

Quantitative Histochemical Analysis of Starch, Malate and K^+ , together with the Activity of Phospho-enolpyruvate Carboxylase along an Elongating Primary Leaf of *Hordeum vulgare*

R. Hampf

Institut für Biologie I, Universität Tübingen, D-7400 Tübingen 1, Bundesrepublik Deutschland

W. H. Outlaw Jr.

Biology I, Florida State University, Tallahassee, FL 32306, USA

and

H. Ziegler

Lehrstuhl für Botanik, Technische Universität München, D-8000 München 2, Bundesrepublik Deutschland

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The content of starch, malate, potassium and the activity of phospho-enolpyruvate carboxylase (PEPC) were analyzed by quantitative histochemistry in mesophyll cells of different zones along the axis of lyophilized primary elongating leaves of barley. The concentrations of potassium and malate were low in the region of the intercalary meristem (close to the point of grain attachment), but concentrations of solutes increased abruptly and stoichiometrically (equivalent basis) in the elongation zone (2 to 10 mm from the leaf base), where they contributed approximately -0.17 megapascal to the solute potential. Although this solute concentration represents only a minor portion of the reported solute potential, the specific nature of the change, its correlation with a decrease of starch content, and the distribution of the activity of PEPC indicate cell expansion in barley could be augmented by a potassium malate osmoregulatory system that utilizes starch as a source of carbon skeletons.

Introduction

Leaves of graminaceous plants develop from basal intercalary meristems, which are surrounded by sheaths of fully developed leaves (review: ref. [1]). Cell elongation occurs in a zone adjacent to the meristem that is also enclosed by sheaths of fully developed leaves. Thus, there is a continuum of cellular development ranging from achlorophyllous heterotrophic cells near the leaf base to fully expanded exposed cells. Because of its simplicity, this anatomical arrangement has permitted study of various aspects of chloroplast development [2–4].

Graminaceous leaves have also been used to study the relationship between growth and water potential

[5–12]. Under normal conditions, leaf extension may exceed by up to several-fold the length of the cell elongation zone, depending on species. As growth requires water influx and turgor pressure [13], high growth rates must be coupled to rapid accumulation of solutes. Rapid water influx associated with high growth rates may resemble osmoticum-driven plant movements, which involve K^+ and various anions such as malate [14, 15].

Water relations from the cell elongating region cannot be inferred from data obtained from fully expanded regions of the leaf blade [6]. This observation indicates that factors influencing growth should be studied at a scale where it actually occurs. Therefore, we have examined the distribution of possible key metabolites (malate, K^+ , starch) and of phospho-enolpyruvate carboxylase (PEPC) on a single cell level along expanding primary leaves of barley. We report that potassium and malate accumulate stoichiometrically in the elongation zone. Our data are consistent with the idea that malate is synthesized from carbon skeletons derived from starch.

Abbreviations: PEPC, Phospho-enolpyruvate Carboxylase, $\psi_{p,s,w}$, Hydrostatic, Solute, Water Potential.

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Materials and Methods

Seeds of *Hordeum vulgare* L. cv Gerbel were germinated in moist peat (tap water) in darkness at 26 °C. After three days, the seedlings were transferred to continuous light (approx. 9 W m⁻² provided by Osram HQLS (400 W) lamps; 22 °C). Primary leaves of seven-day-old seedlings were used in all experiments.

Biochemical studies were conducted using lyophilized tissue of five zones of the leaf, numbered sequentially from the leaf base (Zone I, 0–1 cm; Zone II, 1–2 cm; Zone III, 2–3 cm; Zone IV, 4.5–5.5 cm; Zone V, 9.5–10.5 cm; see Fig. 1). Zones Ia and Ib were the basal and apical halves of Zone I, respectively. Zone V contained fully expanded cells.

The zone of cell elongation (about 2 to 10 mm away from the point of grain attachment) was identified according to Matsuda and Riazi [5]. A series of small pinholes were made through the coleoptile, spaced 1.5 mm apart at a distance of 2 to 17 mm from the leaf base. After 30 h the distance between the pinholes of the leaves was remeasured from seedlings that grew at normal rates.

