Chloride Availability Affects the Malate Content and its Control by the Circadian Clock in Pulvini of *Phaseolus coccineus* L.

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In soil-grown 3- to 4-weeks-old *Phaseolus coccineus* L. plants the chloride content changed antagonistically in the extensor and flexor of the laminar pulvinus during the circadian leaf movement in continuous light. This is as expected for an osmoticum involved in the volume changes of pulvinar cells. However, the malate content of extensor and flexor cells was not altered in a circadian manner. Furthermore, during light/dark cycles the malate content in both, extensor and flexor, was higher in the light than in the dark. This indicates that malate was not used in the osmotic motor of leaf movement and that its level was not controlled by the circadian clock in 3- to 4-weeks-old soil-grown plants.

When leaves were cut from 14-days-old soil-grown plants and cultured in distilled water the pulvini were depleted of chloride and the malate content was increased. In these chloride deprived leaves malate, and to a lesser extent citrate (about 1/10 of malate), changed antagonistically in a circadian manner in the extensor and flexor, indicating that these organic anions were now involved in the osmotic motor and under the control of the circadian clock. The similar properties of pulvinar and stomatal movements of starch-containing guard-cells are evident: in both cases, depending on the availability of chloride, Cl^- and/or organic anions are used for the compensation of the electrical charge of K^+.

Introduction

Leaf movement mediated by pulvini results from osmotic effects. These are based on antagonistic changes of the content of K^+ salts in extensor and flexor cells of the pulvinus. Chloride is the most important anion in pulvini of plants grown in soil [1, 2]. However, the mobile fraction of K^+ is only partially (between 40–80%) balanced by Cl^-, indicating that other anions must participate in balancing the electric charge of the K^+. For *Phaseolus vulgaris* plants, grown in vermiculite and watered with a complex salt solution, changes in the pulvinar content of Cl^- and NO_3^- as well as in the total number of carboxyl groups of organic acids were correlated with those of K^+ [3]. When plants were cultured in a solution containing either 10 mM KCl or 10 mM KNO_3 changes in potassium pools were largely compensated by the respective anion [4]. However, culture in distilled water or in 10 mM KH_2PO_4 resulted in a complete lack of charge compensation by Cl^-, NO_3^- or inorganic phosphate [4], indicating a role of organic anions.

For starch-containing guard cells it has been shown that the availability of Cl^- determines whether Cl^- or organic anions, mainly malate, are used as counterions during K^+ induced swelling [5, 6]. Since the mechanism of guard cell movement appears to be comparable with that of pulvinar motor cells, malate could be involved in a similar way.

In this study we have measured the content of malate and Cl^- of extensor and flexor tissues dissected from the laminar pulvinus of primary leaves of *Phaseolus coccineus* L. The results were correlated to the different leaf positions during the second circadian leaf movement cycle of Cl^--supplemented or Cl^--deprived plants.

The results indicate that malate compensates the mobile K^+ ions if the availability of Cl^- is reduced.

Materials and Methods

Phaseolus coccineus L., cv. Preisgewinner, was grown in a greenhouse at approximately 23 °C and 9 h light/15 h dark cycles (darkness from 4.30 p.m. to 7.30 a.m.). During the light period the natural day light was supplemented with that of fluorescent lamps (Osram L 65/25 S and L 65/77 R). The plants were cultured in soil and well watered with tap water. In the experiments illustrated by Figs. 1 and 2 three- to four-weeks-old soil-grown plants were used. The angle between epicotyl and petiole of the primary leaf was fixed in a frame at 135° one day

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prior to the transfer of the plants into the growth chambers (Fig. 1). For the experiments shown in Fig. 3 and 4 the primary leaves of 14-days-old soil-grown plants were excised above the primary pulvinus and the cut ends of their petioles submersed in distilled water. They were kept under controlled conditions (greenhouse) for another 8 days (Fig. 4) or until the pulvini were dissected (Fig. 3).

