

Nitrogen deficiency inhibits leaf blade growth in *Lolium perenne* by increasing cell cycle duration and decreasing mitotic and post-mitotic growth rates

MONIKA KAVANOVÁ, FERNANDO ALFREDO LATTANZI & HANS SCHNYDER

Lehrstuhl für Grünlandlehre, Technische Universität München, Am Hochanger 1, D-85350 Freising, Weihenstephan, Germany

ABSTRACT

Nitrogen deficiency severely inhibits leaf growth. This response was analysed at the cellular level by growing *Lolium perenne* L. under 7.5 mM (high) or 1 mM (low) nitrate supply, and performing a kinematic analysis to assess the effect of nitrogen status on cell proliferation and cell growth in the leaf blade epidermis. Low nitrogen supply reduced leaf elongation rate (LER) by 43% through a similar decrease in the cell production rate and final cell length. The former was entirely because of a decreased average cell division rate (0.023 versus 0.032 h⁻¹) and thus longer cell cycle duration (30 versus 22 h). Nitrogen status did not affect the number of division cycles of the initial cell's progeny (5.7), and accordingly the meristematic cell number (53). Meristematic cell length was unaffected by nitrogen deficiency, implying that the division and mitotic growth rates were equally impaired. The shorter mature cell length arose from a considerably reduced post-mitotic growth rate (0.033 versus 0.049 h⁻¹). But, nitrogen stress did not affect the position where elongation stopped, and increased cell elongation duration. In conclusion, nitrogen deficiency limited leaf growth by increasing the cell cycle duration and decreasing mitotic and post-mitotic elongation rates, delaying cell maturation.

Key-words: kinematics; leaf growth; leaf growth zone.

INTRODUCTION

Nitrogen is an essential constituent of amino acids, proteins, nucleic acids and secondary metabolites (Marschner 1995). Because of its ubiquity, nitrogen deficiency affects most aspects of plant functioning, from metabolism to resource allocation, and development (Stitt 1999; Crawford & Forde 2002; Wang *et al.* 2003; Scheible *et al.* 2004). In particular, the availability of nitrogen is a crucial factor governing the leaf area expansion rate (Volenc *et al.* 1983; Gastal & Nelson 1994; Fricke, McDonald & Mattson-Djos 1997; Roggatz *et al.* 1999; Tóth *et al.* 2002). Leaf growth results from an interplay between the concerted processes of cell

proliferation and cell expansion. Yet, our understanding of the effects of nitrogen status on leaf growth at the cellular level is far from complete (Longnecker 1994; Lawlor, Lemaire & Gastal 2001).

The epidermis of grass leaf blade is a model system of choice for unravelling cellular processes underlying growth responses. This is because of the presence of a developmental gradient along the leaf axis that spatially separates cell proliferation from cell expansion and maturation, allowing thus their simultaneous evaluation. Moreover, cell expansion is predominantly unidirectional, and the analysis can thus be largely simplified to that of elongation rates. The leaf growth zone is located at the base of the growing leaf (Kemp 1980), and has two distinct regions. In the basal cell division zone, meristematic cells undergo a number of cell cycles accompanied by mitotic growth. In the adjacent elongation-only zone, cells undergo a phase of post-mitotic expansion during which they attain their mature length. Several studies on nitrogen deficiency attributed the decrease in leaf elongation rate (LER), that is, the flux of leaf tissue out of the leaf growth zone (mm h⁻¹), to a lower flux of cells into the elongation-only zone, that is, cell production rate (cell h⁻¹), while only minor or no changes in final cell length (mm per cell) were observed (Volenc & Nelson 1983; Gastal & Nelson 1994; Fricke *et al.* 1997; Tóth *et al.* 2002). Yet, it is unknown if the observed decrease in the cell production rate resulted from a reduction in cell division rate (cell per cell h⁻¹), or from a decreased number of meristematic cells in the division zone. In addition, the role of the mitotic growth rate, which may be affecting the cell division rate, has not been evaluated. Similarly, the final cell length integrates not only the relative elongation rates, but also the duration of post-mitotic growth, and the balance between cell growth and division during the proliferative phase (Beemster *et al.* 2006). Thus, if the molecular regulation of cell proliferation and cell growth under nutrient stress is to be elucidated, it is first necessary to discriminate between processes that are actually reacting to nitrogen deficiency and those that are not.

