or was metabolized to CO_2. Acetate, as a permeable weak acid, may enable rapid Ca-uptake by stimulating proton extrusion at the plasmalemma and by serving as a counterion during Ca-accumulation in the vacuole, but is unlikely to function as the principal substrate for oxalate synthesis.

Key words: Acetate – Calcium uptake – Calcium oxalate crystals – Crystal-cell induction – *Gleditsia.*

Introduction

Calcium-oxalate crystals are common in cells and tissues of higher plants, yet the mechanisms involved in calcium accumulation and crystal formation, and the role of calcium excretion with respect to the mineral economy of the whole plant have received only little attention (see reviews by Arnott and Pautard 1970; Franceschi and Horner 1980).

In the course of the growing season, the leaflets of *Gleditsia triacanthos* L. (honey locust) accumulate large quantities of calcium-oxalate crystals in a highly specific temporal and spatial pattern (Borchert 1984). The time course and pattern of crystal formation in *Gleditsia* leaflets indicate that differentiation of crystal-forming cells (crystal cells or crystal idioblasts) might be induced by high concentrations of extracellular Ca^{2+} in the leaf tissues.

When isolated, peeled leaflets of *Gleditsia* seedlings are floated on 0.3–2 mM Ca-acetate, increasing numbers of Ca-oxalate crystals are deposited in the mesophyll in a characteristic, concentration-dependent, spatial pattern (Borchert 1985). Crystals are not induced by Ca-chloride or nitrate. Three phases were identified in the induction of crystals: an initial period of *adaptive aging*, during which Ca^{2+} is not required and crystal induction is not possible; a 48-h *induction period*, during which exposure to 1–2 mM Ca-acetate induces the differentiation of mesophyll cells into crystal cells; and *crystal growth*, which begins 72 h after the start of crystal-cell induction (Borchert 1985).

Cells of terrestrial plants, like those of all eucar-

yotic organisms, maintain very low cytoplasmic concentrations of Ca^{2+} (Marmé and Dieter 1983; Hepler and Wayne 1985). Elimination of surplus Ca^{2+}, which enters plants with the water absorbed by the roots and is left behind when water evaporates in transpiration, must therefore represent a major physiological problem for these plants. As inducible sinks for the orderly excretion of surplus Ca^{2+} in inert form, crystal cells may represent the solution of this problem and thus play a role in the plant's Ca-metabolism which has not been fully recognized in the past.

In the analysis of Ca-uptake during crystal induction described in this paper, it was found that acetate and to a lesser extent lactate and carbonate, but not other anions, facilitated the absorption and accumulation of relatively large quantities of Ca^{2+} by isolated, peeled *Gleditsia* leaflets.

Material and methods

Experimental material; microscopic observation of Ca-oxalate crystals. Seedlings of *Gleditsia* were grown and leaflets were peeled as described in Borchert (1985). For microscopic observation and microphotography in polarized light, leaflets were fixed, destained, and imbedded as also described in Borchert (1985).

Measurement of Ca-uptake. For short-term (24–48 h) measurements of Ca-uptake, peeled leaflets were aged at 25° C for 12 or 24 h under constant illumination (400 $mmol \cdot m^{-2} \cdot s^{-1}$; fluorescent F40PL Plant Light; General Electric, Cleveland, O., USA) on 0.1 mM Ca-acetate (40 leaflets per 10-cm-diameter Petri dish containing 15 ml solution). Normally, 10 leaflets per experimental treatment were used. Sets of 10 peeled leaflets weighed between 36 and 45 mg. The fresh weight of 10 peeled leaflets was assumed to be 40 mg in all calculations, because weighing peeled leaflets for each experiment was found to be impractical, and the leaflets varied not only with respect to size (fresh weight and leaf area), but also with respect to the fraction of leaf surface actually peeled. Leaflets were treated in small (6 cm diameter) disposable plastic Petri dishes containing 2 or 3 ml experimental solution with $10 \cdot 10^5$–$15 \cdot 10^5$ $cpm \cdot ml^{-1}$ ^{45}Ca (added as $CaCl_2$; Amersham Corporation, Arlington Heights, Ill., USA) and shaken at 100 rpm under continuous light (see above). Two 10-µl aliquots were pipeted at the indicated times onto 1-cm^2 pieces of filter paper, which were then inserted into 3-ml Nalgene Filmware tubes (Nalge Co., Rochester, N.Y, USA) containing 1 ml scintillation liquid (Scintiverse E; Fisher Scientific Co., Fairlawn, N.J., USA). Samples were counted in a liquid scintillation counter (Packard Tricarb; United Technologies Packard, Downers Grove, Ill., USA). Using a program written in BASIC, the following values were calculated on a microcomputer for each sampling time from the disappearance of ^{45}Ca from the experimental solutions: Ca-uptake as $\mu M\ Ca^{2+} \cdot (10\ leaflets)^{-1}$ and $\mu M\ Ca^{2+} \cdot (g\ FW)^{-1}$ (equivalent to the $[Ca^{2+}]$ in mM in the tissue), rate of Ca-uptake as $\mu mol\ Ca^{2+} \cdot h^{-1} \cdot (g\ FW)^{-1}$, and percent uptake of ^{45}Ca in the original experiment solution. In each experiment, individual treatments were done in duplicate, and experiments were repeated at least three times. In important experiments, errors were calculated from three or four identical experimental treatments in one experiment.