Recording of the circadian leaf movement

Intact plants (Fig. 1) or leaves (Fig. 4) were transferred to growth chambers with continuous light (19 $W \cdot m^{-2}$, fluorescent lamps; Osram L 65/25 S) and constant temperature (23 ± 1 °C). Lamina movement was recorded with kymographs. The pulvini were dissected during the 2nd circadian cycle at laminar positions that were identified from the recordings.

Determination of fresh weight and dry weight

The fresh weight of the tissues analyzed was determined immediately after dissection, the dry weight either after drying at 80 °C for 12 h (Cl^- samples) or freeze-drying (malate samples).

Determination of solutes

For the determination of Cl^- one whole pulvinus (Fig. 3) or 2 extensor or flexor sections (Fig. 1 and 4) were extracted with boiling water. Cl^- concentration of the extracts was measured (at least two independent readings) with a Cl^--specific electrode (Metrohm, Herisau, CH).

Malate and citrate were assayed from whole pulvini and extensor or flexor sections that were freeze-stopped in liquid nitrogen and freeze-dried. For the experiment shown in Fig. 2 40 flexor and extensor sections were homogenized, extracted with boiling water, and used for malate determination [7]. For the other assays 4 to 9 extensor or flexor sections (Fig. 4), individual pulvini (Fig. 3) or individual extensors and flexors (Fig. 1) were used. The pulvinar tissues were sliced and extracted with 0.1 N HCl on ice. Malate and citrate were determined enzymatically according to Lowry and Passonneau [8] and Hampp *et al.* [9]. The technique of "alkali enhancement" was used for the determination of citrate (Table I) and "enzymatic cycling" was used, to measure the small amounts of malate in individual extensors and flexors (Fig. 1) [8, 9]. All assays were internally standardized.

Results

Malate and chloride content of extensor and flexor from soil-grown plants. Correlation with the leaf position during the 2nd circadian movement cycle

Fig. 1 A shows the typical time course of the circadian movement of the primary leaf laminae of 3- to