Another open question is whether the cellular responses observed under nitrogen deficiency are unique, or are common to other nutrients. In a previous study that analysed cell dynamics in the leaf growth zone of *Lolium perenne*

Correspondence: H. Schnyder. Fax: +49 (0)8161-713243; e-mail: schnyder@wzw.tum.de

under phosphorus deficiency (Kavanová *et al.* 2006b), we used a kinematic analysis – a method to translate the spatial pattern of growth variables along the leaf axis into the developmental history of an individual cell (Green 1976; Silk & Erickson 1979; Kavanová *et al.* 2006b), combined with a direct estimation of the position where cell division stopped (Beemster *et al.* 1996; Kavanová *et al.* 2006b). This approach allowed to derive rates and durations of mitotic and post-mitotic growth, as well as the average division rate of meristematic cells, and the average number of division cycles that the progeny of the initial cell went through before losing meristematic activity (Ivanov & Dubrovsky 1997). The current study was undertaken to provide a similarly comprehensive analysis of the cellular responses to nitrogen deficiency in *L. perenne* leaf blades. The hypothesis was tested that nitrogen deficiency affects the rates of cell division, and mitotic and post-mitotic elongation, but that other processes (such as the maintenance of meristematic activity) are independent of the plant internal nitrogen status.

MATERIALS AND METHODS

Plant material and growth conditions

Surface-sterilized seeds of *L. perenne* L. cv. Acento were sown individually in pots (diameter 5 cm; height 35 cm) on quartz sand with a drainage at the bottom. Plants grew in a growth chamber (E15; Conviron, Winnipeg, Canada) with 20 °C and 85% relative air humidity. Continuous light of 275 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density at plant height was provided by cool-white fluorescent lamps. The chamber was equipped with an automatic irrigation system flooding pots eight times per day with a modified half-strength Hoagland's solution containing either 1 mM NO_3^- (low nitrogen) or 7.5 mM NO_3^- (high nitrogen). The composition of the nutrient solution was as follows: (1) high nitrogen: macronutrients: 2.5 mM $\text{Ca}(\text{NO}_3)_2$, 2.5 mM KNO_3 , 1 mM MgSO_4 , 0.18 mM KH_2PO_4 , 0.21 mM K_2HPO_4 , 0.5 mM NaCl , 0.4 mM KCl , 0.4 mM CaCl_2 ; micronutrients: 125 μM Fe–ethylenediaminetetraacetic acid, 46 μM H_3BO_3 , 9 μM MnSO_4 , 1 μM ZnSO_4 , 0.3 μM CuSO_4 , 0.1 μM Na_2MoO_4 . (2) Low nitrogen: macronutrients: 1 mM KNO_3 , 1 mM MgSO_4 , 0.18 mM KH_2PO_4 , 0.21 mM K_2HPO_4 , 0.5 mM NaCl , 0.7 mM K_2SO_4 , 2 mM CaCl_2 ; micronutrients were the same as in high-nitrogen plants.

Leaf elongation rate

Leaf elongation rate (mm h^{-1}) and its components were analysed in tillers with a similar sheath length of the youngest fully expanded leaf (82 ± 2 mm). To this end, LER was measured 30 d after sowing in high-nitrogen plants, and 58 d after sowing in low-nitrogen plants. This ensured that the direct effects of nitrogen deficiency were not confounded with the indirect effects of a smaller tiller size (Kavanová *et al.* 2006a).

Representative tillers with at least three fully expanded leaves were selected. The LER was determined on the

