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Leaf development in *Ricinus communis* during drought stress: dynamics of growth processes, of cellular structure and of sink–source transition

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Abstract

Dicot leaf growth is characterized by partly transient tip-to-base gradients of growth processes, structure and function. These gradients develop dynamically and interact with dynamically developing stress conditions like drought. In *Ricinus communis* plants growing under well-watered and drought conditions growth rates peaked during the late night and minimal values occurred in the late afternoon. During this diurnal course the leaf base always showed much higher rates than the leaf tip. The amplitude of this diurnal course decreased when leaves approached maturity and during drought stress without any significant alteration of the diurnal pattern and it increased during the first days after rewatering. Unique relationships between leaf size and cytological structure were observed. This provided the framework for the analysis of changes in assimilation, transpiration and dark respiration, chlorophyll, protein, carbohydrate, and amino acid concentrations, and of activities of sink–source-related enzymes at the leaf tip and base during leaf development in well-watered and drought-stressed plants. Gas exchange was dominated by physiological rather than by anatomical properties (stomatal density). Tip-to-base gradients in carbohydrate concentrations per dry weight and sink–source-related enzymes were absent, whereas significant gradients were found in amino acid concentrations per dry weight. During drought stress, growing leaves developed source function at smaller leaf size, before specific physiological adaptations to drought occurred. The relevance of the developmental status of individual leaves for the drought-stress response and of the structural changes for the biochemical composition changes is discussed.

Key words: Drought, leaf growth, sink–source transition, diurnal growth patterns, carbohydrate, amino acid, chlorophyll, protein, sucrose phosphate synthase, sucrose synthase.

Introduction

Growing tissues are characterized by the simultaneous development of structure and function (Kühn *et al*., 1996; Schurr, 1998*a*; Walter and Schurr, 2000). In many cases the relevance of the interaction of structure and function is straightforward as, for example, in gas exchange where leaves have to develop functional stomata in order to actively control water and CO_2 exchange with the atmosphere. In sink–source transition plasmodesmatal and phloem structures have to be adapted to the export function (Oparka and Turgeon, 1999). On the other hand analysis of concentrations of substances (per weight, area or many other reference parameters) require sound information on the structural basis in order to be correctly interpreted: the ratios between tissue layers (Heckenberger *et al*., 1998; Roggatz *et al*., 1999), between veins and interveinal areas and between vacuoles and cytoplasm change during development. This may have a direct impact on the composition of a sample, because compartments differ in their principal components. This adds to the general problem of analysing an expanding tissue: the region sampled at a certain point in time changes in size during expansion, and therefore deposition over time (net fluxes into or out of a growing region) can only be calculated on the basis of kinematic growth analysis (Silk, 1992; several other papers in this issue). These simple examples illustrate that structural information is crucial in order to interpret functional data in

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Abbreviations: LML, length of the middle lobe of the *Ricinus* leaf; MOPS, 3-(*N*-morpholino)propane sulphonic acid; EDTA, ethylene diamine tetraacetic acid; EGTA, ethyleneglycol-*bis*-(2-aminoethyl-)-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulphonylfluoride.

growing tissues correctly as already pointed out by Körner *et al.* (Körner *et al.*, 1989).

Structure and function do not build up homogeneously in growing leaves, but develop significant spatial gradients that change dynamically (Maksymowych, 1973; Heckenberger *et al.*, 1998; Granier and Tardieau, 1998; Walter and Schurr, 2000): cell division and elongation cease first in the leaf tip (with a time lapse of several days depending on the species). This allows the tip to mature several days before the leaf base, in its functional relations too (Turgeon, 1989). Strong discrepancies of structure and function develop between the leaf base and tip that are usually transient and disappear when maturity is reached.

Stress can alter leaf structure considerably. Leaves from stressed plants usually reach apparently smaller final sizes and their cytological structure can be altered in comparison to controls (Heckenberger *et al.*, 1998; Granier and Tardieau, 1998). The dynamic of the stress interacts with the dynamic of the development of structure and function in growing tissues, resulting in very different responses to stress in leaves of different developmental situations (Roggatz *et al.*, 1999). Therefore, the parallel analysis of structure and function is of special importance in the analysis of stress responses in growing tissues.

The interaction of structure and function in growing leaves during drought is a complex field. This paper therefore can only highlight some aspects. It intends also to encourage interdisciplinary approaches in this complex field between different areas of botany, as data from different scales have to be combined to understand these complex processes. Additionally, co-operation with other disciplines can supply new techniques and methodology leading to new approaches to this central field of plant physiology.

Materials and methods

Growth of Ricinus communis

Ricinus communis plants were grown as described previously (Heckenberger *et al.*, 1998). In short, seeds of *Ricinus communis* L. (var. Carmencita) were germinated in vermiculite and transplanted to special planting pots (4470 cm^3) filled with a sandy loam in order to use equivalent conditions for the experiments with the root pressure chamber (Schurr, 1998*b*) to study the impact of water potential on leaf growth. Plants were cultivated for 3 weeks in a growth cabinet at a day/night cycle of 12/12 h (300 μmol photons m^{-2} s^{-1}), a constant air temperature of 25 °C and a relative humidity of 60/45% during the light and dark periods, respectively. Plants used in the experiments had developed one pair of green cotyledons, one pair of primary leaves and one fully developed main leaf (Heckenberger *et al.*, 1998). Until the start of the drought experiments, the gravimetric soil water content was kept above 0.20 g g^{-1} by repeated watering 2 h after the onset of light with modified Hoagland solution (in mol m^{-3}: K^+ 3.0, NO_3^- 4, $H_2PO_4^-$ 1, Ca^{2+} 1, SO_4^{2-} 2, Mg^{2+} 2; in mmol m^{-3}: NH_4^+ 0.12, Fe^{3+} 18, BO_4^{3-} 1, Mn^{2+} 0.09, Zn^{2+} 0.035, $Mo_7O_{24}^{6-}$ 0.02).

Drought stress and rewatering treatments

The experiment was designed to study the course of changes occurring in successive growing leaves during drought stress. A detailed description of the drought stress conditions has already been given (Heckenberger *et al.*, 1998). In short, one-half of the plants was subjected to drought stress from day 18 after germination onwards, while control plants were kept well watered. *Ricinus* plants are highly tolerant to drought stress as even after the complete cessation of watering for up to 20 d leaves did not wilt. Samples from the leaf base and the leaf tip of leaves 2, 3 and 4 were taken from water-stressed and control plants at day 24 (no apparent decline in growth rate), day 26 (significant decline in growth rate), day 29 (cessation of areal growth), and day 35 (growth stopped for 6 d) after germination. Control plants were probed additionally on day 32. In the rewatering experiments water was added to field capacity to drought-stressed plants after 3 d of complete cessation of growth (equivalent to day 32 after germination).

Diurnal variation of leaf growth

Diurnal variation of leaf expansion of *Ricinus* was analysed during the development of leaf 3 in control and water-stressed plants by a continuous length detection device based on an angle meter (Schurr, 1998*a*). In short, the leaf base was fixed by rods in its position. Twine was attached to the leaf tip and led over a roll to a metal rod connected perpendicular to the rotation axis of the angle meter (Novotechnik, Germany). The weight (15 g) on the opposite side kept the leaf midrib in a straight position. Arrays of nylon twines parallel to the midrib enclosed the leaf from top and bottom and fixed the leaf in a plane of the midrib to minimize non-planar movements. The electrical output of the angle meter was digitized every second. For diurnal patterns of expansion hourly means were calculated.

Diurnal variation of leaf growth distribution

Distribution of growth rates at high temporal and spatial resolution was determined in a separate experiment. Image sequences of leaves of a fast and a slow growing *Ricinus* plant were taken at day/night cycles of 12/12 h. Plants were soil grown at constant temperatures of 25 °C and constant relative humidities of 80%. The fast growing plant was exposed to 500 μmol photons m^{-2} s^{-1}; the slow growing plant was exposed to 200 μmol photons m^{-2} s^{-1}. The observed leaves grew with relative growth rates of 17% and 4% d^{-1}, respectively, during observation.