Electron microscopy

One-mm² pieces of leaf were fixed in 6.5% (w/w) glutaraldehyde in 60 mM K-phosphate buffer (pH 7.3) for 2 h at room temperature. Then the tissue was washed four times with phosphate buffer and postfixed with 2% (w/w) OsO₄ in buffer for 2 h. After three more washings in buffer, the pieces of tissue were dehydrated stepwise in acetone and embedded in resin according to Spurr [16]. Sections were cut with an LKB ultramicrotome and examined in a Zeiss EM 9 electron microscope.

Biochemical assays

Leaves were removed from the coleoptile, cut into segments corresponding to different developmental zones, frozen in melting N₂ and freeze-dried at -35 °C [17]. Individual mesophyll cells were dissected and weighed on a quartz fiber balance (20–40 ng; see ref. [18] before analysis. The oil well technique [17] and enzymic cycling procedures were used to assay starch [19] and malate [20] concentrations. Potassium was measured using K-stimulation of pyruvate kinase [21]. The activity of PEPC was assayed microfluorometrically [22].

Results

Structural observations

Electron microscopy (not shown) was used to study ultrastructural properties of different developmental zones along primary *Hordeum* leaves as described in Fig. 1. Plastids of Zone Ia (close to the meristem) were small (ca. 1 µm in diameter). They lacked extensive thylakoid development but partly included large starch grains. Internal membrane proliferation was apparent in plastids located in Zones II and III, resulting in the development of granum stacks; however, the number of starch grains contained in plastids of these zones had decreased (see also Fig. 3). The complex thylakoid system of plastids in Zone V was an indication of fully developed chloroplasts.

Biochemical characterization

The malate and potassium concentrations in the basal 0.5 cm of the leaves were low (10 to 44 mmol·(kg dry mass)⁻¹, respectively, Fig. 2). Over the next 0.5 cm, however, the concentrations of these substances increased dramatically, by approximately 175 milliequivalents·(kg dry mass)⁻¹. Importantly, the concentration of these ions increased in parallel and equally, if expressed on a charge basis. From these high concentrations, the amount of malate then declined steadily with distance from the leaf base to approximately 20% of the maximum value. In contrast, the decrease in potassium concentration was abrupt, more variable, and smaller (to approximately 75% of its maximum value).

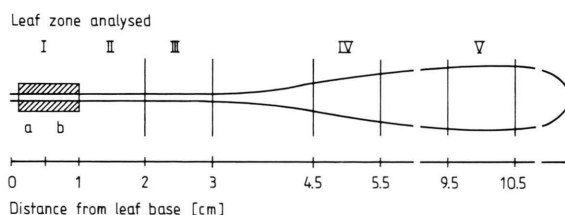


Fig. 1. Schematic presentation of an elongating primary leaf of barley. The basal end of the leaf is the point of grain attachment. The zone of leaf elongation was located between 2 and 10 mm from the basal end (marked area). Samples from frozen-dried tissue were dissected from zones I to V and analyzed for their content of potassium and malate (Fig. 2), starch (Fig. 3), and the activity of phospho-enolpyruvate carboxylase (Fig. 4).

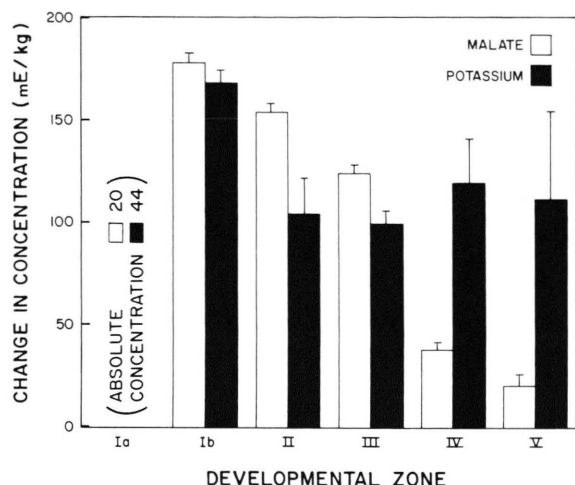


Fig. 2. The changes in concentration of malate and potassium (milliequivalents·(kg dry mass)⁻¹) within developing leaf blades of barley. The concentrations (20 and 44 mEq·(kg dry mass)⁻¹ for malate and K⁺, respectively) in Zone Ia, most probably the intercalary meristem, were the references. Error bars are SE; *n* = 25. (For this comparison, the relevant concentration unit is equivalents, but in the text where the conversion of starch to malate is discussed and where the calculations for water potential are given, the relevant concentration unit is moles).