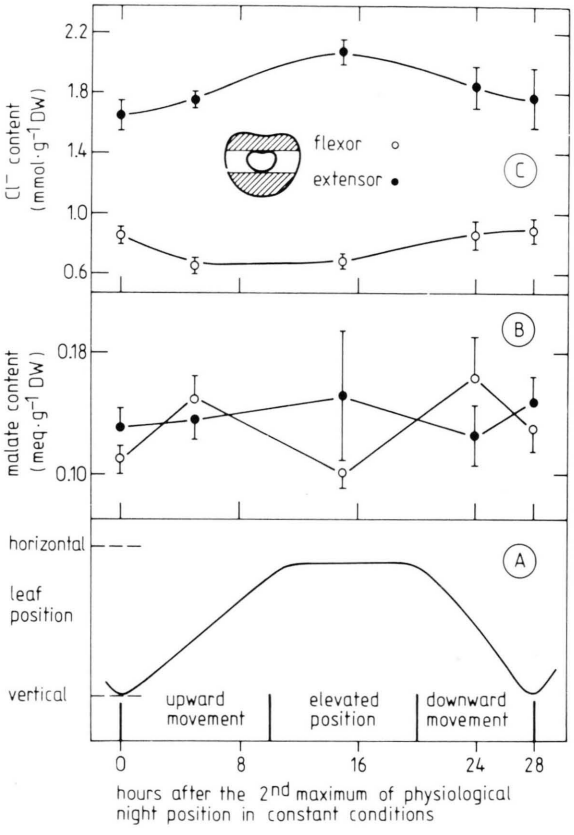

Fig. 1. Malate and Cl^- content in extensor and flexor of laminar pulvini (dissected as indicated in the inset) in relation to the leaf position during the 2nd circadian leaf movement cycle in continuous light (19 $W \cdot m^{-2}$) and constant temperature (23 ± 1 °C). The pulvini were dissected from the primary leaves of 3- to 4-weeks-old soil-grown plants. The leaf movement was recorded after the transfer of leaves into constant conditions (growth chamber). The malate and Cl^- was determined in two separate experiments. A: Typical time course of the circadian movement of the lamina caused by volume changes of extensor and flexor cells of the pulvinus. The volume of the extensor cells increases during upward movement and decreases during downward movement of the lamina. The volume of the flexor cells changes antagonistically to that of extensor cells. B: Malate content. The values are means (± SE, $n = 6$) of samples of one extensor or flexor. C: Cl^- content. The values are means (± SE, $n = 6$ to 15) of samples of 2 extensors or flexors.

4-weeks-old *Phaseolus* plants. The approximately vertical position (hours "0" and "28") corresponds to the small, the nearly horizontal position (hours "10" to "20") to the large volume of the extensor cells. Flexor cells behave inversely [10]. In order to determine the malate and Cl^- content of extensor and flexor, the pulvini were cut at the times indicated in the individual recordings of leaf movement.

The amount of extractable Cl^- (Fig. 1C) changed as expected for an osmoticum involved in the osmotic motor. It increased during volume increase (extensor: hours "0" to "15"; flexor: hours "20" to "28") and decreased during cell shrinking (extensor: hours "20" to "28"; flexor: hours "0" to "15"). In absolute terms changes in the amount of Cl^- were about 0.35 mmol · g^{-1}DW (extensor) and 0.2 mmol · g^{-1}DW (flexor).

The malate content (Fig. 1B) of extensor and flexor was small (0.1 and 0.16 meq · g^{-1}DW or 1/10 and 1/5, respectively, of the Cl^- level) and its variation was not correlated with the circadian swelling or shrinking of the respective tissue.

Diurnal changes of the malate content of extensor and flexor tissue from soil-grown plants

In Fig. 2 the changes of the malate content of motor tissues from 3- to 4-weeks-old plants are illustrated. The pulvini were collected from soil-grown plants during a 9/15 h light/dark cycle. In both, extensor and flexor, malate increased during illumination and decreased upon darkening. The increase, however, was larger in the extensor than in the flexor.

Pools of chloride and malate in whole pulvini after the transfer of leaves to distilled water

Hosokawa and Kiyosawa [4] reported evidence that organic, instead of inorganic, anions take part in the osmotic motor when plants were transferred to H_2O. In order to investigate whether malate could be one of the organic anions postulated, we incubated leaves *via* their petioles in distilled water and measured the content of Cl^-, malate, and of water in whole pulvini for a period of 23 days (Fig. 3).

The decrease in pulvinar water, shown in Fig. 3C, indicates that the growth of pulvini, *i.e.*, the production of dry mass, is continued. Thus content and concentration of substances which are not synthesized

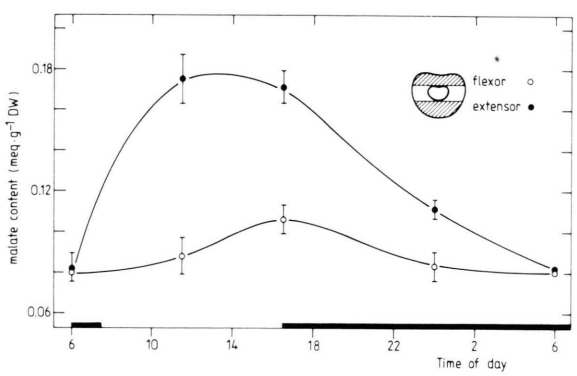

Fig. 2. Diurnal changes of the malate content in extensor and flexor of laminar pulvini. The sections (see inset) were obtained from 3- to 4-weeks-old soil-grown plants. The leaf movement was not recorded in this experiment. The values are means (± SE, n = 3 to 8) of preparations with 40 pooled extensors or flexors.