The analysis of distribution of leaf growth was done by image sequence analysis according to Schmundt *et al.* (Schmundt *et al.*, 1998). In the context of this technique, 'growth' means the increase of area. In short, image sequences in the near infrared (>930 nm) were digitized from standard CCD cameras directly. The leaf fixed in the focal plane of the camera system was illuminated day and night by an IR diode array emitting at 950 nm. Motion in the captured sequences was analysed using the structure tensor algorithm (Haussecker and Spies, 1999) in an implementation of the image sequence analysis software HEURISKO (Aeon, Hanau, Germany) previously described in detail (Schmundt *et al.*, 1998). The resulting velocity vector fields were interpolated and local growth rates were calculated from the local divergence in the velocity maps (Schmundt and Schurr, 1999). The growth maps were then used to determine growth within the natural reference system of the leaf, the leaf vein system (Walter and Schurr, 1999), by processing growth rates at defined positions along the midvein of the analysed leaves (Walter and Schurr, 2000).

Analysis of cytological status of Ricinus leaves

The length of the middle lobe of the leaf was used as a measure of leaf size (Heckenberger *et al.*, 1998; Roggatz *et al.*, 1999). Cytological parameters (cell density, cell density in individual cell layers, height of palisade cells, exposed surface area of epidermal cell, stomatal densities) were determined in leaf discs (4 mm diameter) taken from the leaf base and tip of leaves 2, 3 and 4 of well-watered and drought-stressed *Ricinus* plants. Detailed descriptions of the methods used have been given (Heckenberger *et al.*, 1998). In short, cell density was determined using a Neubauer chamber after complete separation of the cells by enzymatic digestion. The height of palisade cells was determined by microscopic analysis of the isolated palisade cells in the cell suspension. The exposed surface area of epidermal cells and stomatal density was determined from FormVar replicas of the leaf surface and quantified using digital image analysis software (SigmaScan, Jandel Scientific, Erkrath, Germany).

Analysis of gas exchange in growing leaves

Gas exchange (assimilation, transpiration, dark respiration) was analysed by cuvette measurements of *Ricinus* leaves. For cuvette measurements of the leaf tip and base of growing leaves, a special cuvette was designed which clamps only 2.7 cm^2 of the leaf area. This cuvette was attached to the porometer head of a commercial porometer (Walz, Effeltrich, Germany). The air temperature inside the cuvette was maintained at 25 °C and a leaf-to-air vapour pressure difference of 12.7 $Pa\,kPa^{-1}$ was provided. CO_2-concentrations were at ambient growth conditions. Light measurements were obtained at ambient light conditions in the growth chamber (300 μmol photons $m^{-2}\,s^{-1}$).

Analysis of the composition of growing leaves

For the analysis of the composition of growing leaves it is important to choose a reference unit, which allows suitable interpretation and which is suitable for technical reasons. This is especially relevant in growing tissues, as the cytological structure changes significantly and thereby the relative contribution of different cellular compartments might affect the concentrations measured in the different regions without alteration of the concentration within the individual cellular compartments (cf. Roggatz *et al.*, 1999). Composition data are expressed here on a dry weight basis as calculated from the fresh weight to dry weight ratio determined with equivalent leaf discs.

Leaf samples (3 discs of 1 cm diameter each) were taken from the leaf base and tip of growing leaves of *Ricinus* plants, respectively. In very small leaves (LML <4 cm) a separation of leaf base and tip was not possible and one sample (3 leaf discs) per leaf was taken. All leaf discs were immediately frozen in liquid nitrogen and stored at -80 °C until extraction. One leaf disc was extracted in ethanol (see below), the other two were used for analysing enzymatic activity (see below).

For ethanol extraction, the frozen leaf disc was transferred into 900 μl of 80 °C ethanol/water (50:50, v/v) for 20 min. The supernatant was stored and the extraction was repeated twice with 200 μl ethanol/water (80:20, v/v) until the leaf disc was pale. The supernatants were combined and used for analysis of chlorophyll, glucose, fructose, sucrose, and amino acid concentration. The pale leaf disc was used for starch analysis.

Chlorophyll concentration was analysed photometrically at 652 nm after Arnon (Arnon, 1949). Hexoses and sucrose were analysed after Jones *et al.* (Jones *et al.*, 1977) by enzymatic analysis. Starch concentration was determined enzymatically as described previously (Matt *et al.*, 1998). Amino acid concentrations and patterns were determined by HPLC as described previously (Gerendas and Schurr, 1999). Proline concentrations were determined separately after Bates *et al.* (Bates *et al.*, 1973) by toluol-fractionation of ninhydrin derivates.

Enzymatic activities were determined in an extract prepared as follows: Two leaf discs were ground in liquid nitrogen and taken up in 700 μl extraction buffer (50 mM MOPS (pH 7.4), 12 mM MgCl, 5 mM EDTA, 5 mM EGTA, 1 mM benzamidin/ε-aminocapronic acid, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF). The extract was centrifuged at 12 000 rpm for 4 min. The supernatant was passed through a G-25 column equilibrated before with 50 mM MOPS (pH 7.4), 12 mM $MgCl_2$ and 1 mM DTT. The eluate was used immediately for the determination of enzymatic activities.

Sucrose phosphate synthase was determined after Reimholz *et al.* (Reimholz *et al.*, 1994) at non-selective (maximal) and selective conditions. In non-selective conditions the phosphorylated and non-phosphorylated form of SPS was determined. In selective conditions only the activated form of SPS was analysed. Activation state was calculated as the ratio of selective and non-selective activity of SPS in the extracts. Sucrose synthase (SuSy) was determined after Dancer *et al.* (Dancer *et al.*, 1990) by measuring the formation of UDP-glucose by SuSy in the extracts. Protein concentrations were determined after Bradford (Bradford, 1976) from these extracts.

Results and discussion

Temporal and spatial scales relevant for growth analysis in well-watered and water-stressed leaves

Before individual parameters of growth can be analysed it is necessary to recall the importance of spatial and temporal aspects of growing leaves especially in their interaction with dynamically developing stress conditions such as drought.

Growing leaves are characterized by dynamic gradients of growth processes and functional development. Different temporal scales interact in a complex manner. In well-watered *Ricinus* leaves, expansion rates varied by a factor of more than 6 during the day with a distinct maximum during the second half of the dark period (Fig. 1A, B). In young leaves, expansion was detected throughout 24 h, periods of zero expansion were observed only in almost mature leaves. Most of the expansion took place during the few hours around the day–night transition. A distinct smaller peak of expansion was consistently detected at the beginning of the night. This peak was still present even in almost mature leaves. A local minimum of expansion of similar amplitude was observed at the beginning of the day. These minor patterns were closely associated with the closure and the opening of stomata (data not shown).

In many species diurnal variation of leaf expansion has been reported (for an overview see Walter and Schurr, 2000). It is beyond the scope of this paper to analyse the mechanisms underlying this diurnal course, however, des-

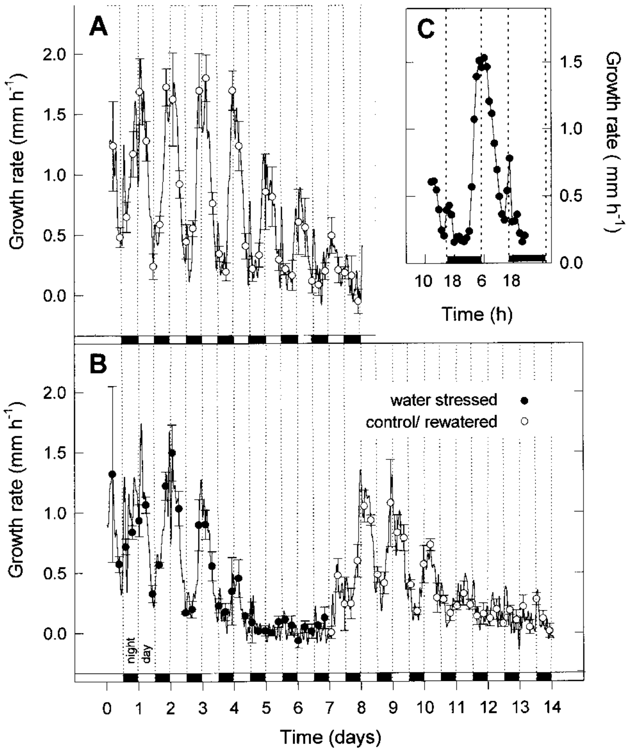

Fig. 1. Growth rates of leaves of (A) well-watered ($n=4$) and (B) water-stressed and rewatered (day 7, $n=3$) *Ricinus* plants. Values are hourly means from continuous length analysis (see Materials and methods). For every 5th data point the bars indicate standard errors. In well-watered plants, the length of the middle lobe of the measured leaf 3 was 5.8 ± 0.3 cm at the beginning of the measurement and reached 19.1 ± 0.3 cm at final leaf size. Leaves of water-stressed plants had an initial length of the middle lobe of 5.1 ± 0.2 and reached a final size of 18.0 ± 0.6 cm after rewatering. (C) Continuous expansion analysis of leaf 3 maintained at maximal water potential in a well-watered plant by means of pressure application to the root system in a root pressure chamber.