For measurement of long-term Ca-uptake (3–6 d), 10 leaflets per experimental group were floated under constant illumination (see above) on 25–150 ml experimental solution in stoppered 125- or 250-ml Erlenmeyer flasks and stirred at 100 rpm with magnetic stirrers. Two 50-µl aliquots were taken at the indicated times, added to 1 ml scintillation liquid in 3-ml Nalgene Filmware tubes, counted, and evaluated as described above.

Uptake of [^{14}C]acetate. Methods for uptake of [^{14}C]acetate (Na-acetate; Amersham Corp.) were identical to those described above for Ca-uptake. To determine the release of $^{14}CO_2$ during long-term uptake of acetate, a 3-ml plastic cup containing 5 cm^2 filter paper soaked with 0.5 ml 20% KOH (w/v) was suspended in the air space of a tightly sealed (Parafilm plus rubber stopper) Erlenmeyer flask; at the end of the experiment, the filter paper was dried, placed into 5 ml scintillation liquid, and the radioactivity counted as described above.

Distribution of ^{45}Ca and [^{14}C]acetate in tissue fractions. After the determination of the uptake of Ca^{2+} or acetate, leaflets were washed for a minimum of 6 h in 10 ml of the washing solutions described in *Results*. Aliquots (2 ml) of the washes and, ultimately, the washed leaflets were added to 4 ml scintillation liquid and counted in 7-ml scintillation vials as described above. To verify the formation of Ca-oxalate crystals in the leaflets during long-term uptake experiments, groups of leaflets were treated like the corresponding experimental groups, but without radio isotope, and were examined microscopically as described in Borchert (1985).

Results

During the experimental induction of calcium oxalate crystals in isolated *Gleditsia* leaflets, crystal formation was observed to begin 3 d after exposure of the leaflets to the inductive Ca-solution (Borchert 1985). In the present study, uptake and accumulation of Ca^{2+} during the induction period preceding crystal formation has been analyzed, but the criterium for optimum induction remains crystal formation following induction.

Table 1. Calcium-oxalate-crystal formation in peeled *Gleditsia* leaflets as a function of Ca-concentration and total available Ca^{2+} in the experimental solution. Ten leaflets per treatment were floated for 6 d on variable volumes of Ca-acetate containing the indicated amounts and concentrations of Ca^{2+}. Scoring of crystal formation: 0–no crystals present; 1–few, scattered crystals; 2–many crystals along the veins, but few in the mesophyll; 3–crystals in up to 50% of mesophyll area; 4–mesophyll densely packed with crystals (see Fig. 1 B)

$[Ca^{2+}]$	Total Ca^{2+} (µmol)			
	2.5	5	10	20
0.25 mM	0–1	1–2	1–2	
0.5 mM	0–1	2–3	3–4	4
1.0 mM		1–2	3–4	3–4
2.0 mM			3	3–4