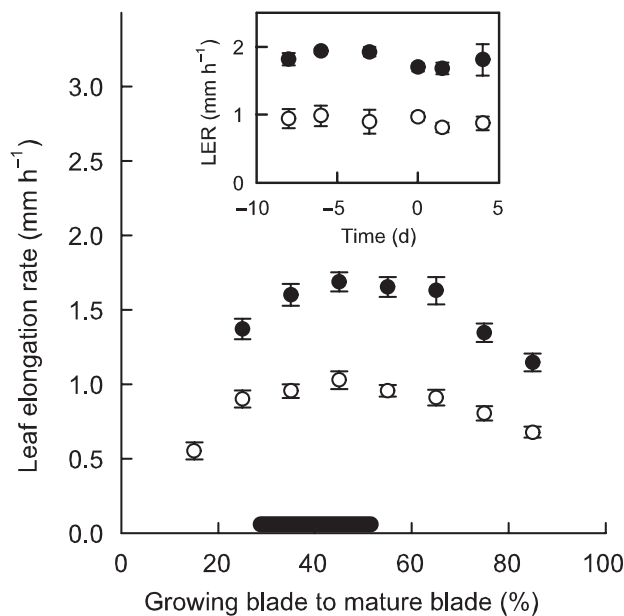


Figure 1. Effects of nitrogen supply and leaf developmental stage on the leaf elongation rate (LER). *Lolium perenne* plants were grown at high (7.5 mM, ●) and low (1 mM, ○) nitrogen supply. Leaf developmental stage is expressed as the percentage of the growing blade length to the blade length of the youngest expanded leaf. The thick line indicates the stage when the kinematic analysis was performed. Data points are means of 2 to 26 plants (\pm SE). Inset shows LER of leaves in the sampled stage measured before and after the sampling date (day 0).

youngest, most rapidly growing blade, during the phase of maximal growth, when LER was near constant. The blade length of the analysed leaf was 30–50% of the length of the youngest expanded leaf (Fig. 1). During this developmental stage, leaf growth is due exclusively to the activity of the blade growth zone, and cell divisions in the blade meristem and blade expansion are approximately steady (developmental stage A→B in Schnyder *et al.* 1990). The LER was calculated as the rate of change of the distance between the tip of the elongating blade and the ligule of the youngest fully expanded leaf measured with a ruler every 24 h.

Sampling and nitrogen analysis

Fourteen plants per treatment were sampled 34 d after sowing in high-nitrogen, and 57 d after sowing in low-nitrogen plants. Leaf growth zones were dissected from leaves similar to those used for LER measurements. Samples were frozen in liquid N_2 , freeze-dried, weighed, ground and stored at -25 °C. Nitrogen concentration was determined on 0.7 mg samples with an elemental analyser (NA1500; Carlo Erba Instruments, Milan, Italy).

Cell length measurement

The growing blades of the leaves used for leaf elongation measurement were carefully freed from surrounding older

leaves 30 d after sowing in high-nitrogen plants, and 58 d after sowing in low-nitrogen plants. Leaves were excluded if the ligule was situated more than 1 mm from the leaf insertion, in order to ensure that only the blade growth zone (i.e. above the ligule) was contributing to leaf elongation (Schnyder *et al.* 1990). The contribution of meristematic cells in the sheath (i.e. below the ligule) to LER was negligible, because during this stage the post-mitotic cell expansion in the sheath growth zone has not started yet. In five leaves per treatment, a transparent replica of the abaxial epidermis along the basal 50 mm of the growing leaf was taken as described by Schnyder *et al.* (1990). Briefly, a thin layer of 4% (w/w) of polyvinylformaldehyde (Formvar 1595 E; Merck, Darmstadt, Germany) in chloroform was spread along the basal part of the growing leaf. Then, the film was transferred with a transparent adhesive tape to a microscope slide.

Images were captured using a digital camera (Camedia C-5050Z; Olympus Corp., Tokyo, Japan) fitted to an optical microscope (Olympus BX50, Olympus Corp.). Starting from the base of the growing blade (i.e. the ligule), images were taken every 1 mm (0–10 mm from the base) or 2 mm (>10 mm from the base). Images were captured at magnifications of 400 to 40× (according to increasing cell lengths), and subsequently analysed in Sigma Scan Pro 5.0 (SPSS, Chicago, IL, USA). The mean epidermal cell length at each distance from the base was determined by measuring the length of 20–80 cells in cell files located midway between files containing stomata.

In addition, a sequence of overlapping images was taken along the basal 2–3 mm (starting from the ligule), and composite images were created. The length and distance from the ligule base of every cell in 8–10 cell files located midway between files containing stomata were recorded in each leaf. In each cell file, we recorded the most distal position of a newly formed (visually thinner) perpendicular cell wall, which was used as a marker for the distal end of the cell division zone (Beemster *et al.* 1996; Kavanová *et al.* 2006b).