pite the common increase in water potential and sometimes turgor (Boyer, 1968; Bunce, 1977) it seems unlikely that increased water status is responsible for the higher growth activity during the night hours (Schurr, 1998*a*), especially as maintaining the shoot water potential at maximum by means the root pressure chamber did not alter the diurnal course of leaf growth (Fig. 1C). Expansion rates are more likely to be controlled by the expansibility of the cell wall, as the spatial distribution of turgor does not fit the pattern of expansion (Tomos and Pritchard, 1994) and the transient increase in the expansion rate after an increase in turgor by the short-term application of pneumatic pressure is due to unphysiological spatial patterns of expansion and is only transient despite higher turgor pressures being maintained (Schmundt *et al.*, 1998). In contrast, the spatial patterns of cell wall properties along growth zones fit well with their potential role in growth regulation (overview in Schurr, 1998*a*; Wu and Cosgrove, 2000). Little is known about the diurnal patterns of cell wall properties, but more data on this topic have to be sampled in order to elucidate the mechanism behind the spatio-temporal patterns of leaf expansion in the diurnal course.

The transient peaks at the beginning of the dark and the light phases have been linked to turgor changes due to stomatal activity and a transient imbalance of turgor, as the height of these peaks can be affected by the vapour pressure deficit (Christ, 1978; Cutler *et al.*, 1980) and transient alterations of expansion rate can be induced by alterations of light and air humidity (Shakel *et al.*, 1987; McDonald and Davies, 1996; Clifton-Brown and Jones, 1999). However, even when water potential in the leaves was held constantly high by controlled application of pneumatic pressure to the root system, short-term changes in growth rate occurred during the light–dark transition (Fig. 1C).

Drought stress mainly affected the amplitude of the diurnal growth peak with little influence on the overall diurnal pattern and the number of days with significant growth activity (Fig. 1B). Zero growth was first detected temporally at day 4 after the onset of the analysis, but the peak activity of expansion rate was still significant (0.48 mm h^{-1}). Even at severe drought stress, when almost no expansion was detected any more (Fig. 1B, days 5 and 6), the small expansion peak at the beginning of the night was detected. Thus, from the diurnal pattern of expansion rate, leaves from drought-stressed plants mimicked more mature leaves of control plants at smaller leaf size.

Rewatering (at the beginning of day 7, Fig. 1B) immediately increased the growth rate, showing that there was still the potential to grow in these leaves 2 d after the complete cessation of expansion. Significant expansion was regained within 30–40 min after rewatering (data not shown), but expansion rates comparable to well-watered rates were only detected a day later and maintained for one additional day (Fig. 1B). Thereafter leaves reduced their expansion rate again for developmental reasons.

The restoration of expansion rates comparable to well-watered controls of the actual leaf size was fastest in the smallest leaves (data not shown). Final leaf size in rehydrated plants was reduced most in leaves that were small at the onset of drought. However when the length of the middle lobe of a leaf (LML) was above 12 cm at the onset of drought, rehydration within 2 d after complete growth stop allowed full recovery of the final leaf size. This leaf size compares well with the one at which cell division stopped entirely (Heckenberger *et al.*, 1998). This result hints to a limitation of cell division as the cause of a reduced final leaf size in these cases. Cell division has already been identified to be of central importance for the cytological stress response of growing leaves (Granier and Tardieau, 1998), as has been found previously in roots (Sacks *et al.*, 1997) and under nitrogen limitation (Roggatz *et al.*, 1999).

Spatial heterogeneity is another essential feature characteristic for different classes of growing tissues (Schurr, 1998*a*). Dicot leaves are characterized by their tip-to-base gradients in growth rates which occur transiently during development (Maksymowych, 1973; Taylor *et al*., 1994) and respond to drought stress (Granier and Tardieau, 1998). Biochemical and molecular control of expansion processes acts in the range of minutes to hours (Hsiao and Xu, this issue) rather than in the range of days. Therefore, the temporal resolution that is relevant to analyse spatio-temporal relationships of the biochemical and molecular mechanisms responsible for, for example, variation of cell wall properties, was not available until recent technical developments based on image sequence analysis (Schmundt *et al*., 1998; Schmundt and Schurr, 1999; Walter and Schurr, 2000; for some details see Materials and methods). Previous approaches were limited to monocot leaves (Schnyder *et al*., 1988) with the problem that the growth zone in monocots is enclosed in the leaf sheath and is thus not optically accessible for the analysis of growth distribution prior to sampling. In dicots, the spatial and temporal resolution of the growth analysis was much too low to do such an analysis (Dale, 1988). The new imaging techniques have not yet been applied to drought-stressed plants, but distinct differences in the spatio-temporal patterns of relative growth rate between leaves growing at high and low growth rates due to light conditions have been observed (Fig. 2). Essentially, the diurnal variation reflects the pattern observed by continuous length detection systems (see above).It is clear from these first data in *Ricinus* that the tip-to-base gradients change significantly. During the diurnal course in fast-growing leaves, areas that grow with rates of 5% h^{-1} at peak activity, completely cease growth in the middle of the day (Fig. 2A). Even in slow-growing *Ricinus* leaves distinct patterns of diurnal growth distribution were observed. The diurnal peak activity was still observed in the late night and entirely located at the leaf base (Fig. 2B).

This technique will be used in the future to elucidate different mechanism of stress response in dicot leaves. In roots, it has already been shown that the decrease of overall growth rate in response to stress is due either to variation in amplitude or to the spatial distribution of expansion rate (roots: Silk, 1992; Sharp *et al*., 2000; monocot leaves: Durand *et al*., 1995). With the strong spatio-temporal patterns present, growth in dicot leaves needs to be studied on a diurnal scale in order to link the underlying biochemical (and molecular) mechanisms acting in that time scale. Additionally, quantitative analysis of deposition rates of substances in growing tissues (kinematic growth analysis, Silk, 1992; Granier and Tardieu, 1998) require techniques which analyse the spatio-temporal patterns of growth, as the presented data show that the assumption of steady growth in dicot leaves throughout the day is wrong. A relevant analysis of

