Ca$^{2+}$ as developmental signal in the formation of Ca-oxalate crystal spacing patterns during leaf development in *Carya ovata*  
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**Abstract.** Changes in the spacing patterns of Ca-oxalate crystals during enlargement of *Carya ovata* Mill. leaves were quantified by computerized image-analysis. Single Ca-oxalate crystals form in the vacuoles of young mesophyll cells transformed into crystal cells. Crystals are very small in newly induced crystal cells and increase in size throughout leaf development. Crystal patterns thus reflect both induction and relative age of crystal cells. Shortly after the emergence of young leaves from the bud, very small crystals are formed in the mesophyll at high density. As leaves expand, these crystals grow larger and become separated by increasing distances. New small crystals appear in the gaps between the older, larger crystals. Later crystal patterns consist of widely spaced, larger crystals only. Finally, clusters of small crystals are formed again in the gaps between large crystals. No crystals were observed in young leaves expanding in a moist chamber, but large numbers of crystal cells were induced experimentally in sections of immature leaves floating on 4 mM Ca-acetate. The observations support the following mechanism of crystal-pattern formation: Ca$^{2+}$ carried into leaves with the transpiration stream acts as the developmental signal inducing transdifferentiation of a few mesophyll cells into crystal cells when apoplastic [Ca$^{2+}$] rises. Crystal cells precipitate absorbed Ca$^{2+}$ as oxalate and, acting as Ca$^{2+}$ sinks, inhibit crystal-cell induction in their vicinity by depleting apoplastic Ca$^{2+}$. This prevents close spacing of crystal cells. New crystal cells form in the gaps between the depletion zones of older crystal cells when these move apart during leaf expansion. Later changes in crystal patterns result from increasing sink strength of crystal cells, lowered inducibility of mesophyll cells, and increased Ca$^{2+}$ influx into leaves during intensive transpiration. Throughout leaf development, spacing of crystal cells permits rapid secretion of apoplastic Ca$^{2+}$ as Ca-oxalate.

**Key words:** Calcium-oxalate crystals – *Carya* – Leaf development – Spacing patterns

**Introduction**

Most specialized cells in plant leaves such as stomata, hairs, gland cells and veins are arranged in characteristic, species-specific spatial patterns (Bünning 1965). The regular, more or less equidistant spacing of stomata in the leaf epidermis of many plants has been the classical object of studies aimed at elucidating the developmental mechanisms responsible for the formation of such spacing patterns (Bünning 1965; Korn 1981; Sachs 1978, 1984). Bünning (1965) postulated that stomata and other differentiated cells inhibit cell differentiation in their vicinity, and new cells can differentiate in response to the respective developmental signal only when gaps arise between the inhibition zones in the course of leaf expansion (Fig. 1). The characteristic minimum distances between differentiated cells in spacing patterns would thus result from the interaction between developmental signals, inhibition by differentiated cells, and growth of the tissue. This mechanism of pattern formation has been widely accepted because of its compelling simplicity, but there is little direct experimental evidence to support it. Neither the developmental signal inducing differentiation of stomata nor the chemical basis of inhibition by differentiated stomata are known.

Leaves of most higher plants contain numerous Ca-oxalate crystals, often arranged in species-specific spatial patterns (Napp-Zinn 1973, p. 437 et seq.). For example, crystals may be found exclusively in the bundle-sheath cells along the veins or may be scattered in the mesophyll. In developing leaves of *Gleditsia triacanthos* crystal patterns change characteristically during leaf development (Borchert 1984). When exposed to Ca-acetate, mesophyll cells of young, peeled *Gleditsia* leaflets form Ca-oxalate crystals, i.e., they transdifferentiate into Ca$^{2+}$-accumulating crystal cells (Borchert 1985, 1986).
results support a mechanism of pattern formation similar to that proposed for the spacing of stomata.

Material and methods

Leaf material. For the analysis of crystal patterns in young leaves of increasing age and for the experimental induction of crystal cells, series of immature leaves of increasing size were obtained from young shoots of shagbark hickory (Carya ovata) within two weeks after bud break between April 20 and May 5, 1988 and 1989 in woodlands near Lawrence, Kan. Mature leaves were collected later in the growing season.