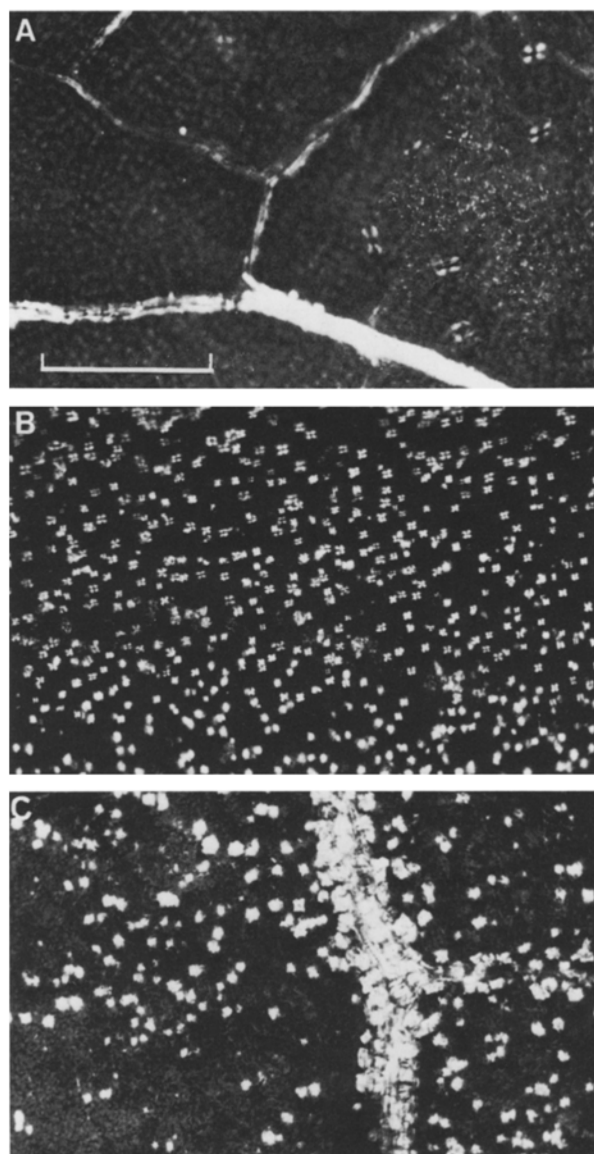

Fig. 1 A–C. Distribution of calcium oxalate crystals in peeled, cleared *Gleditsia* leaflets, as seen in polarized light. ×240; bar = 100 μm. **A** Untreated, partially peeled leaflet with small terminal veins (*top*) and major vein (*bottom*). Birefringent guard cells of stomata and minute calcium oxalate crystals, located in the lower epidermis, are visible in the unpeeled leaf portion at *right*. **B** Ca-oxalate crystals induced in the palisade parenchyma under optimum experimental conditions (see Table 1). **C** Formation of many, relatively large Ca-oxalate crystals in the unpeeled portions of a leaflet (*right half* including vein) and of relatively few, small crystals in the peeled portion (*left*)

Optimization of crystal formation. Crystal formation on small, constant volumes of experimental solutions of variable [Ca^{2+}] was observed to be irregular. Since crystal formation is likely to vary as a function of both [Ca^{2+}] and total Ca^{2+} in the medium, crystal formation was determined using variable volumes of experimental solutions

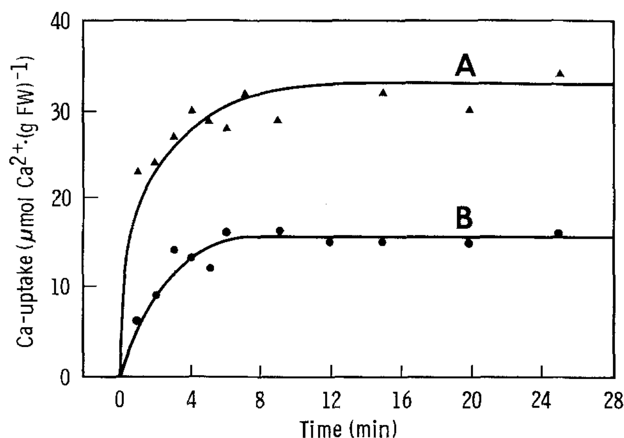

Fig. 2. Calcium uptake from 1 mM [^{45}Ca]acetate by peeled *Gleditsia* leaflets during the first 30 min of experimental treatment. *Curve A*, freshly peeled, 25-d-old leaflets; *curve B*, freshly peeled, 45-d-old leaflets. Ten leaflets were placed into 2 ml experimental solution, two 10-μl aliquots were taken at the indicated intervals, and Ca-uptake was calculated from the disappearance of ^{45}Ca from the experimental solution as described in *Material and methods*

(Table 1). Combinations of 0.5–2 mM Ca^{2+} and 2 μmol Ca^{2+} per leaflet (= 50 μmol Ca^{2+} · (g FW)$^{-1}$) were found to be optimal for crystal formation (Fig. 1 A, B) and were therefore used in long-term Ca-uptake studies.

Uptake of Ca from Ca-acetate. In measurements of Ca-uptake by plant tissues, ionic binding of Ca^{2+} by the negatively charged cell-wall matrix must be distinguished from Ca-uptake into the protoplast (Macklon 1984). When placed on 1 mM Ca-acetate, peeled, aged or non-aged *Gleditsia* leaflets rapidly absorbed Ca^{2+} into the cell-wall free space (Fig. 2). Within 10–12 min [Ca^{2+}] in the tissue reached 15–35 μmol · (g FW)$^{-1}$. Consistently, initial Ca-uptake by older leaflets (40–50 d old) was significantly lower than by young (22–27 d old) leaflets (Fig. 2). In numerous Ca-uptake experiments, [Ca^{2+}] in young leaflets measured 30 or 60 min after initial exposure to ^{45}Ca was in the range of 20 to 35 μmol · (g FW)$^{-1}$ (sources of variability in the experiments are discussed in *Material and methods*), and only Ca-uptake above this range may therefore be considered as Ca-uptake into the protoplast.

Uptake of Ca from 0.5 and 2 mM Ca-acetate, measured at 12-h intervals, was rapid for 3 d and then began to level off (Fig. 3A: I, II). The maximum rate of Ca-uptake was 2.2 μmol $Ca^{2+} \cdot h^{-1}$ · (g FW)$^{-1}$ and the final [Ca^{2+}] in the tissue was between 250 and 350 μmol · (g FW)$^{-1}$ in all experiments (compare Table 2).