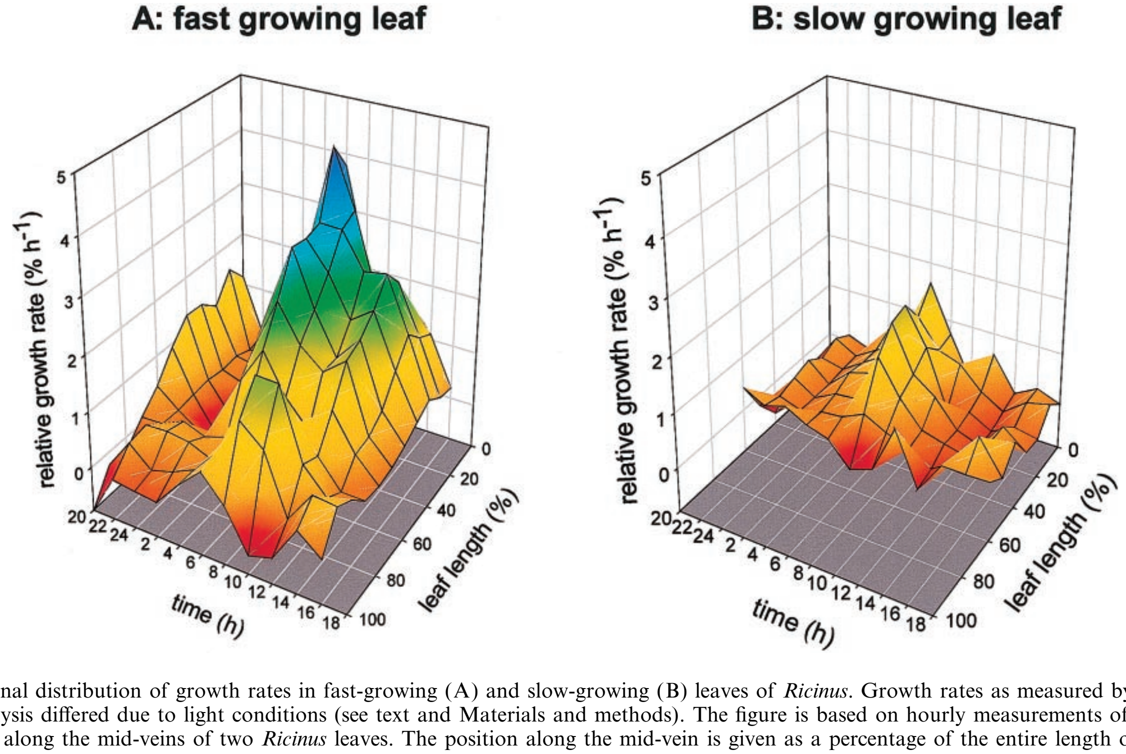

Fig. 2. Diurnal distribution of growth rates in fast-growing (A) and slow-growing (B) leaves of *Ricinus*. Growth rates as measured by optical leaf growth analysis differed due to light conditions (see text and Materials and methods). The figure is based on hourly measurements of growth rate distribution along the mid-veins of two *Ricinus* leaves. The position along the mid-vein is given as a percentage of the entire length of the middle lobe. In (A) it was possible to image the entire middle lobe of the *Ricinus* leaf, in (B) the analysis was concentrated to the basal 60% of the length of the middle lobe (i) in order to be able to analyse the low growth rates and (ii) as the distal part of the leaf did not show any significant growth rates any more.

growth in stressful conditions needs to take into account that (i) processes directly involved in growth or in its control must be functional at the correct time and space and (ii) that development and stress vary within the same time scale.

The cytological status as a framework for functional analysis

The dynamic nature of leaf growth requires a thorough analysis of potential reference systems, as common reference units like area, weight or chlorophyll change dynamically in growing tissues. A number of possible reference systems have been proposed (Erickson and Michelini, 1957; Poethig and Sussex, 1985; Hejnowicz and Karaczewski, 1993; Granier and Tardieu, 1998; Walter and Schurr, 1999; Tardieu *et al.*, 2000), but a single one matching all requirements may be impossible to find (Erickson and Michelini, 1957).

Cytological status is attractive as a framework for functional analysis as it circumvents problems with variation in cell size and relative share of cells to the tissue samples (see Introduction), which is especially evident for the biochemical analysis of plant material taken from different cytological states due to the stress of other treatments (Schurr, 1998*a*; Walter and Schurr, 2000). However, a complete analysis of the cellular status during development with spatial and temporal resolution is complex and tedious (Taylor *et al.*, 1994; Granier and Tardieau, 1998; Heckenberger *et al.*, 1998; Roggatz *et al.*, 1998).

Leaf size, as expressed by the length of the middle lobe of the *Ricinus* leaf (LML), has been proposed as a potential reference system for the analysis of interaction of structure and function due to its links to cellular parameters (Heckenberger *et al.*, 1998; Roggatz *et al.*, 1999). In drought-stressed plants subjected to the indicated conditions, unique relationships were found in leaf 3 between the cytological parameters, as well as their tip-to-base gradients to leaf size in water-stressed and well-watered plants (Heckenberger *et al.*, 1998). Very similar relationships between leaf size and all the cytological parameters analysed were found for successive leaves (Fig. 3). Even the relative share of tissue layers (palisade and spongy parenchyma, upper and lower epidermis) was identical at a given leaf size (data not shown). This makes 'leaf size' an especially good reference system for structure–function relationships, as leaves of identical size can be compared for 'functional analysis' without the need for a complex analysis of the cytological structure just on the basis of simple leaf size measurements. This may not be the case in all drought stress treatments or under other stress conditions (e.g. nutrient stress: Roggatz *et al.*, 1999), especially when cell division is affected. However, it opens direct approaches to study the interaction of structure and function.

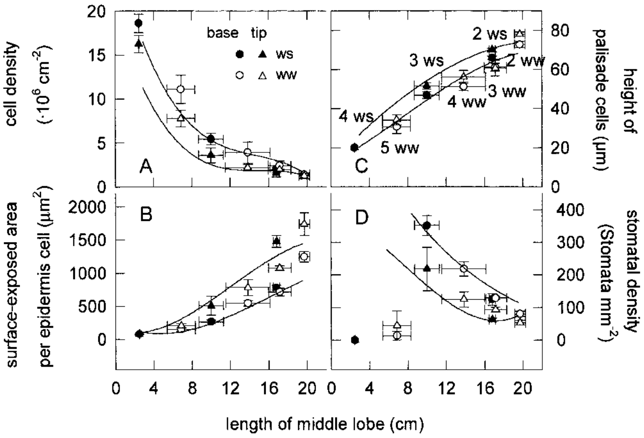

Fig. 3. Relation of cell density (A), surface-exposed area of epidermis cells (B), height of palisade cells (C), and stomatal density (D) at base and tip of leaves in relation to leaf size (indicated as the length of the middle lobe) in five successive leaf storeys of *Ricinus* plants 35 d after germination. Water was withheld from day 18 after germination from water-stressed plants. ($n = 3 \pm$ standard error). The closed lines indicate the relation obtained for leaf 3 in well-watered plants during its development (as indicated in Heckenberger *et al.*, 1998). Numbers in (C) indicate the leaf storey, from which the samples were taken: 'ww' indicates well-watered and 'ws' water-stressed plants. Leaf 5 did not developed to a significant size in water-stressed plants.

Sink–source transition in growing leaves

In parallel to cytological changes, sink–source transition alters physiological processes in the developing leaf (Turgeon, 1989) ranging from gas exchange to composition (concentration and relative) and to the enzymatic apparatus of the leaf regions. The following will mainly concentrate on carbon metabolism but, additionally, simultaneously and strongly interlinked, other aspects of primary metabolism (e.g. nitrogen metabolism) and secondary metabolism change fundamentally during sink–source transition.

Gas exchange

Mature stomata are required for a functional gas exchange (Kebede *et al.*, 1994) and thus for sink–source transition. In Heckenberger *et al.* it has been shown that the production of stomatal complexes and the 'dispersal' of these by the expanding epidermal cells (Fig. 3) interact to produce a characteristic spatio-temporal pattern of stomatal density in growing leaves (Heckenberger *et al.*, 1998). Mature stomata were present at a leaf size of approximately 5 cm (LML) and 8 cm (LML) at the leaf tip and base, respectively. This corresponds to a developmental delay of 2–3 d at the base relative to the leaf tip. Stomatal density decreased with increase in leaf size after the maximum due to the increase of the functional ratio between leaf area expansion and formation of stomatal complexes. Stomatal density in the leaf base peaked again approximately 2 d later (LML 11 cm). This pattern of development is consistent within successive leaves (Fig. 3D).

Drought stress (as described in Heckenberger *et al.*, 1998) did not significantly alter the relationship between stomatal density and leaf size in leaf 3 from which gas exchange parameters were analysed (Fig. 3). Therefore, leaves of similar size were equipped with the same structural apparatus at a given leaf size, irrespective if they were well-watered or water-stressed (Heckenberger *et al.*, 1998). The delay in development caused leaves from water-stressed plants at a given size to have higher stomatal densities than leaves from control plants at a given point in time. Variations in stomatal density and distribution in response to environmental stresses need therefore to be interpreted on the basis of the acceleration or deceleration of development (Taylor *et al.*, 1994).

In control plants, transpiration rates per area increased with leaf size and barely differed between the leaf base and the leaf tip and (Fig. 5). In contrast, assimilation rates were significantly higher at the leaf tip than at the leaf base in small leaves (Fig. 4A). Assimilation rate became positive at a LML of 5 cm and 10 cm at the leaf tip and base, respectively. When the leaf at the base just started to gain carbon on its own photosynthetic activity, the assimilation rate of the leaf tip was already half the mature rate (Fig. 4A). Assimilation rate was highly and linearly correlated with cell density, with the leaf base and the leaf tip showing the same relationship (Fig. 4B). Dark respiration per area dropped significantly with increase in leaf size (Fig. 4C), with the leaf base declining at larger leaf sizes than the leaf tip. At maturity no gradients of dark respiration between tip and base were present any more. The gradients were predominantly due to differences in cell density between leaf base and leaf tip (Fig. 4D), as dark respiration per cell at the leaf base and leaf tip were similar throughout the development of the leaf.