Kinematic analysis of cell proliferation and growth

Kinematic analysis was performed as described previously (Kavanová *et al.* 2006b).

Analysis of cell elongation

Final cell length (L_f ; mm) and leaf growth zone length (L_{LGZ} ; mm) were determined by fitting a Richards function (Morris & Silk 1992) to plant cell length profiles (Table-Curve 2D; SYSTAT, Point Richmond, CA, USA):

$$y = e + a \frac{1}{[1 + \exp^{(b-cx)}]^{\frac{1}{d}}}, \quad (1)$$

where y is cell length; x is distance from the leaf base; $e + a$ is the asymptotic final cell length; e is the average

meristematic cell length; and b , c and d are constants. Because a reaches the maximum value only at an infinite distance, L_f was estimated as 95% of the value of a , and L_{LGZ} as the position where this was reached. Alternatively, L_{LGZ} was determined using the pin-pricking method on an independent set of plant tillers (Schnyder, Nelson & Coutts 1987; Kavanová *et al.* 2006a).

Cell flux (F ; cells h^{-1}), the rate at which cells are displaced past a particular position, was estimated at the distal end of the elongation-only zone from LER and final cell length (L_f):

$$F = \frac{LER}{L_f} \quad (2)$$

Under steady-state growth, when the LER and the cell length profiles do not change with time, cell flux is uniform beyond the division zone, and equal to cell production rate (Silk 1992).

In the elongation-only zone, the displacement velocity of a cell at a certain position is the result of the elongation of all cells located more basally in the growth zone. Therefore, displacement velocity increases with distance from the leaf base and finally becomes constant and equal to LER. Under steady-state growth, there is a strict correspondence between local cell length [$L(x)$, mm] and local displacement velocity [$v(x)$, mm h^{-1}] in the elongation-only zone (Morris & Silk 1992; Silk 1992):

$$v(x) = \frac{L(x)}{L_f} \times LER \quad (3)$$

The relative elongation rate in the elongation-only zone (R_e ; mm $mm^{-1} h^{-1}$; synonymous terms that have been used before are 'strain rate', 'relative elemental growth rate' and 'segmental elongation rate') was estimated by differentiating numerically the displacement velocity with respect to position. This parameter provides a measure to compare the magnitude of elongation rate independently from the absolute cell length at a given position (Silk 1992).

The average relative elongation rate in the elongation-only zone ($\overline{R_e}$; mm $mm^{-1} h^{-1}$) was calculated as:

$$\overline{R_e} = \frac{v_e - v_d}{L_e} \quad (4)$$

where v_e and v_d are displacement velocity (mm h^{-1}) at the end of the elongation-only and division zone, respectively, and L_e is the elongation-only zone length (mm).

The spatial profiles of cell length, displacement velocity and relative elongation rate were transformed in temporal profiles by calculating the trajectory function that describes the time it takes for a cell located at position x to be displaced to the end of the elongation-only zone (Silk, Lord & Eckard 1989):

$$t(x) = c \times N_e(x) \quad (5)$$

where c , the cellochron (h per cell), is the time required to displace a cell forward by one position in a cell file within the elongation-only zone, and is equal to the inverse of cell flux, and $N_c(x)$ is the number of cells present between position x and the distal limit of the elongation-only zone.

The average elongation duration (i.e. the residence time of a cell in the elongation-only zone; T_c ; h) was then calculated as:

$$T_c = \frac{N_c}{F} \quad (6)$$

where N_c is the total number of cells present in the elongation-only zone, and F is the cell flux.

Analysis of cell division

The average cell division rate in the whole meristem can be determined by relating the cell production rate to the number of cells in the division zone (Ivanov & Dubrovsky 1997). This estimation assumes that all cells in the meristem are proliferative, which is supported by studies showing that the proliferative fraction is close to one (Ivanov & Dubrovsky 1997; Ivanov, Dobrochaev & Baskin 2002, and references therein). In addition, an approximate constancy of division rate along the meristem has been shown (Beemster *et al.* 1996), and discussed (Baskin 2000).

The average cell division rate (D ; cell per cell h⁻¹) was calculated as:

$$D = \frac{F}{N_{\text{div}}} \quad (7)$$

where F is the cell production rate, and N_{div} is the number of cells in a meristematic cell file in the division zone (Green 1976; Ivanov & Dubrovsky 1997).