To determine the distribution of absorbed Ca^{2+} in the leaf cells, the following washes were

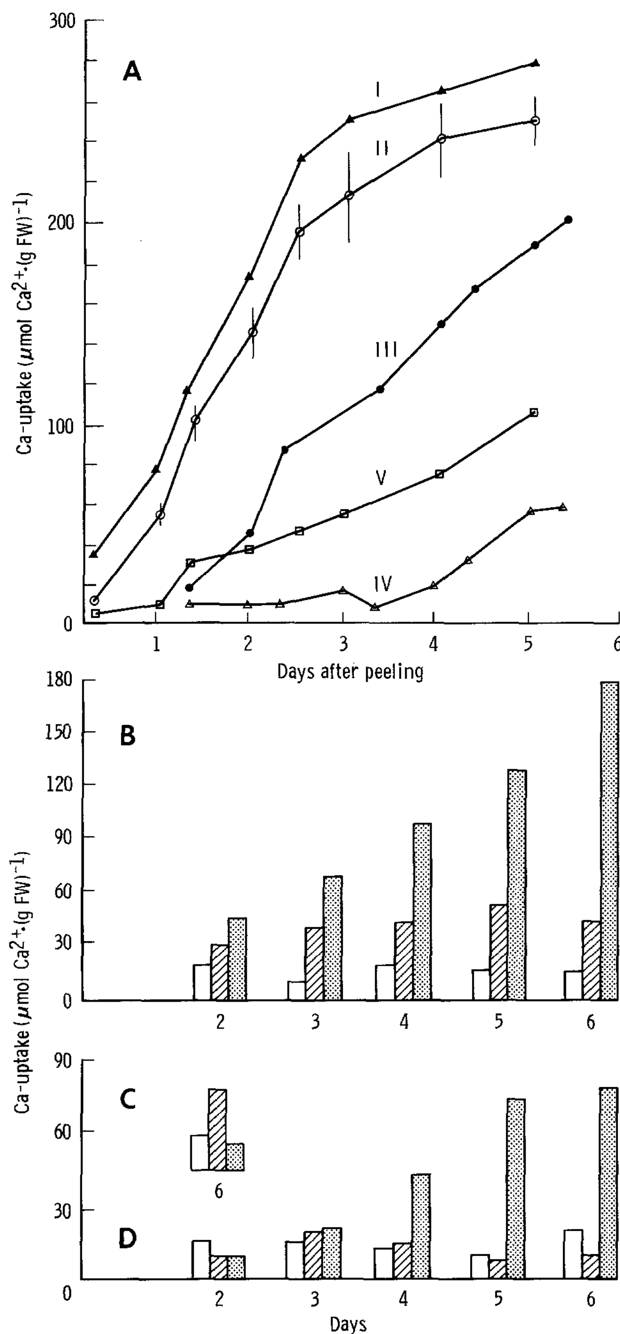

Fig. 3A–D. Long-term Ca-uptake by *Gleditsia leaflets* (**A**) and distribution of absorbed Ca^{2+} in three major cell compartments (cell-wall, vacuole, Ca-oxalate crystals; **B–D**). **A** Uptake of Ca by peeled leaflets from: I. 0.5 mM Ca-acetate (▲——▲); II. 2 mM Ca-acetate (○——○); III. 2 mM Ca-lactate (●——●); IV. 2 mM Ca-chloride (△——△); V. saturated (0.16 mM) Ca-carbonate (□——□). Uptake of Ca was measured with leaflets floating on 25–100 ml stirred solution in 150-or 250-ml Erlenmeyer flasks. Aliquots of 50 µl were taken at the indicated times, and Ca-uptake was calculated from the disappearance of ^{45}Ca as described in *Material and methods*. **B** Distribution of ^{45}Ca in major cell compartments after treatment of peeled *Gleditsia* leaflets with 2 mM Ca-acetate for 2–6 d. Leaflets were incubated on ^{45}Ca-solutions as described for **A** above, sampled at the indicated days, and washed in the following washes as described in *Material and methods*. *Left bars*: cell-wall-bound Ca^{2+}, washed out with 3 mM Na-EDTA. *Center bars*: soluble (mostly vacuolar) Ca^{2+}, released by 95% ethanol + 10 mM Ca-chloride. *Right bars*: insoluble Ca^{2+} (Ca-oxalate) released by 2 N HCl + 10 mM $CaCl_2$. After this wash, only minute amounts of Ca^{2+} remained in the leaflets. **C** Distribution of Ca in peeled leaflets treated with 2 mM Ca-chloride for 6 d. **D** Distribution of Ca in unpeeled leaflets with two parallel, longitudinal cuts, treated with 2 mM Ca-acetate

Table 2. Uptake of ^{45}Ca and ^{14}C-acetate by peeled *Gleditsia* leaflets during the formation of calcium-oxalate crystals. Data are the means of five replicates taken after 5 d incubation of 10 leaflets on 50 ml 0.5 mM Ca-acetate