Microscopic analysis of crystal patterns. Sections from the terminal leaflet of the compound Carya leaf were cleared with 5% Naohyochlorite for 4–6 h, washed in 70% ethanol and water, and imbedded in glycerin-jelly (Fisher Scientific, Pittsburgh, Pa., USA) on microscope slides. From each cleared leaf section two to four adjacent crystal fields containing 50–200 Ca-oxalate crystals were photographed at the appropriate magnification in partially polarized light with a Zeiss (Oberkochen, FRG) Photomicroscope II. To maintain comparability, all micrographs were printed at the same enlargement, and magnification was determined from micrographs of a micrometer scale photographed and printed like the leaf samples.

Electron microscopy. Leaf samples (2×5 mm²) were fixed in 3% glutaraldehyde overnight, postfixed in 1% OsO₄ for 2 h, dehydrated in ethanol and xylol, and embedded in Spurr’s medium (Spurr 1969). Thin sections were poststained with lead citrate, mounted on formvar-coated grids, and examined with a Philips (Eindhoven, The Netherlands) transmission electron microscope in the scanning mode.

Quantification of crystal patterns. X- and y-coordinates of the following components of each crystal field were collected using a sonic digitizer (Model GP7 Grafbar; Science Accessories Corp., Southport, Conn., USA): (1) corners of the crystal field; (2) corners of vein islets (=intercostal fields); (3) two points delimiting a diameter across each crystal. Data were analyzed on a microcomputer (Zenith 158; Zenith Data Systems Corp., St. Joseph, Mo., USA) using programs written in BASIC. To verify the correctness of data entry, an image of the recorded crystal field was generated from the raw data by high-resolution computer graphics on the video screen and compared with the original micrograph (Figs. 2, 3–7A). Crystal fields were captured from the screen and printed on a LaserJet Printer (Hewlett-Packard Co., Boise, Idaho, USA) using the PC-Draft II program (Natural Software, St. Charles, Ill., USA). Patterns with random crystal size and distribution were computer-generated for comparison with natural crystal patterns (Fig. 8). Statistical methods developed for the evaluation of spatial distribution of uniform differentiated cells (e.g. stomata) in a tissue or of organisms in an ecosystem (Picou 1974; Sachs 1984) were not suited for this study, because crystal spacing patterns are characterized both by size distribution of crystals and minimum distances between crystals. Patterns were therefore quantified as described below.

The prediction to be tested was that small crystals in newly induced crystal cells should form outside the depletion zone of older crystal cells, i.e., at a certain minimum distance from larger crystals (Fig. 1). Three sets of variables were calculated to describe differences between patterns in crystal fields and to test the proposed mechanism of pattern formation.

1) Crystal size is a measure of relative age of crystal cells. Crystal diameters were obtained as the distance between the two points recorded for each crystal; crystal volumes were calculated from diameters assuming that the aggregate (druse) crystals have spherical shape. The following measures of crystal size are given for

Thus Ca²⁺ is the developmental signal inducing crystal-cell differentiation when present at concentrations above the threshold for induction. In turn, Ca²⁺ is being absorbed and secreted by crystal cells functioning as Ca²⁺ sinks. Crystal cells thus deplete the developmental signal, Ca²⁺, in the depletion zones surrounding older crystal cells. Changes in the pattern of A resulting from expansion of the tissue and growth of the inhibition zones with age of the differentiated cells. For explanation see text.

Fig. 1. A Formation of a spacing pattern by inhibition of cell differentiation within inhibition zones (dotted circles) around differentiated cells (large black dots) and cell differentiation (small black dots) in the gaps between inhibition zones. In crystal-spacing patterns, inhibition of crystal-cell differentiation is the result of depletion of the developmental signal, Ca²⁺, in the depletion zones surrounding older crystal cells. B Changes in the pattern of A resulting from expansion of the tissue and growth of the inhibition zones with age of the differentiated cells. For explanation see text.

The mechanism of crystal-pattern formation has not been analyzed so far. Hickory (Carya ovata Mill.) leaves were chosen as the experimental material for this study because they contain unusually large, evenly-spaced Ca-oxalate crystals (Arnott 1980) and the dynamics of pattern formation can be easily observed in cleared leaves during the 400-fold leaf expansion from bud break to maturity. Crystal patterns in growing leaves were quantified by computerized image analysis and modified experimentally by variation of [Ca²⁺] in the leaf tissues. The