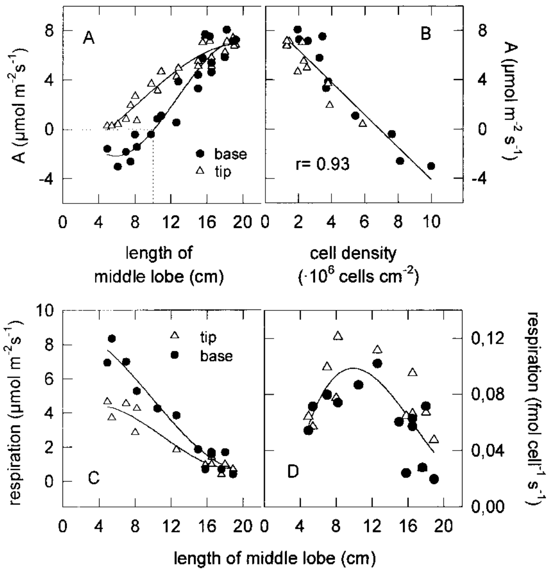

Fig. 4. Assimilation and respiration rates at the leaf base and tip in growing leaves of well-watered *Ricinus* plants. (A) Assimilation rate in relation to leaf size. The dotted lines indicate the compensation points at the leaf base and tip, respectively, (B) relationship between assimilation rate and cell density, (C) relationship between respiration rate per area, and (D) respiration rate per cell and leaf size.

Assimilation and transpiration rate did not follow stomatal density. Therefore physiological parameters dominated gas exchange at the stomatal level. This is in contrast to findings by Kebede *et al.* (Kebede *et al.*, 1994). The gas exchange gradients studied here were rather stable, in contrast to short-term fluctuations of stomatal conductance and assimilation rate as reported for mature leaves (Lawson and Weyers, 1999). However, in contrast to their approach, temperature and humidity conditions in these experiments were maintained as constant as possible during the measurements. Mapping transpiration rates by thermography (Kümmerlen *et al.*, 1999) will be a valuable tool to analyse the developmental aspects of gas exchange in growing leaves further. The close relationship between assimilation rate and cell density and the transient maximum of dark respiration per cell in mid-sized leaves underline the importance of cellular development for the photosynthetic capacity in growing leaves.

Drought stress affected the development of assimilation rate later than transpiration rate (Fig. 5). While transpiration rates at the leaf base and tip were significantly lower than controls 8 d after withholding water, with no difference between leaf base and tip (Fig. 5A), assimilation rates of leaves from drought-stressed plants increased in a similar way to the controls due to ongoing maturation for at least two more days (Fig. 5B). At day 11 of withholding water, assimilation rates at the leaf tip declined strongly and reached values similar to the ones of the less mature leaf base. In the leaf base the developmental increase of assimilation rates was only stopped by

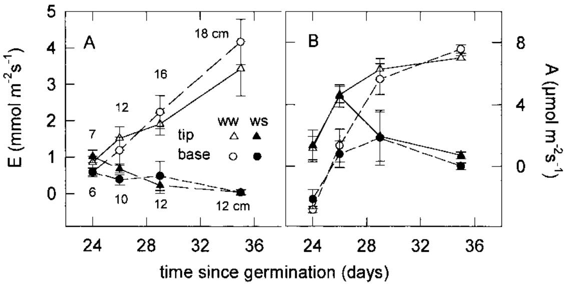

Fig. 5. Course of transpiration (A) and assimilation rates (B) at the leaf base and the leaf tip of growing leaves during a drought stress cycle in water-stressed and well-watered *Ricinus communis* plants. Numbers in (A) indicate the size of the leaf (length of the middle lobe) at the time (days after germination) indicated. The data points in (B) correspond to the same leaves. ($n=3$ for age 26, 29 and 35 d, $n=2$ for age 24 d). Watering was stopped on day 18 after germination: 'ww' indicates well-watered and 'ws' water-stressed plants.

drought after more than 11 d of withholding water. Even at the strongest drought stress assimilation rate was still positive and no net respiration was observed during the light period. Upon rewatering the assimilation rate again recovered faster than transpiration rate and both reached values similar to controls after 2 d (data not shown). Dark respiration was strongly reduced during drought stress at the leaf base and tip in comparison to control plants (Fig. 6). However, rates of dark respiration in mature leaves were similar in well-watered and water-stressed plants.

The higher sensitivity of transpiration to drought in comparison to assimilation rate has often been observed in mature leaves (for an overview see Schulze and Hall, 1982). Chlorophyll fluorescence analysis showed that non-photochemical quenching processes increased in *Ricinus* during drought stress to dissipate excess energy (U Heckenberger and U Schurr, unpublished results). In stress conditions the physiological control again strongly overrode the structural pattern, as gas exchange declined irrespective of the higher stomatal density in drought-stressed plants (Heckenberger *et al.*, 1998). In contrast to assimilation and transpiration rates, dark respiration was closely associated with the earlier maturation of stressed leaves.

Determination of biochemical composition in growing and stressed tissues

Due to the dynamic situation in growing tissues, it is difficult to find a single reference unit, which allows easy interpretation (for a more detailed analysis see Walter and Schurr, 2000). Under the conditions of these experiments, ratios of dry weight over fresh weight were not statistically different between leaf positions and between water-stressed and well-watered leaves (well-watered: leaf 2, 0.24 ± 0.06; leaf 3, 0.23 ± 0.06; leaf 4, 0.229 ± 0.04; water stressed: leaf 2, 0.27 ± 0.05; leaf 3, 0.28 ± 0.07; leaf 4, 0.3113 ± 0.07). The maximal difference obtained between values on the fresh weight and the dry weight basis was in leaf 4 between well-watered and water-stressed plants resulting in a maximal change by 30%. Nevertheless, concentrations were expressed on a dry weight basis in order to circumvent any problems of interpretation in the drought-stress treatments.

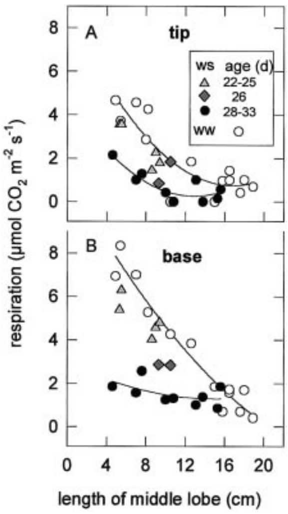

Fig. 6. Dark respiration rates at the leaf tip (A) and base (B) of growing leaves of well-watered and water-stressed *Ricinus* leaves. The different symbols indicate the age (d) of the water-stressed plants since germination. The closed lines indicate the relation obtained for well-watered and drought-treated plants respectively during their development: 'ww' indicates well-watered and 'ws' water-stressed plants.

Chlorophyll and protein concentrations

Alterations in chlorophyll and protein concentrations are essential steps in sink–source transition. Chlorophyll concentration per unit dry weight increased almost linearly with the increase in leaf size and stayed constant after the final leaf size was reached (Fig. 7). The difference in chlorophyll concentration between the leaf base and the leaf tip was small, with the leaf tip having a slightly higher concentration than the leaf base in mid-sized leaves. As the leaf tip and base had different cell densities (Fig. 3) the leaf tip had an approximately 1/3 higher amount of chlorophyll per cell than the leaf base throughout most of the development of the leaf. This increase in chlorophyll concentration is typical during the development of dicot leaves (Harn *et al.*, 1993; Merlo and Passera, 1991). Protein concentration per dry weight increased slightly with leaf size (Fig. 7B). No significant

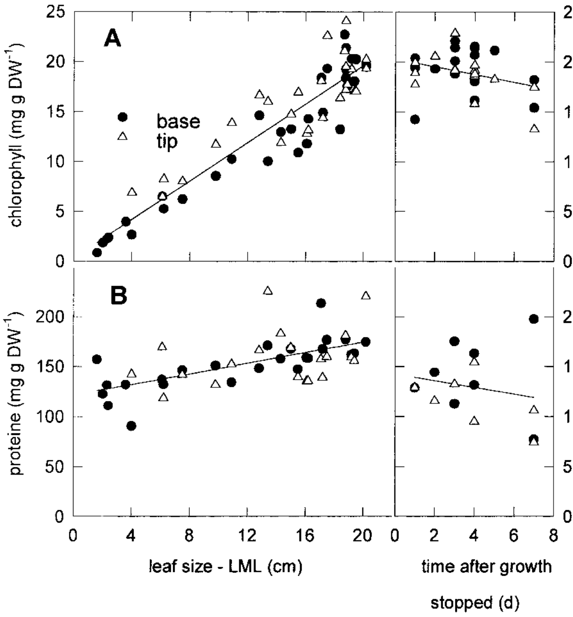

Fig. 7. Chlorophyll (A) and protein concentrations (A) per unit dry weight in leaf base and tip of leaves from well-watered *Ricinus* plants during leaf development. The panels on the right indicate the course of concentrations after the final size of the leaves was reached.

differences were present between the leaf base and the leaf tip despite significant differences in cell density (Fig. 3).