	^{45}Ca	SD	^{14}C	SD
Solute uptake (µmol)	12.9	1.5	31.4	2.8
[Solute] in the tissue (µmol)·(g FW)$^{-1}$	323.3	36.6	776.4	75.0

used (Fig. 3B–D): (1) 3 mM Na_2-ethylenediaminetetraacetate (Na-EDTA), which effectively binds cell-wall-bound Ca^{2+}, but cannot penetrate the plasmalemma to chelate soluble Ca^{2+} within the protoplast; (2) 95% ethanol + 10 mM Ca-chloride (to displace ^{45}Ca) releases all soluble Ca^{2+} from the various cell compartments by rendering membranes permeable. In view of the quantities of absorbed Ca^{2+}, it is likely that most soluble Ca^{2+} is stored in the vacuole; (3) 2 N HCl dissolves Ca-oxalate crystals and thus releases Ca^{2+} insoluble in the ethanol wash. Between 83 and 96% of the previously absorbed ^{45}Ca was recovered in the washes in most experiments. Throughout the period of Ca-uptake by peeled leaflets, cell-wall-bound Ca^{2+} constituted the smallest and, as expected, rather constant fraction of absorbed Ca^{2+}; soluble Ca^{2+} increased moderately to 40–50 µmol Ca^{2+}·(g FW)$^{-1}$, but Ca^{2+} precipitated as Ca-oxalate increased dramatically with time (Fig. 3B).

During peeling of *Gleditsia* leaflets most crystal cells located in the bundle sheath of major veins are removed with the lower epidermis (Fig. 1A). The number of Ca-sinks in peeled leaflets is thus significantly reduced until new ones have formed during experimental induction of crystal cells (Borchert 1985). If Ca-uptake by unpeeled leaflets is made possible by means of longitudinal cuts with a razor blade rather than by peeling, the number of functional Ca-sinks should not be affected and cut leaflets should effectively convert absorbed Ca^{2+} to Ca-oxalate in the existing Ca-sinks. Compared with peeled leaflets, Ca-uptake by cut leaflets was relatively low, and the soluble fraction remained very small – usually below 15 µmol Ca^{2+}·

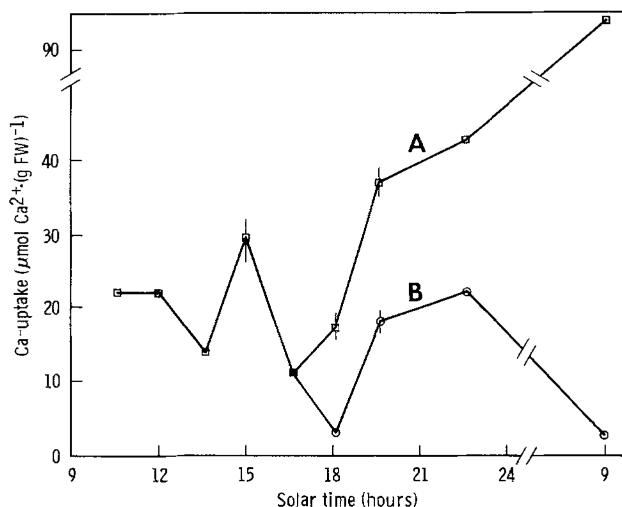

Fig. 4. Uptake of Ca by peeled *Gleditsia* leaflets from 2 mM [^{45}Ca]acetate between 24 and 48 h after peeling. *Curve A.* 2 mM Ca-acetate (□——□); *curve B*, 2 mM Ca-chloride (o——o). Leaflets were aged for 24 h on 0.1 mM Ca-acetate, then treated on 3 ml experimental solution per 10 leaflets. Two 10-μl aliquots were taken at the indicated times and Ca-uptake was calculated as described in *Material and methods*

(g FW)$^{-1}$ – throughout the experiment (Fig. 3D). The absorbed Ca^{2+} was deposited mostly in existing crystal cells along the veins, where small Ca-oxalate crystals grew notably during the experimental treatment; only few crystal cells were newly induced in the palisade parenchyma.

The time course of long-term Ca-uptake as described in Fig. 3A indicates that Ca-uptake proceeds steadily at relatively high rates for several days. However, measurement of Ca-uptake by aged and non-aged leaflets at 1- or 2-h intervals showed that during the first 10 h of treatment with 1 or 2 mM Ca-acetate, periods of Ca-uptake alternated with periods during which a large fraction of the previously absorbed Ca^{2+} was released into the experimental solution (Fig. 4). When leaflets were aged for 24 h and treated with Ca-solutions in the morning, there was always a moderate release of Ca^{2+} between 12:00 and 14:00 h and an almost complete release of previously absorbed Ca^{2+} during the late afternoon (17:00–19:00 h) of the leaflets' first day of exposure to Ca^{2+}. Leaflets placed on Ca-acetate during the early afternoon showed only the second period of Ca-release. Essentially the same time course of Ca-absorption was observed with leaflets placed on 1 mM Ca-acetate right after peeling (data not shown). During the following days Ca-uptake from Ca-acetate proceeded at high, rather constant rates (Fig. 4A), but leaflets treated with Ca-chloride eventually released all previously absorbed Ca^{2+} into the medium (Fig. 4B).