The number of cells in a meristematic cell file (N_{div}) was directly counted from the basal end of the division zone (i.e. the ligule) to the position of the last recently formed perpendicular cell wall. Meristematic cell length was determined as the average cell length between the basal and the distal end of the cell division zone.

The average cell cycle duration (T_c ; h), the time from a cell's formation to the next cytokinesis that yields two daughter cells, was calculated as follows (Green 1976; Ivanov & Dubrovsky 1997):

$$T_c = \frac{\ln(2)}{D} \quad (8)$$

The real residence time for an individual cell in the division zone is equal to T_c . Yet, it is possible to estimate the time needed for a perpendicular cell wall situated at the basal end of the division zone to reach the distal end of it. The residence time in the cell division zone (T_{div} ; h) is then related to the number of division cycles necessary to form all cells in the division zone (Korn 1993; Beemster & Baskin 1998):

$$T_{\text{div}} = T_c \times \log_2(N_{\text{div}}) \quad (9)$$

The average number of division cycles of the progeny of a cell formed by the division of the initial cell at the base of the meristem (i.e. the number of division cycles necessary to displace a transversal cell wall from the basal to the distal boundary of the division zone) was determined as follows (González-Fernández *et al.* 1968):

$$N = \log_2(N_{\text{div}}) \quad (10)$$

Statistical analysis

Differences between treatments were tested by Student's t -test (Statistica 6.0, Statsoft, Tulsa, OK, USA). The error associated with parameters calculated from averages was estimated by Gaussian error propagation. Results are shown as means \pm SE.

RESULTS

Leaf elongation rate

Lolium perenne plants grew at low (1 mM) or high (7.5 mM) nitrogen supply. Growth at low nitrogen supply reduced the nitrogen concentration in the leaf growth zone by 40% ($P < 0.001$; Table 1) and the LER by 43% ($P < 0.001$; Table 1 & Fig. 1). The latter was because of changes of similar magnitude in both the cell production rate (-28%) and final cell length (-20% ; Table 1).

Grasses growing under nutrient deficiency have smaller tillers, because LER and final leaf length are positively correlated (Fournier *et al.* 2005). Because a close correlation exists between the tiller size and the leaf growth zone length (Kemp 1980; Arredondo & Schnyder 2003; Kavanová *et al.* 2006a), we used an experimental design to ensure that the treatment effect on LER was entirely because of differences in nitrogen status and not in plant size (Kavanová *et al.* 2006b). The selected tillers of low- and high-nitrogen plants thus did not differ in size: the sheath of the youngest fully expanded leaf had similar length in both treatments (82 ± 2 mm SE), and the tillers held a similar number of green leaves (four to five).

Further, growing leaves were sampled at the same developmental stage, when they were one-third to one-half of the youngest mature blade length. During this stage, LER was approximately constant (Fig. 1), and only the blade meristem was active. Furthermore, plants were grown under continuous light and temperature in order to minimize the day-night fluctuations in growth parameters (Schnyder & Nelson 1988). This all ensured that an essential assumption of the kinematic analysis – a system in a steady state – was met. Importantly, not only LER should be in steady state, but also the cellular process determining it (Silk & Erickson 1979; Ivanov & Dubrovsky 1997). In older blades, an approximately constant LER results from a declining activity of the blade meristem and an awakening sheath

Parameter	High nitrogen	Low nitrogen	Difference (%)
Nitrogen in the growth zone (mg g ⁻¹ dry weight)	36.4 ± 1.2	21.9 ± 0.6	-40***
Leaf elongation rate (mm h ⁻¹)	1.70 ± 0.06	0.97 ± 0.06	-43***
Cell production rate (cell h ⁻¹)	1.67 ± 0.07	1.21 ± 0.03	-28**
Cell division rate (cell per cell h ⁻¹)	0.032 ± 0.003	0.023 ± 0.004	-28**
Cell cycle duration (h)	22 ± 2	30 ± 3	+40**
Number of cells per meristematic cell file	52.5 ± 3.3	53.1 ± 5.5	NS
Number of division cycles	5.7 ± 1.7	5.7 ± 2.4	NS
Division zone length (mm)	1.37 ± 0.04	1.35 ± 0.07	NS
Final cell length (mm)	1.03 ± 0.06	0.82 ± 0.06	-20*
Average cell length in the meristem (μm)	25.6 ± 0.8	26.5 ± 2.7	NS
Post-mitotic relative elongation rate (mm mm ⁻¹ h ⁻¹)	0.049 ± 0.01	0.033 ± 0.003	-33***
Elongation-only zone length	34 ± 1	31 ± 2	NS
Number of cells in the elongation-only zone	116 ± 8	101 ± 3	NS