During drought stress, chlorophyll concentrations per dry weight increased in leaves 2, 3 and 4 of water-stressed plants at smaller leaf sizes than in well-watered plants (Fig. 8A). However, concentrations at the smaller final size of the leaves under stress were not significantly different from those of mature leaves of well-watered plants. Protein concentration per dry weight of leaves from drought-stressed plants were not significantly different from controls at a given leaf size.

The rewatering experiments (Fig. 9) supported these findings for water-stressed plants, with chlorophyll concentrations in drought-stressed plants being similar to the concentrations at maturity of well-watered controls, but at a smaller leaf size. Leaves of rewatered plants increased their leaf size, but maintained their chlorophyll concentration at mature levels. This indicates the induction of chlorophyll biosynthesis to counteract the dilution due to regained leaf expansion. Protein concentrations were not analysed in the rewatering experiment.

Chlorophyll and protein content per dry weight at the final leaf size were similar in leaves of well-watered and drought-stressed plants. As leaf size and cellular parameters were closely associated, the protein and chlorophyll content per cell increased during drought. The functional development of the leaf continued during stress and similar 'mature' concentrations per dry weight of major constituents equivalent to the mature control leaves were reached. A fixed protein and chlorophyll concentration per dry weight was characteristic for a functional mature leaf. Even during the rewatering experiments, when leaf size increased considerably, the 'mature' concentration of chlorophyll was maintained. These findings support the hypothesis that the content of these constituents per dry weight is closely controlled in developing leaves with an apparent upper limit.

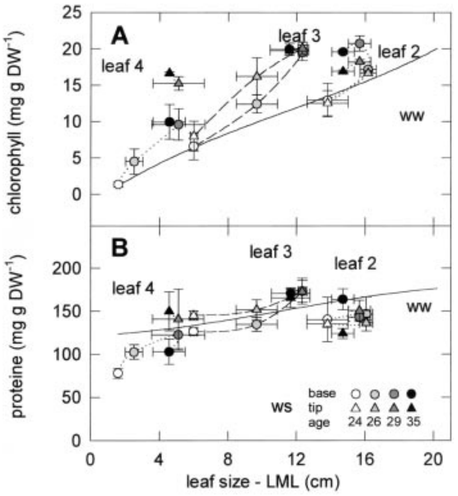

Fig. 8. Chlorophyll (A) and protein concentration (B) per dry weight at the leaf tip and base of leaves of drought-stressed *Ricinus* leaves in relation to the leaf size (length of middle lobe (LML), cm) of leaves 2, 3 and 4 subjected to drought stress (means ±standard error, $n = 3$, except for tip of leaf 4 and age class 24: $n = 2$). In leaf 4 a differentiation between leaf tip and base was not possible for age 24 and 26 d after germination. The closed lines ('ww') indicate the relation obtained for leaf 3 in well-watered plants during their development.

In other experiments chlorophyll concentrations have been reported to decline during drought stress, which might be related to membrane disintegration due to oxidative stress in less drought-tolerant species (Moran *et al.*, 1994). Additionally, the nitrogen form used by the plant can cause considerable differences in the response of chlorophyll concentration during drought stress (Mihailovic *et al.*, 1992).

Carbohydrate concentrations

Glucose (Fig. 10A) and fructose (Fig. 10B) content per dry weight increased in parallel with leaf size by a factor of 7, but no tip-to-base gradients were observed. In mature leaves, hexose concentrations tended to decline with time after the final size had been reached. Sucrose concentrations were significantly higher than glucose and fructose concentrations in small leaves, but were similar to hexose concentrations in mature leaves (Fig. 10C). Sucrose concentration increased by only a factor of 2 during leaf development. No tip-to-base gradients were present in sucrose concentration, even though assimilation rates were significantly higher at the tip (Fig. 4). Sucrose concentrations remained constant after the cessation of growth. Starch was hardly quantifiable in small leaves and increased to a mean value of 300 μmol hexose equivalents g^{-1} dry weight in mature leaves. After the cessation of growth, variation between leaves increased significantly in all measured carbohydrates, even though samples were taken at fixed times of day. The increase of sucrose-to-hexose ratios is common during leaf development and due to the interaction of sucrose-phosphate synthase, sucrose synthase and invertase activities during sink–source transition (Weber *et al.*, 1996).

During drought stress, glucose and fructose concentrations increased at a smaller leaf size up to values equivalent to or slightly above those of mature leaves of well-watered controls in leaves 2, 3 and 4 (Fig. 11A, B). When these mature values were reached, glucose and fructose concentrations decreased slightly in leaf 2, but declined 3-fold in leaf 3. Leaf 4 did not reach mature concentrations during the experiment. Sucrose concentrations also reached mature concentrations (Fig. 11C), but concentrations increased thereafter, equivalent to the decline of hexoses. Starch concentrations increased slightly in leaves of water-stressed plants (Fig. 11D).

The rewatering experiment supported the findings of the drought-stress experiment as, during drought, hexose

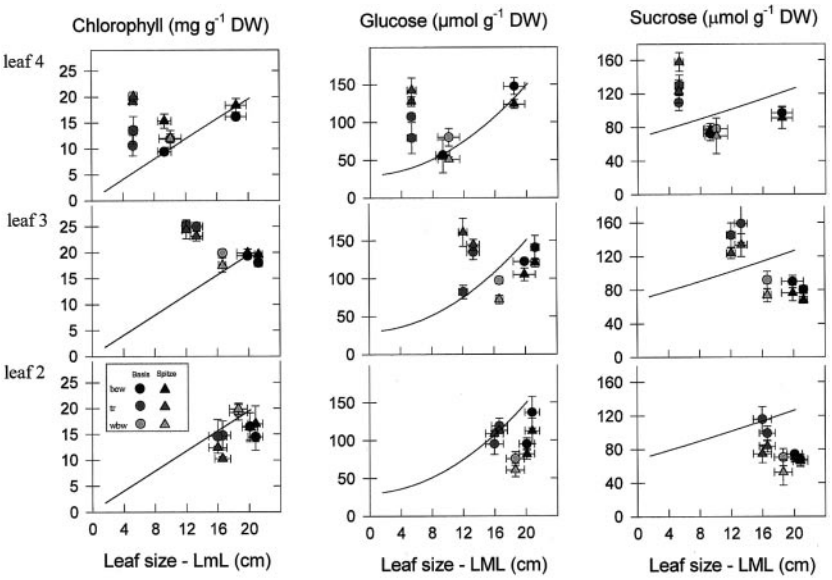

Fig. 9. Chlorophyll (left panel), glucose (middle panel) and sucrose concentration per dry weight in leaf storeys 2, 3 and 4 of drought-stressed, rewatered and well-watered plants of *Ricinus communis*. The closed lines indicate the relation obtained for leaf 3 in well-watered plants during their development