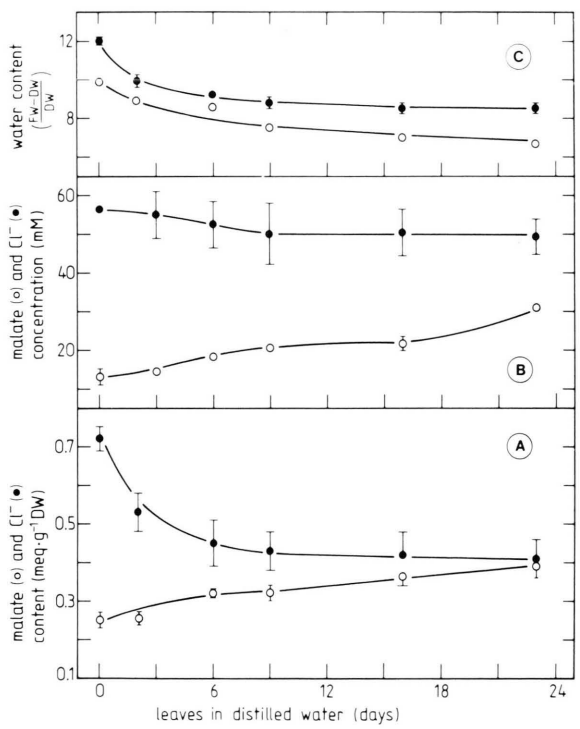

Fig. 3. Changes of water, malate and Cl^- content, resp. concentration, of whole laminar pulvini after the transfer of the leaves to distilled water. The leaves were cut from 14-days-old soil-grown plants and kept under 9 h light/15 h dark cycles after transfer to distilled water. A: Malate and chloride content (means ± SE, n = 6). B: Malate and chloride concentration (means ± SE, n = 4). C: Water content (means ± SE, n = 4).

within or transported into the pulvini should decrease with time, when referred to weight. On a dry weight basis, Cl^- content (Fig. 3A) and Cl^- concentration (Fig. 3B) decreased in parallel to the decrease of the water content (Fig. 3C), while malate exhibited an inverse behaviour (Fig. 3A and B).