Table 1. Kinematic parameters characterizing leaf blade growth and nitrogen status of the growth zone

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Lolium perenne plants were grown for 30 d at high (7.5 mM) and 58 d at low (1 mM) nitrogen supply. Kinematic data are averages for abaxial epidermal cells of five plants (\pm SE), along with the significance of the difference based on a *t*-test.

NS, not significant, $P > 0.05$.

meristem (Schnyder *et al.* 1990). Accordingly, during the following stage of leaf development, the LER was still constant, but a transition between the activities of blade to sheath meristem occurred (data not shown).

Cell proliferation: number of meristematic cells and their division rate

Nitrogen deficiency did not affect the average number of epidermal cells in the division zone nor their average length ($P > 0.1$; Table 1). In consequence, the length of the division zone also remained unaffected by nitrogen status ($P > 0.1$; Table 1). The number of division cycles necessary to displace a transversal cell wall from the proximal to the distal boundary of the division zone was derived from the number of cells in the division zone (González-Fernández *et al.* 1968). This parameter represents the average number of division cycles of the initial cell's progeny as it traverses the cell division zone. Nitrogen deficiency did not affect the maintenance of meristematic activity of epidermal cells, expressed as the average number of division cycles ($P > 0.1$; Table 1).

Although nitrogen deficiency did not affect the number of meristematic cells, it decreased the cell production rate, that is, the total flux of cells out of the division zone (determined from the LER and final cell length; Silk 1992), by 28% ($P < 0.01$; Table 1). Because nitrogen deficiency did not reduce the number of meristematic cells, the 28% decrease in the cell production rate in low-nitrogen plants was because of a 28% lower average cell division rate ($P < 0.01$; Table 1), that is, the number of cells produced per cell in the division zone per unit time (Ivanov & Dubrovsky 1997). This implies that the average cell cycle duration was 8 h

longer in low-nitrogen plants (Table 1). As a consequence of an unaltered number of division cycles per cell and a longer cell cycle duration, the average cell residence time in the division zone increased under nitrogen stress (124 ± 12 h at high versus 174 ± 28 h at low nitrogen). Thus, nitrogen-deficient cells maintained meristematic activity for a longer period of time.

Cell proliferation: relative elongation rate of meristematic cells

The cell division zone in *L. perenne* is short and enclosed in older leaves. Thus, the relative elongation rate within the cell division zone could not be independently measured by neither destructive nor non-destructive methods (Fiorani & Beemster 2006). However, the size of meristematic cells in itself provides information on the balance between the relative rates of division and elongation (Green 1976; Baskin 2000; Ivanov *et al.* 2002). The spatial profile of cell length in the division zone did not differ between high- and low-nitrogen plants (Fig. 2). Cell length was typically constant along each individual meristematic cell file until the position of the fifth division cycle of the initial cell progeny, that is, in the proximal half of the division zone (Fig. 2, inset). In the distal half of the cell file, the average cell length increased by 30 and 38% in low- and high-nitrogen plants, respectively, but the increase in the minimal cell length was less (Fig. 2). Cells situated in the distal half of the division zone are undergoing the last (sixth) division cycle, and would be displaced past the limit of the division zone during one cell cycle duration (Ivanov & Dubrovsky 1997).