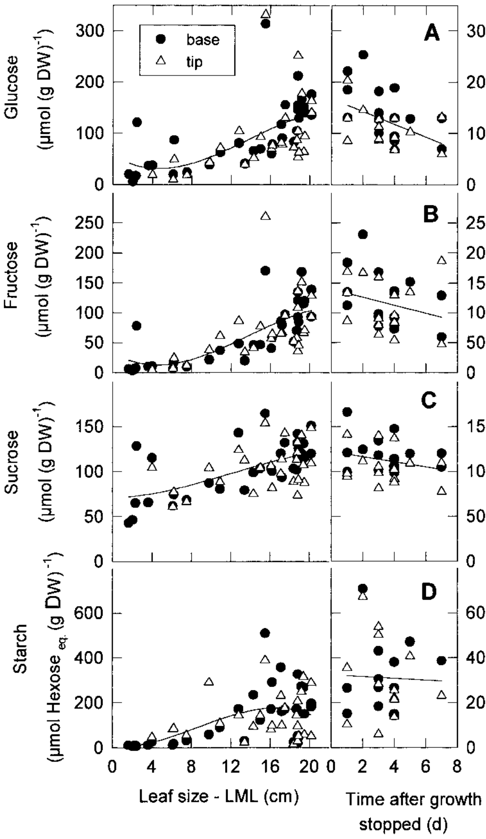

Fig. 10. Concentrations of (A) glucose, (B) fructose, (C) sucrose, and (D) starch in the tip and base of leaf 3 of well-watered *Ricinus* plants during development. Left panel: during expansion growth (in relation to leaf size). right panel: time-course after end of leaf expansion (in relation to time after cessation of growth).

and sucrose concentrations increased to mature levels (Fig. 9B) in leaves 3 and 4, while they stayed in the same range as in controls in the almost mature leaf 2. The decline in hexose concentrations in leaf 3 was also observed, but was coincident with rewatering the plants. The rewatering response of soluble carbohydrates was clearly different to the drought response: upon rewatering both hexose and sucrose concentrations declined to the concentrations of leaves equivalent to leaves of that size in controls.

Again, these findings support the hypothesis that the leaf finishes sink–source transition at a smaller leaf size during drought stress. It is clear from the results presented here that the impact of drought on the speed of development has to be taken into account in growing leaves for understanding the changes of biochemical composition. Rewatering allowed the leaf to expand and to regain the ability to expand its source function.

Amino acids

Amino acid concentrations per dry weight varied strongly in growing leaves (range 50–350 µmol g^{-1} DW). In small leaves, tips had significantly higher amino acid concentrations than the leaf base, but decreased consistently with leaf size (Fig. 12A). During this decline, the amount of amino acids per cell remained constant, indicating that the decline was mainly due to cell expansion. At a leaf size (LML) of 16 cm the tip-to-base gradient had vanished and amino acid concentration fell to values of 30 µmol g^{-1} DW, which were maintained constant in the mature tissues (data not shown). During this decline the content of amino acids per cell decreased. This was mainly due

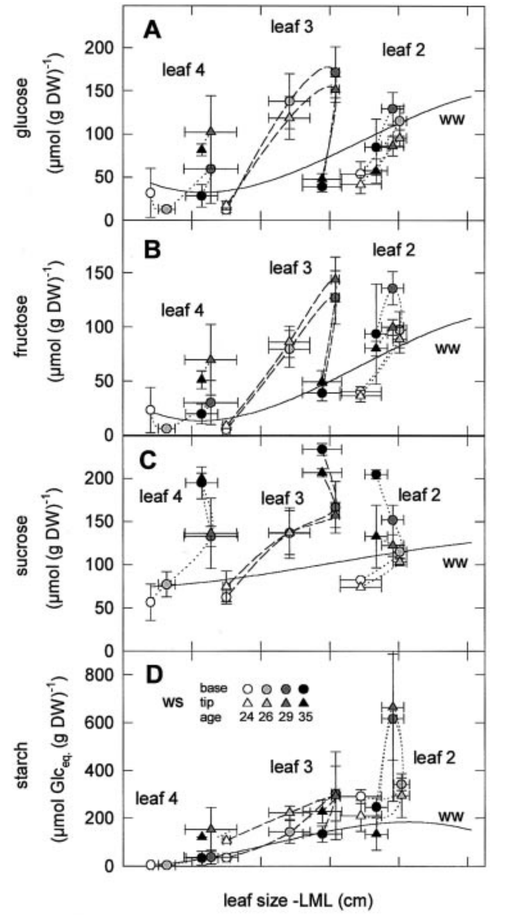

Fig. 11. Concentrations of (A) glucose, (B) fructose, (C) sucrose, and (D) starch in the tip and base of leaf 2, 3 and 4 of water-stressed *Ricinus* plants during development. (means ±standard error, $n=3$, except for tip of leaf 4 and age class 24: $n=2$). In leaf 4 a differentiation between leaf tip and base was not possible for age 24 and 26 d after germination. The closed lines ('ww') indicate the relation obtained for leaf 3 in well-watered plants during their development.

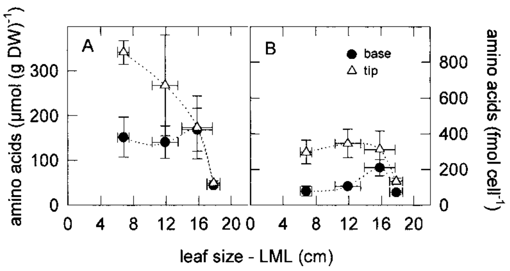

Fig. 12. Concentration of amino acids in leaf tip and base of leaf 3 of well-watered *Ricinus* plants in relation to (A) dry weight and (B) per cell (means ±standard error, $n=3$).

to falling concentrations in glutamine and arginine, which were the two major amino acids in growing leaves (Fig. 13B, C).

The developmental change in amino acid composition is a common phenomenon associated with the transition of the growing leaf from a sink to a source for amino acids. In particular, the decline in glutamine as the major transport (and storage) form is characteristic. Amino acids showed the most prominent tip-to-base gradients in all measured leaf constituents.

During drought stress, amino acid concentrations decreased at smaller leaf sizes until final leaf area was reached (Fig. 13A). Strong tip-to-base gradients were observed. This decline was similar to the maturation in control plants, as (i) the concentration at final leaf size was 50–100 µmol g^{-1} DW, (ii) it was mainly due to falling concentrations of glutamine and arginine (Fig. 13B, D) and (iii) transient tip-to-base gradients were present in leaf 3 (Fig. 13B, D). However, when the final leaf size was reached, amino acid concentrations increased again (Fig. 13A). This increase was mainly (40% of the entire increase) due to arginine (Fig. 13B), which increased its concentration more than 4-fold compared to the concentration at the day when growth ceased first. Proline concentration was low and almost constant in leaves of well-watered plants during development (Fig. 13C solid line). During drought stress it increased strongly by factors of 10, 3 and 4 in leaves 4, 3 and 2, respectively. By far the most dramatic increase took place in the youngest leaves (leaf 4), where proline concentrations reached values close to the concentrations of glutamine.

The decline of amino acid again suggests that sink–source transition occurs at smaller leaf size (see above). This hypothesis is supported by the transient yet strong tip-to-base gradients in amino acid concentrations, which also occur at a smaller leaf size during drought stress. The increase after reaching the final leaf size is clearly a different physiological stage, similar to the changes in carbohydrates after cessation of growth. The strong increase in proline concentration has often been reported in drought-stressed plants (Heuer, 1994) and has been interpreted as osmotic adjustment with proline being a compatible solute or as increased storage of unused nitrogen (Heuer, 1994; Lawler and Leech, 1985).

Sink–source transition: enzymatic level

The biochemical (and molecular) basis of sink–source transition is the alteration of the enzymatic apparatus of the leaf. The activities of sucrose-phosphate synthase and sucrose synthase activity in leaves are reported. Analysis of invertases (acid and alkaline) in the same material (data not shown) supported the general conclusion (see below). Again it has to be emphasized that leaves of a given size in this experiment had a similar cytological structure in well-watered and drought-stressed plants and thus a change in activity of an enzyme in response to drought cannot be related simply to a change in the number of cells.