The concentration of starch was highest (95 mmol·(kg dry mass)⁻¹) in Zone Ia (Fig. 3; the mol basis is calculated on anhydroglycosyl moieties). Starch concentration decreased by nearly two-thirds over the next 2 cm of the leaf. The initial decline in

starch concentration, observed between Zones Ia and Ib, was 40 mmol·(kg dry mass)⁻¹. On a mol basis, this decline could account for approximately one-half the increase in malate concentration (Fig. 2).

The specific activity of PEPC (dry mass basis) was highest in Zone Ib (Fig. 4), which also had the highest malate concentration (Fig. 2).

Discussion

We report parallel increases in the concentrations of potassium and malate within the elongation zone of *Hordeum* leaves (between 2 and 10 mm from the basal end). In this region, we found a reciprocal change in starch concentration. The zone having the highest concentration of malate also had the highest specific activity (dry weight basis) of PEPC. The ensheathed location of cells in the elongation zone and the plastid ultrastructure (only very few thylakoid membranes) indicate that malate was not formed directly by photosynthesis. Thus, although our data do not constitute proof, a simple interpretation could be that starch degradation leads to phospho-enolpyruvate formation and, by subsequent carboxylation, to malate accumulation. As the cells reach their final size (more than 1.5 cm from the base), malate concentration declined to about 10% (Zone V) of its highest value, and the stoichiometric relationship between malate and potassium was lost. These general features are consistent with the participation of

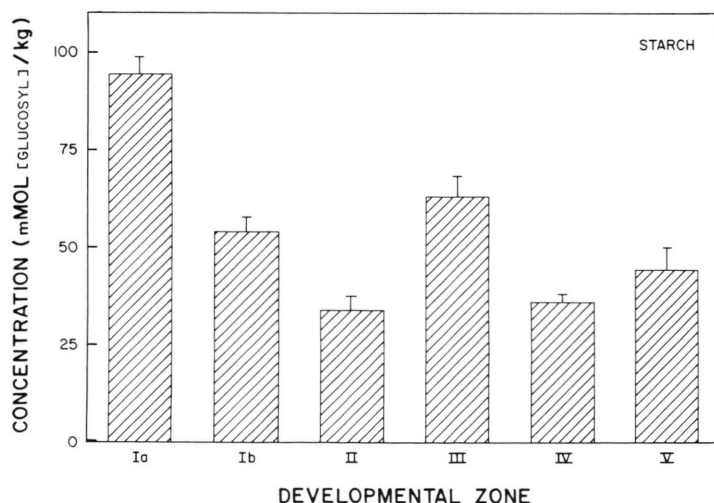


Fig. 3. The concentration of starch (millimoles anhydroglycosyl units·(kg dry mass)⁻¹) of different developmental zones of elongating leaf blades of barley. Error bars are SE; *n* = 25.

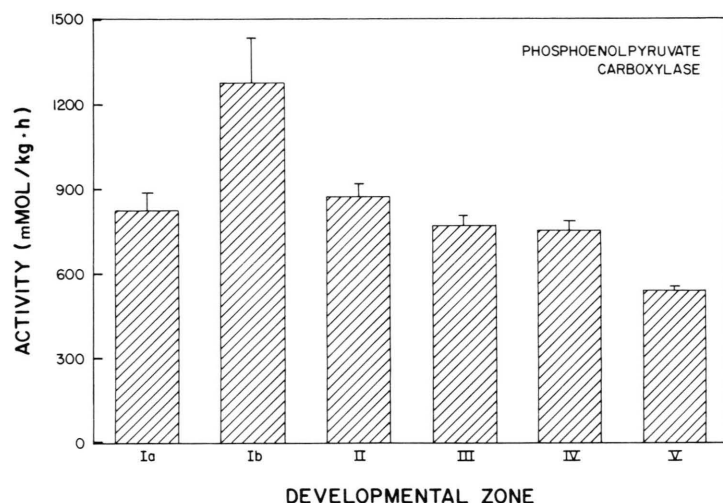


Fig. 4. The activity of phosphoenolpyruvate carboxylase (millimoles of substrate consumed·(kg dry mass·h)⁻¹) in the different developmental zones of elongating leaf blades of barley. Error bars are SE; *n* = 25.

potassium malate in an osmoregulatory system, existing in the lower 1 cm of the primary leaf of these barley plants.