The stability of the average cell length during the major part of the proliferative phase means that relative rates of

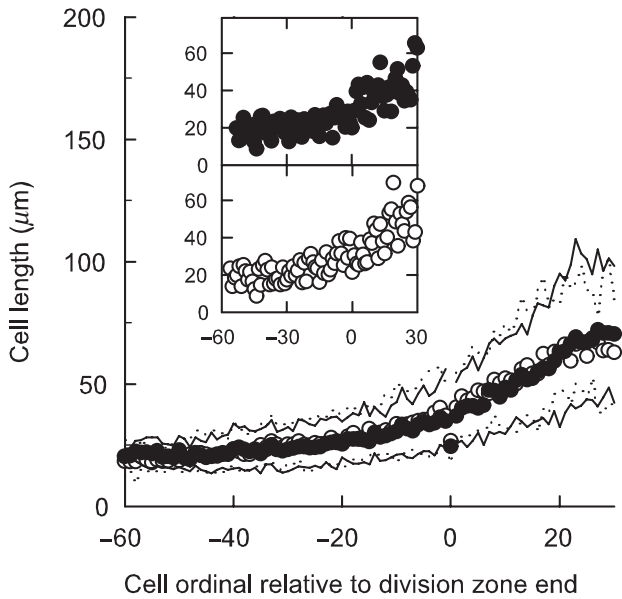


Figure 2. Spatial profile of the average epidermal cell length along the basal part of the leaf growth zone. *Lolium perenne* plants were grown for 30 d at high (7.5 mm, ●) and 58 d at low (1 mm, ○) nitrogen supply. The average length of the shortest and the longest cell at each cell rank along the division zone is indicated by continuous lines (7.5 mm nitrogen) and dashed lines (1 mm nitrogen). Data are means of five plants. The inset shows the raw cell length data for an individual cell file of a high- (●) and low- (○) nitrogen plant.

meristematic cell elongation were very close to average cell division rates. In other words, nitrogen-sufficient meristematic cells had 28% higher rates of cell division and 28% higher rates of elongation than nitrogen-deficient cells.

Spatial analysis of post-mitotic elongation

The difference in nitrogen status did not largely affect the position where post-mitotic cell elongation started and stopped (Table 1 & Fig. 3a). This result was independently confirmed by the pin-pricking method which revealed that the leaf growth zone length was $38 \pm 2\%$ of the sheath length of the youngest fully expanded leaf in low-nitrogen, and $42 \pm 4\%$ in high-nitrogen plants ($P > 0.1$). Similarly, the number of cells that were elongating simultaneously was not affected by low-nitrogen status ($P > 0.1$; Table 1). Thus, the higher cell production rate in high-nitrogen plants did not lead to a proportional increase in the length of the elongation-only zone.

Under steady-state growth conditions, the spatial distribution of cell length can be used to derive the profile of displacement velocity along the elongation-only zone (Silk 1992). The derivative of the velocity profile gives the spatial distribution of relative elongation rates ($\text{mm mm}^{-1} \text{h}^{-1}$), a measure to compare the magnitude of elongation rate independently from the absolute cell length (Silk 1992). Relative elongation rates did not differ in the proximal 7 mm, but were lower at all distal positions in low-nitrogen plants

($P < 0.05$; Fig. 3c). Interestingly, relative elongation rate in the first millimetres of the elongation-only zone was equal to the estimated mitotic elongation rate of meristematic cells in high-nitrogen plants, but was 26% higher in low-nitrogen plants (Table 1 & Fig. 3c). Nitrogen deficiency changed the shape of relative elongation rates profile, slightly shifting the position of the maximal relative elongation rate towards the leaf base. Averaged over the whole elongation-only zone, relative elongation rate was 33% lower in low-nitrogen plants ($P < 0.05$; Table 1).