Sucrose phosphate synthase (SPS) is predominantly

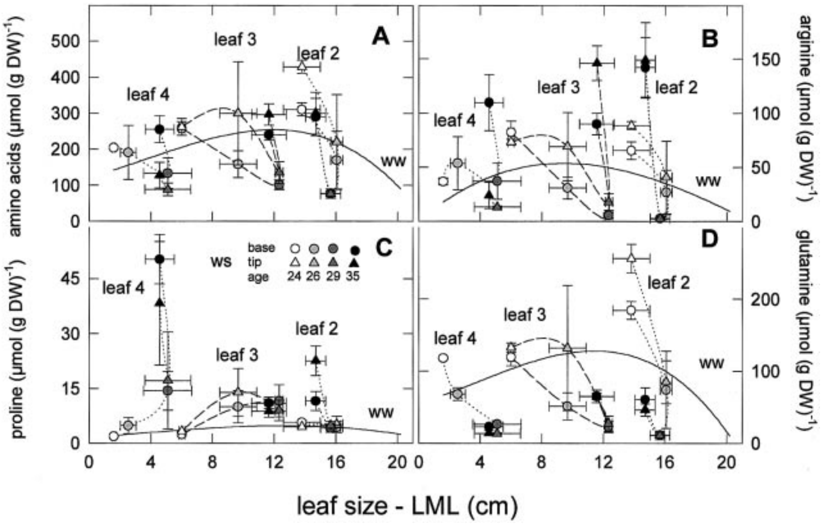

Fig. 13. Concentration of amino acids (A), arginine (B), proline (C), and glutamine (D) in relation to leaf size of leaves 2, 3 and 4 in leaves of water-stressed *Ricinus* plants. (means ☐ standard error, $n=3$, except for tip of leaf 4 and age class 24: $n=2$). In leaf 4 a differentiation between leaf tip and base was not possible for age 24 and 26 d after germination. The closed lines indicate the relation obtained for leaf 3 in well-watered plants during their development, 'ww' indicates well-watered and 'ws' water-stressed plants.

involved in source activity, providing sucrose for export (Weber *et al.*, 1994; Harn *et al.*, 1993). Maximal SPS activity, correlated to SPS protein (Walker and Huber, 1989), increased during the second half (in relation to leaf size) of the leaf development in well-watered control plants (Fig. 14A). Tip-to-base gradients in SPS activity were not present despite the considerable difference in assimilation rate. Due to the different cell density in the leaf base and the leaf tip, SPS activity per cell was higher in the leaf tip than in the leaf base throughout most of the development of the leaf (data not shown). SPS-activity declined to levels of activity similar to those in small leaves soon after final leaf size was reached. SPS is activated by phosphorylation and its activation state can be measured *in vitro* (Huber *et al.*, 1989; Reimholz *et al.*, 1994). SPS activation increased with leaf size by a factor of 3 (Fig. 14B) and, in contrast to the maximal activity, was maintained constant in mature leaves. Therefore, the increase in SPS function in mature source tissues in *Ricinus* was predominantly due to an increased activation state. However, no tip-to-base gradients were present in SPS-activation that would relate to sink–source gradients in the growing tissues. This is the same on a per cell basis, as the activation is calculated from the ratio of the maximal and the selective activity of SPS.

Drought stress caused an increase of SPS activity at smaller leaf size in leaf 3, while leaf 4 did not reach mature SPS activities even at the end of the experiments (Fig. 15A). The effect of drought on the SPS activation state was more pronounced and again caused an increase of activation at smaller leaf size (Fig. 15B). Hence SPS activity reached mature levels at a smaller leaf size indicating a sink–source transition at smaller leaf size. It is clear

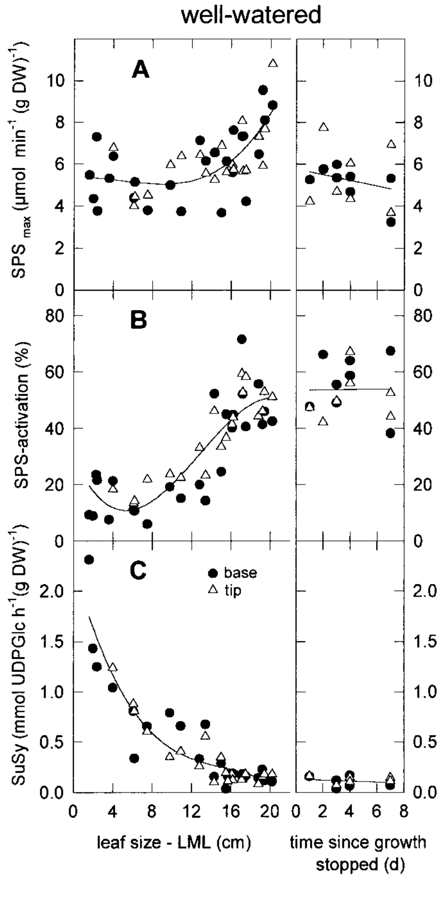

Fig. 14. Maximal activity (A) and activation state (B) of sucrose phosphate synthase (SPS) in leaf 3 of well-watered *Ricinus* plants during leaf development (left panel) and during the first 7 d after final leaf size was reached (right panel).

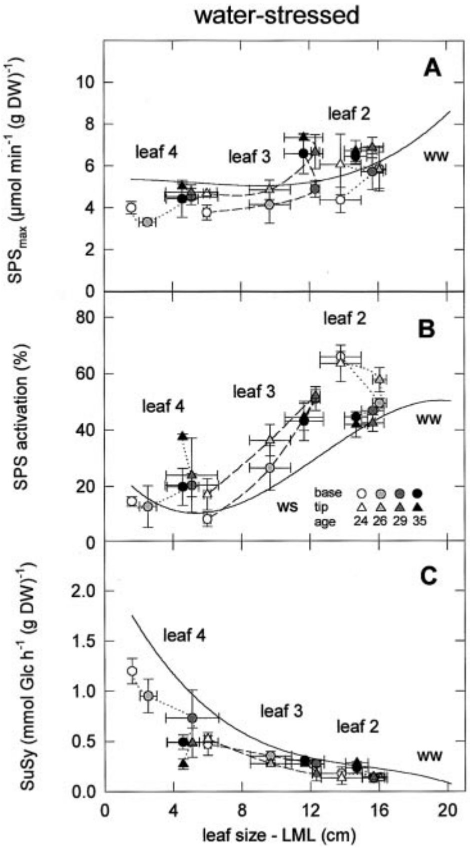

Fig. 15. Maximal activity (A) and activation state (B) of sucrose phosphate synthase (SPS) in leaves 2, 3 and 4 of water-stressed *Ricinus* plants during leaf development. (means ±standard error, $n=3$, except for tip of leaf 4 and age class 24: $n=2$). In leaf 4 a differentiation between leaf tip and base was not possible for age 24 and 26 d after germination. The closed lines indicate the relation obtained for leaf 3 in well-watered plants during their development, 'ww' indicates well-watered and 'ws' water-stressed plants.

from these data that SPS is more related to sink–source transition than to growth and that the deduction of a central role for SPS in growth from the close correlation with elongation (Seneweera *et al.*, 1995) is an oversimplification. This analysis of SPS during development and drought did not take into account that different isoforms of SPS have been reported to be expressed during the different developmental stages of the leaf and during stress response (Reimholz *et al.*, 1997).

Sucrose synthase (SuSy) is central for the activation of sucrose imported from the phloem into sink leaf (Weber *et al.*, 1994) and is generally found markedly up-regulated in developing leaves (Sturm *et al.*, 1995). Sucrose synthase activity declined with leaf size (Fig. 14C). The decline with leaf size was predominantly due to the expansion of the cells and thus 'dilution' of the activity, as the SuSy activity per cell decreased only by a factor of 2 with development (data not shown). Again no tip-to-base gradients in enzyme activity were found on a dry weight basis, making the SuSy activity per cell in the tip higher than at the base. Drought stress did not significantly alter the relationship between SuSy activity and leaf size, again emphasizing the role of cell expansion (dilution effect) in the decline in SuSy activity. Sucrose synthase has been reported to increase during drought (Keller and Ludlow, 1993), but in those experiments sucrose concentrations decreased and hexose concentrations increased during the stress.

Conclusion

In this paper, data from the whole leaf to the biochemical level have been summarized to illustrate the complexity and interdependency of structure and function in growing leaves and its interaction with drought stress. The results indicate that (i) on a macroscopic scale, growth rate and its distribution vary strongly in a diurnal manner, (ii) cytological structure alters strongly during development and this provides a framework for the analysis of function in growing leaves in combination with leaf size as a reference system, (iii) gas exchange properties of a developing leaf are dominated by the physiological properties with the stomatal development providing the basic structures, (iv) tip-to-base gradients in carbohydrate concentrations and activities of enzymes linked to sink–source transition were not strongly developed, in contrast to gradients of amino acids, (v) drought stress (and rewatering) caused a maturation of leaves at a smaller leaf size with a complete syndrome in growth dynamics, carbohydrate and amino acid concentrations as well as sink–source related enzymes. Finally, these data sets show the complexity of the interaction of structural and functional development in a dynamically changing environment.

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