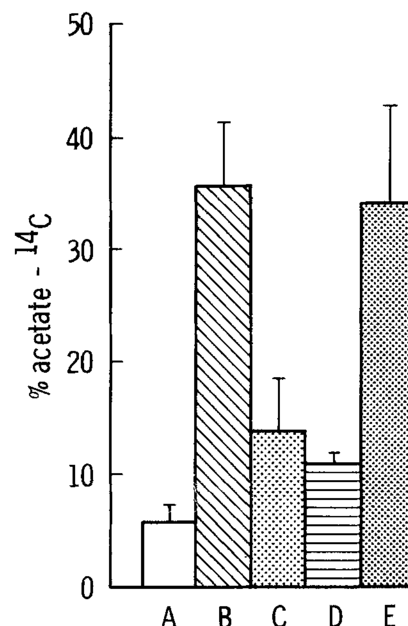

Fig. 5. Distribution of ^{14}C absorbed from 0.5 mM Ca-acetate in various fractions of *Gleditsia* leaflets after 5 d of experimental treatment. *Bars A–C*: acetate-^{14}C located in the cell-wall (*A*), in the soluble fraction of the protoplast (mostly vacuole; *B*), and in Ca-oxalate (*C*) (washes as described in Fig. 3B); *bar D*: ^{14}C remaining in the leaflets after the last wash; *bar E*, ^{14}C in CO_2 trapped by KOH in the airspace above the experimental solution

The role of acetate in the induction of calcium-oxalate crystals. Enhancement of Ca-uptake by acetate (Fig. 3, Table 2) indicates that acetate may make Ca-uptake possible by serving as a counterion which permeates the plasmalemma in its protonated form, enters the vacuole, and balances the charges of Ca^{2+} accumulating in the vacuole. Acetate might also function as a substrate for oxalate synthesis, and acetate-^{14}C should then be incorporated preferentially into the acid-soluble Ca-oxalate fraction. In either case, 2 mol of acetate should be taken up for each mole of Ca^{2+} absorbed. A comparison between the uptake of ^{45}Ca and [^{14}C]acetate shows that acetate uptake during 5 d of treatment was approx. 25% higher than the predicted stoichiometry (Table 2).

The distribution of absorbed ^{45}Ca and acetate-^{14}C in the various tissue fractions was compared using the washes described above (Fig. 3B); in addition, retention of ^{14}C in the acid-washed leaflets and release of $^{14}CO_2$ into the gas phase of the experimental vessel was determined (Fig. 5). In contrast to the distribution of ^{45}Ca observed after treatment with Ca-acetate (Fig. 3B, 6 d), very little ^{14}C was found in the cell-wall fraction, but a major part remained in the soluble protoplast fraction; less than 20% ^{14}C was incorporated into Ca-oxa-

Table 3. Uptake of Ca by peeled *Gleditsia* leaflets from solutions of the Ca-salts of various organic and inorganic anions. Leaflets were aged for 24 h on 0.1 mM Ca-acetate, then placed on 3 ml 2 mM Ca-solutions adjusted to pH 6.8 with $Ca(OH)_2$. Uptake of ^{45}Ca was measured as described in *Material and methods*

	Experimental treatment	µmol $Ca^{2+} \cdot (g\ FW)^{-1}$ after ...		
		3 h	9 h	24 h
	Organic onions:			
A	Acetate	36	71	131
B	Lactate	39	47	53
C	Glycolate	14	14	22
D	Glyoxalate	15	14	17
E	Citrate	24	13	12
F	Malate	15	8	11
G	Ascorbate	25	20	13
	Inorganic anions:			
H	Chloride	26	10	0
I	Nitrate	15	5	0
J	Sulfate	12	4	0
K	*Ca-uptake by cell walls after 30 min*	20–28		

late, but substantial amounts were retained in the acid-washed leaflets (presumably in starch, suberin and other polymers synthesized during treatment) or metabolized to CO_2.

Effect of various anions on Ca-uptake. In earlier work it had been found that formation of Ca-oxalate crystals could be induced in peeled *Gleditsia* leaflets by Ca-acetate and Ca-carbonate, but not by inorganic salts such as Ca-chloride, nitrate or sulfate (Borchert 1985). The effect of various inorganic and small organic anions on Ca-uptake was therefore studied (Fig. 3, Table 3).

Uptake of significant amounts of Ca^{2+} during the crystal induction phase occurred, in addition to acetate, only with lactate and carbonate (Fig. 3A, III, V); rates of Ca-uptake from solutions of these latter salts were notably lower than from acetate, and with Ca-carbonate there was a distinct lag phase before any measurable Ca-uptake occurred (Fig. 3A, V). Both anions induced the formation of numerous Ca-oxalate crystals. No Ca^{2+} was absorbed from Ca-chloride during the first 4 d; the small amount of Ca^{2+} taken up during days 5 and 6 (Fig. 3A, IV) remained mostly in the soluble fraction and was not precipitated as Ca-oxalate (Fig. 3C).

During a 24-h treatment of aged, peeled leaflets with 2 mM Ca-salts of various small organic acids, substantial Ca-uptake was observed only with acetate and lactate, whereas virtually no Ca^{2+} was absorbed into the protoplast from solutions of other small organic Ca-salts (Table 3A–G); however, the leaflets retained most of the cell-wall-bound Ca^{2+} taken up initially (20–28 mM Ca^{2+}). Calcium propionate was also tested but found to be toxic to the leaflets. In treatments with inorganic Ca-salts (Table 3H–J) there was no Ca-uptake into the protoplast, and wall-bound Ca^{2+} was completely released within 24 h. If leaflets were transferred to 2 mM Ca-acetate after a 24-h treatment with the above organic or inorganic salts, Ca-uptake proceeded normally on Ca-acetate.