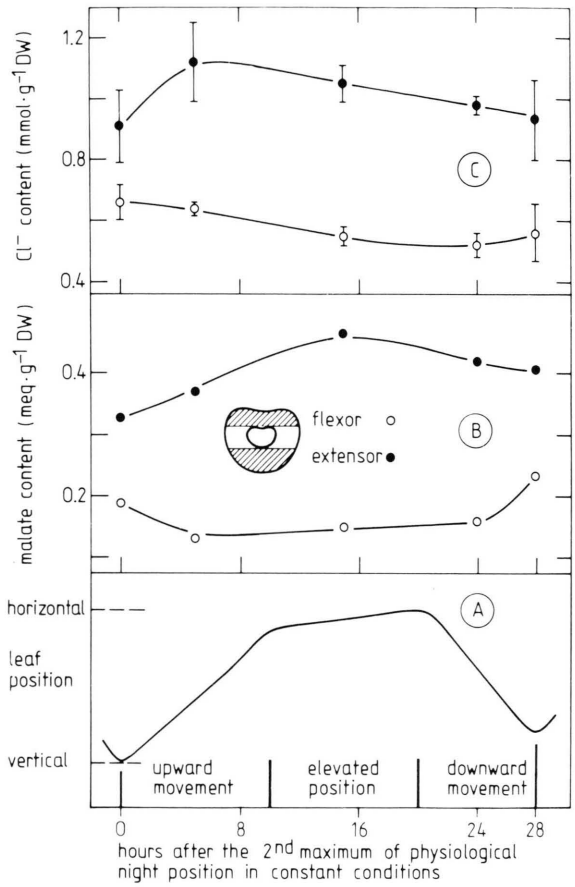

Fig. 4. Malate and Cl^- content of extensor and flexor in relation to the lamina position of Cl^--deprived leaves. The leaves were cut from 14-days-old soil-grown plants and cultured for 8 days in distilled water (9 h light/15 h dark cycles). They were then transferred to constant environmental conditions for recording the circadian leaf movement. The content of Cl^- and malate was determined in pulvini of leaves from one experimental set up. A: Typical time course of the circadian movement of the lamina caused by volume changes of extensor and flexor cells of the pulvinus. B: Malate concentration in one extract (each 4 replicates) as obtained from 9, 6, 9, 6, 4 pulvini parts at hours 0, 5, 15, 24, and 28 respectively. The deviation of the replicates was less than 5%. C: Means (± SE) of n = 5, 3, 5, 3, 3 samples of 2 extensors or flexors at hours 0, 5, 15, 24, and 28 respectively. All other details as in Fig. 1.

Levels of malate, citrate, and chloride in extensor and flexor of chloride-deprived leaves. Correlation with the leaf position during the 2nd circadian cycle

For the experiments shown in Fig. 4 pulvini were used that were in a comparable physiological condition as those analyzed at day 10 in Fig. 3. The typical circadian movement of the respective laminae differed somewhat from leaves of plants grown in soil. The recordings of the time-dependent leaf position indicated a gradual shift towards an increased upward movement. The results given in Fig. 4, compared with those of Fig. 1, show distinct differences between ordinary soil-grown and Cl^--deprived laminar pulvini. Clearly, the pulvinar Cl^- content of water-cultured leaves is generally decreased, but this decrease is most pronounced in the extensor. Consistently, malate is increased in general, but to a higher extent in the extensor tissue. Both, the Cl^- and the malate content in extensor and flexor of Cl^--deprived leaves showed circadian and antagonistic changes. This means, that Cl^- as well as malate were used as osmotica in the osmotic motor for the leaf movements.

In addition to malate citrate was determined from the same extracts given in Fig. 4B. These data, summarized in Table I, show that citrate in the extensor or flexor of the Cl^--deprived leaves only amounts about one tenth of the malate content. However, the circadian and antagonistic changes, recognized best by the differences of the citrate content of extensor and flexor at the different leaf positions, indicate that this acid could take part in K^+ compensation too.

Discussion

Recently Bialczyk and Lechowski [11] reported that the malate content of the laminar pulvini from 14-days-old soil-grown *Phaseolus coccineus* L. plants changed during light/dark cycles antagonistically in extensor and flexor: during the light period it increased in the extensor but decreased in the flexor, while an inverse behaviour was observed upon dark treatment.

Our data on the diurnal changes of the malate content of extensor and flexor of the laminar pulvini from 3- to 4-weeks-old soil-grown plants (same species) differ from these results in that the diurnal fluctuation of the malate pool (high in light, low in the dark) were parallel in both flexor and extensor (Fig. 2). Thus these results indicate that in our 3- to

Table I. Correlation between the citrate content of extensor and flexor and the position of the lamina in Cl^--deprived leaves. The citrate concentration was determined in aliquots of the same extracts (4 parallels) used for the malate determinations in Fig. 4.

Position of the lamina	Citrate content [meq·g^{-1}DW] Extensor	Flexor	Difference of the citrate content of extensor and flexor [meq·g^{-1}DW]
Night position ("0" h)	0.032	0.013	0.019
Upward movement ("5" h)	0.049	0.009	0.040
Day position ("15" h)	0.046	0.013	0.033
Downward movement ("24" h)	0.040	0.019	0.021
Night position ("28" h)	0.041	0.023	0.018

4-weeks-old soil-grown plants malate was not involved in the osmotic motor on which leaf movement is based. This conclusion is supported by the data on the changes of Cl^- and malate levels in extensor and flexor during circadian leaf movement in continuous light. While overall malate was low and rather constant (Fig. 1B), the Cl^- content changed in a circadian fashion and antagonistically in extensor and flexor (Fig. 1C). Only for Cl^- this changes were as expected for an osmoticum participating in the osmotic motor.