The quantitative significance of such an osmoregulatory system has been estimated on the basis of the following assumptions (1) the elongation tissue is 80% water (barley, ref. [9]), (2) the osmotic volume is 90% (assumption for wheat elongating zone, ref. 7), and (3) 40 $\mu\text{mol solute} \cdot (\text{g H}_2\text{O})^{-1} = 0.1 \text{ MPa}$. The solute potential (ψ_s) decrease in Zones Ia and Ib attributable to K_2malate is thus 0.17 MPa, or about 15% of the ψ_s of the barley elongation zone [9]. This change in K_2malate concentration is comparable to that seen in cotton fiber [23], but it is about five-fold smaller than the change that occurs in guard cells of opening stomata [14]. Viewed from this perspective, K_2malate -dependent osmoregulation in this tissue may be relatively unimportant. However, the role of the K_2malate system may be more subtle, as set forth in the following. A characteristic of the elongation zone of graminaceous leaves is the lower (tissue-averaged) water potential (ψ_w) there (1). This lower ψ_w is, however, not a result of a lower ψ_s ; it is, instead, a result of a lower hydrostatic potential (ψ_p ; [11, 12]). Thus in the elongation zone of graminaceous leaves, there appears to be a different relationship between ψ_p and ψ_s . Typical values for ψ_p in the elongation zone of several species are 0.2 to 0.3, ca. 0.6, and ca. 0.4 MPa, respectively for wheat [7], barley [5], and maize [1]. These considerations indicate that ψ_p is potentially more tightly coupled to K_2malate concentration in the elongation zone than a sim-

ple examination of the concentration changes alone would reveal, *i.e.*, the observed changes in the concentrations of potassium and malate could add considerably to the increase in cell volume.

Solute accumulation can be exclusively for turgor development, or turgor development can be an ancillary function of solute accumulation. At the extremes, KCl is accumulated at the nominal "cost" of 1 mol ATP·osmol⁻¹, whereas the overall (synthetic) cost of sucrose accumulation is nominally 108 mol·osmol⁻¹. In broad terms, the cost of K_2malate accumulation is intermediate (approximately 10 mol ATP·osmol⁻¹) and indeed, under (*in vitro*) excess Cl^- conditions, guard cells accumulate Cl^- exclusively as the counter ion to K^+ (24; for pulvinar tissue see also [25]). These large differences in turgor cost are germane to the argument for a special purpose K_2malate osmoregulatory mechanism as a supplement to a basic ψ_s system. The elongation rate of 6 to 7 day old barley leaves was 1.5 to 2 mm·h⁻¹. The photosynthetic rate (20 mg $\text{CO}_2 \cdot \text{dm}^{-2} \cdot \text{h}^{-1}$) of 5 mm² of fully expanded laminae is sufficient to serve as the energy source for the K_2malate system reported here. (In young seedlings, some of the carbon imported into the elongation zone is from the grain (K. Matsuda, pers. comm.), while in older leaves assimilate export from the distal portion of the leaf augments elongation [26]; therefore, this estimate should be considered an upper limit.) This cost is relatively modest, and the difference between it and that for, *e.g.*, sucrose accumulation should be another argument in favour of our hypothesis.

In concluding, we recognize that the chemical nature of osmoregulatory solutes is different in different species, and there are even intraspecific differences [27]. Thus, our data complement and are not in conflict with reports of high concentrations of other substances in the elongation zone of graminaceous leaves (*e.g.* ref. [8, 10]). A limitation of this report is that we have investigated only one aspect of growth-associated water relations, *i.e.*, the possible participation of K_2 malate. On the other hand, this report is unique in that the solute concentrations reported here are derived from quantitative histochemistry at the single cell level. Thus, the morphological resolution is higher than previously reported. These experi-

mental attributes remove inherent sample heterogeneity that can obscure trends in data.

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