Discussion

Phases of Ca-uptake. The following three phases, differing in the kinetics of Ca-uptake, could be distinguished during Ca-uptake by peeled leaflets from 0.5–2 mM Ca-acetate:

(1) Uptake of Ca^{2+} into the cell-wall free space. Like other plant tissues, young, peeled *Gleditsia* leaflets placed on Ca-solutions rapidly absorbed 20–35 µmol $Ca^{2+} \cdot (g\ FW)^{-1}$ into the cell-wall free space (Fig. 2; Marré et al., 1982; Demarty et al. 1984). Similar concentrations of negative charges in the cell-wall have been found in other plant tissues (12 and 16 µmol $\cdot (g\ FW)^{-1}$ for beet root tissue and *Atriplex* leaf slices, respectively; Lüttge and Higinbotham 1979, p. 94). During the saturation of cell walls with Ca^{2+}, protons were displaced and released into the medium, as indicated by a rapid decline in pH from 6.15 to 5.5 during treatment with unbuffered 1 mM Ca-chloride (data not shown).

(2) Induction of the Ca-uptake mechanism. In other plant tissues, short-term, rapid cation uptake into the cell-wall free space is normally followed immediately by a slower, but continuous, active cation transport into the protoplast (Lüttge and Higinbotham 1979, p. 93; Marré et al. 1982). The kinetics of Ca-uptake into peeled *Gleditsia* leaflets differs in two respects from the pattern observed for cation uptake in other plant tissues: (i) for several hours after the initial saturation of the cell-wall free space with Ca^{2+} there is no measurable uptake of Ca^{2+} into the protoplast, and (ii) Ca^{2+} absorbed into the cell-wall is released periodically during the first 8–12 h of treatment of freshly peeled or aged leaflets with 1–2 mM Ca-solutions (Fig. 4). These observations indicate that certain components of the mechanism involved in the subsequent long-term uptake of Ca^{2+} into the protoplast (Fig. 3) and its storage in the vacuole are initially not functional, but are induced by the exposure of the leaflets to 1–2 mM Ca^{2+}. Most lik-

ely, Ca^{2+} released into the medium is displaced from the cell wall by H^+ extruded into the cell-wall space by proton pumps located on the plasmalemma (Lüttge and Higinbotham 1979, p. 130; Sze 1985; Romani et al. 1985). In numerous experiments, this release of Ca^{2+} occurred during the same periods of solar time (12:00–14:00 and 18:00–20:00); Fig. 4), but at different time intervals after the initial exposure of the leaflets to Ca^{2+}. The periodic activity of proton pumps thus appears not to be triggered simply by the initial exposure to Ca^{2+}, but might be also related to a circadian rhythmicity of ion transport as it is manifest in the nyctinastic movements of *Gleditsia* seedling leaflets and leaves of other leguminous trees (for a review, see Satter and Galston 1981).

(3) Continuous, long-term Ca-uptake. After the mechanism for Ca-uptake has become functional, isolated, peeled, illuminated *Gleditsia* leaflets are capable of absorbing Ca^{2+} at relatively high rates and of concentrating the absorbed Ca^{2+} more than 500-fold. These observations are in contrast with the widely held notion, based mostly on studies of Ca-uptake by isolated roots (Macklon 1984), that plant cells absorb Ca^{2+} only slowly and in small quantities.

Precipitation of absorbed Ca^{2+} as Ca-oxalate. If crystal cells are efficient sinks for surplus soluble Ca^{2+} as postulated in the *Introduction*, then Ca^{2+} taken up during experimental treatment of plant tissues containing crystal cells should be rapidly precipitated in these cells as Ca-oxalate. In agreement with this prediction, Ca^{2+} absorbed by isolated, unpeeled, but longitudinally cut *Gleditsia* leaflets was mostly precipitated as Ca-oxalate, and the content in cell-wall-bound and soluble (vacuolar) Ca^{2+} remained low throughout the treatment period (Fig. 3D). In peeled *Gleditsia* leaflets, from which crystal cells have been removed during peeling (Fig. 1A), Ca^{2+} had been found earlier to accumulate in soluble form for 3 d before Ca-oxalate crystals became visible (Borchert 1985). Contrary to these observations, during treatment of peeled leaflets with Ca-acetate, there was a progressive accumulation of Ca-oxalate well before new crystals had appeared (Fig. 3B, 2–4 d), but leaflets also accumulated 40–50 $\mu mol \cdot (g\ FW)^{-1}$ soluble Ca^{2+} (Fig. 3B). These data reflect the fact that actually in "peeled" leaflets variable portions (10–30%) remain unpeeled and thus contain crystal cells capable of precipitating absorbed Ca^{2+} (Fig. 1C). The observed concentrations of soluble, most likely vacuolar, Ca^{2+} are much higher than values reported for other plant tissues such as onion roots (2 mM; Macklon 1984) and various leaves (2–22 mM; Jeschke 1976).