In contrast, leaves that were deprived of Cl^- clearly showed circadian changes of Cl^-, malate, and citrate contents of the extensor and flexor (Fig. 4 and Table I) indicating that all three anions could have functioned as osmotica for the reversible volume changes under this condition. This is in analogy to the situation during stomatal movement of starch-containing guard cells, where – depending on Cl^--availability – malate (or citrate) can substitute the Cl^- in order to compensate for K^+ uptake [12, 13].

The antagonistic and circadian changes in malate content of extensor and flexor in Cl^--deprived pulvini are in accordance with the diurnal changes found by Bialczyk and Lechowski [11] in 14-days-old soil-grown plants. Interestingly, the malate content in the extensor and flexor of their 14-days-old soil-grown plants was even higher than in comparable tissue preparations of our Cl^--deprived leaves. This could indicate that the availability of Cl^- and other inorganic anions is lower in younger tissue. Additional support for this assumption comes from the observation that the Cl^- content of whole pulvini of 10-days-old *Phaseolus coccineus* plants is only about half of that of 22-days-old plants (results not shown). Therefore, it is likely that in younger seedlings organic anions, especially malate, are used for functions that are mediated by Cl^- or other inorganic anions in older ones.

Mechanisms regulating the Cl^-/malate interdependence in motor cells of *Phaseolus* pulvini could be similar to those which operate in starch-containing guard cells, since there is increasing evidence that the K^+ uptake in pulvinar cells is due to a H^+/K^+ exchange as discussed for stomata [14–17]. There is considerable evidence in the literature for the following mechanisms [5, 13, 18, 19]: During opening guard cells export H^+ in exchange for K^+. If available, resulting cytosolic OH^- can be exchanged for Cl^-. If Cl^- is not available, the cytosolic pH will rise, stimulating PEPCase and, in consequence, malate synthesis. Malate synthesis continues as long as its cytosolic concentration is kept low by vacuolar sequestration. As a result, the pH in the cytoplasm is held within a limited range, H^+ excretion and K^+ uptake mechanisms are maintained, and malate as

counterion to K^+ is produced. Taken together, all these events result in an increase of the solute content of the vacuole and finally in stomatal opening.

The control by the circadian clock as well as the effect of light on the malate content of pulvini are different depending on Cl^- availability.

In pulvini with sufficient Cl^- available the malate content increased during light and decreased during dark in both, extensor and flexor (Fig. 2), and a rhythm in continuous light was not detectable (Fig. 1). This indicates that malate metabolism was not controlled by the circadian clock in these pulvini. Comparable diurnal changes in the cellular pools of malate are reported for several plant tissues where volume changes are clearly not involved in their functions [9, 11, 12, 20, 21]. Thus, this light-induced increase of malate could be a consequence of the increased availability of substrates for the PEPCase during photosynthesis.

In contrast to pulvini from Cl^--supplemented plants, extensor and flexor associated malate pools of Cl^--deprived pulvini oscillated in a circadian manner in continuous light. These results show that malate is not an essential component of the circadian clock but that its synthesis, degradation or transport can be coupled to it, depending on the Cl^- availability. We assume that processes similar to those in guard cells connecting malate fluctuations to the H^+/K^+ transport are responsible for the coupling of malate metabolism to the circadian clock in pulvinar cells. According to this assumption the circadian changes of the transport systems involved in the K^+ transport should induce cytoplasmic pH changes which in turn stimulate or inhibit the PEPCase activity. The antagonistic changes of the malate content in extensor and flexor in continuous light (Fig. 4) and during light/dark cycles (flexor, [11]) are a consequence of such a coupling of the malate metabolism to the circadian properties of systems involved in K^+ transport.

Taken together our results, in accordance with those of Hosokawa and Kiyosawa [4], give strong evidence for the high flexibility of pulvinar cells in using different anions in order to balance changes in K^+ levels. Depending on the availability Cl^-, NO_3^-, malate, and to a smaller extent citrate can be used.

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