The accumulation of substantial quantities of soluble vacuolar Ca^{2+} during Ca-uptake into leaf tissues lacking functional Ca-sinks in the form of crystal cells implies that oxalate is not available in these tissues to precipitate Ca^{2+} and that oxalate synthesis may be confined to functional crystal cells. Also, it is unlikely that acetate enhances Ca-uptake by functioning as a specific substrate for oxalate biosynthesis, because the period of maximum uptake of Ca^{2+} and acetate preceded maximum oxalate synthesis by a day or two (Fig. 3) and less than 20% of the absorbed acetate-^{14}C was incorporated into oxalate (Fig. 5).

The role of anions in Ca-uptake. The accumulation of large quantities of soluble Ca^{2+} in the vacuoles of mesophyll cells must be accompanied by the uptake of counterions balancing the positive charges of Ca^{2+}. The marked enhancement of Ca-uptake by acetate compared with other anions (Table 3), indicates that acetate may stimulate Ca-uptake because of its nature as a permeant, weak acid. In several recent studies (Brummer et al. 1984; Hager and Moser 1985; Romani et al. 1985) the uptake of weak permeant acids or their esters was observed to cause hyperpolarization of the plasmamembrane and an increase in K^+-uptake, presumably by stimulating the plasmalemma proton pumps through lowering the cytoplasmic pH. Also, the uptake of divalent cations (not including Ca^{2+}) followed an electrochemical gradient and was balanced by the efflux of cations, mostly protons (Marré et al. 1982). Like other weak acids, acetate is likely to stimulate plasmalemma H^+-transfer ATPases which establish an electrochemical gradient enhancing entry of Ca^{2+} into the cytosol, but acetate may also serve as a readily available counterion balancing the charges of Ca^{2+} accumulating in the vacuole or as a substrate for the synthesis of malate, the counterion commonly produced by plant cells during cation accumulation (Lüttge and Higinbotham 1979, p. 136).

Like acetate, carbonate induces optimum crystal formation and stimulates long-term Ca-uptake (Fig. 3A), probably by serving as a substrate for the synthesis of malate. Lactate should enhance Ca-uptake for the same reasons as acetate, although to a lesser extent because of its larger size and less lipophilic character. Dicarboxylic acids such as malate and citrate are not fully protonated at biological pH and thus, as charged molecules, cross the plasmalemma only slowly. For example, citrate (3 mM, pH 4.5) acidified the cytosol of

maize coleoptile cells noticeably less than acetate (Brummer et al. 1984). Glycolate, glyoxalate and ascorbate, selected for testing because they are known precursors in the biosynthesis of oxalate, were likewise unable to support Ca-uptake (Table 3).

The complete release of cell-wall-bound Ca^{2+} into the medium during treatment with inorganic anions (Fig. 4B; Table 2 H–K) must reflect the release of protons into the cell wall, as it has been frequently observed during uptake of surplus cations (Lüttge and Higinbotham 1979, p. 137). However, in my study, proton release was not preceded or accompanied by a measurable Ca-uptake into the protoplast and occurred in the presence of small, permeable anions, indicating other causes for the inferred proton release than proton/cation exchange. Several divalent cations (Co^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}) were found to be absorbed from a 2 mM sulfate solution into excised corn roots, but Ca^{2+} was not absorbed (Marré et al. 1982), just as it was not taken up by peeled *Gleditsia* leaflets from Ca-sulfate (Table 3). The observed resumption of rapid Ca-uptake upon transfer of leaflets from sulfate and other non-stimulating anions to Ca-acetate indicates that these anions are not toxic to the tissue, but merely fail to facilitate Ca-uptake; this explains the earlier observation that these anions, unlike acetate and carbonate, do not induce the formation of crystal cells in isolated *Gleditsia* leaflets (Borchert 1985).

The role and differentiation of crystal cells. The formation of Ca-oxalate crystals in plant tissues has been studied for many years (for a review, see Franceschi and Horner 1980), but information concerning the function of these crystals remains scarce. In this study it has been demonstrated that crystal cells act as Ca-sinks and enable plant tissues to deposit surplus Ca^{2+} absorbed under experimental – and, implicitly, natural – conditions as Ca-oxalate and to maintain relatively low concentrations of soluble Ca^{2+} in the apoplast and vacuoles of the tissue. If the capacity of existing Ca-sinks is insufficient to precipitate most apoplastic Ca^{2+}, the tissue content in soluble Ca^{2+} will rise and new crystal cells are induced (Fig. 3C; Borchert 1985). The preliminary evidence presented here indicates that differentiation of crystal cells involves the induction of mechanisms for the transport of Ca^{2+} across plasmalemma and tonoplast into the vacuole, the synthesis of organic counterions required for storage of soluble Ca^{2+} in the vacuole, and, lastly, the induction of oxalate synthesis. Both Ca-uptake and the synthesis of organic counterions and oxalate depend on an adequate supply of light energy and photosynthetic products. It should thus come to no surprise that uptake and precipitation of relatively large quantities of Ca^{2+} is possible in isolated, illuminated leaflets, but apparently not in non-photosynthetic organs such as roots (Macklon 1984).

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