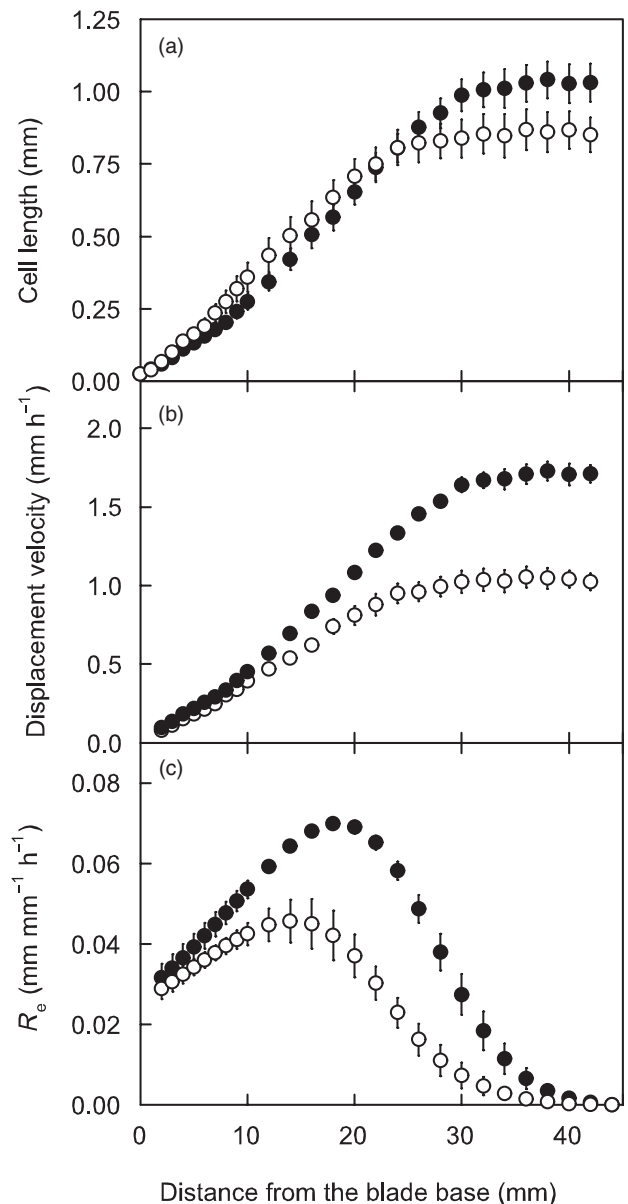


Figure 3. Spatial analysis of the nitrogen supply effect on the epidermal cell length (a), displacement velocity (b) and relative elongation rate (R_e ; c) along the base of the growing leaf. *Lolium perenne* plants were grown for 30 d at high (7.5 mm, ●) and 58 d at low (1 mm, ○) nitrogen supply. Data are means of five plants (\pm SE).

Temporal analysis of post-mitotic elongation

A temporal analysis of the post-mitotic cell elongation was carried out by transforming the spatial profiles of cell length, displacement velocity and relative elongation rate into time profiles using the growth trajectory function, which relates spatial position of a cell to time coordinates (Silk 1992). This revealed that cells expanded for 14 h less under high nitrogen (69 ± 3 h at high nitrogen versus 82 ± 2 h at low nitrogen; $P < 0.05$), because they moved more rapidly through an elongation-only zone of similar length. Thus, the higher relative elongation rate of high-nitrogen plants was associated to shorter elongation duration (Fig. 4).

Final cell length

The final length of epidermal cells was 20% shorter in low-nitrogen plants ($P < 0.05$; Table 1 & Fig. 3a), contributing hence to the reduction in LER under nitrogen deficiency. The final length of a cell is defined by three parameters: the length of the cell when it leaves the division zone and enters the elongation-only zone, the average relative elongation rate during the post-mitotic phase and the duration of this phase. Notably, nitrogen deficiency impaired only relative elongation rates; the cell size at the position where elongation started did not differ between low- and high-nitrogen plants ($P > 0.1$; Fig. 2), and elongation duration actually increased in low-nitrogen plants although not enough as to fully compensate the lower rate of post-mitotic elongation.

DISCUSSION

Nitrogen is the mineral nutrient required in highest quantities, and thus many studies have focused on the effects of nitrogen deficiency on plant functioning. Yet, the cellular bases of the growth reduction in plants are not resolved. The present study shows that the reduction in LER under nitrogen deficiency in the grass *L. perenne* was because of decreases in both the cell division and mitotic elongation rates, which led to a reduction in cell production rate, and post-mitotic elongation rates, which led to a reduction in final cell length. No other parameters responded to nitrogen status, be it the size of meristematic cells, the number of division cycles meristematic cells went through or the positions where post-mitotic elongation started and stopped. But, nitrogen deficiency prolonged the residence time of a cell in the growth zone and delayed its maturation. All these effects were strikingly similar to those observed in the same species in response to phosphorus deficiency (Kavanová *et al.* 2006b), suggesting that at the cellular level, leaf growth responds in the same way to both nutrients.

Nitrogen deficiency decreased cell division rate, but did not affect the coordination between cell division and growth of meristematic cells

The division rate of meristematic cells was positively correlated with the nitrogen status of the growth zone, which, in

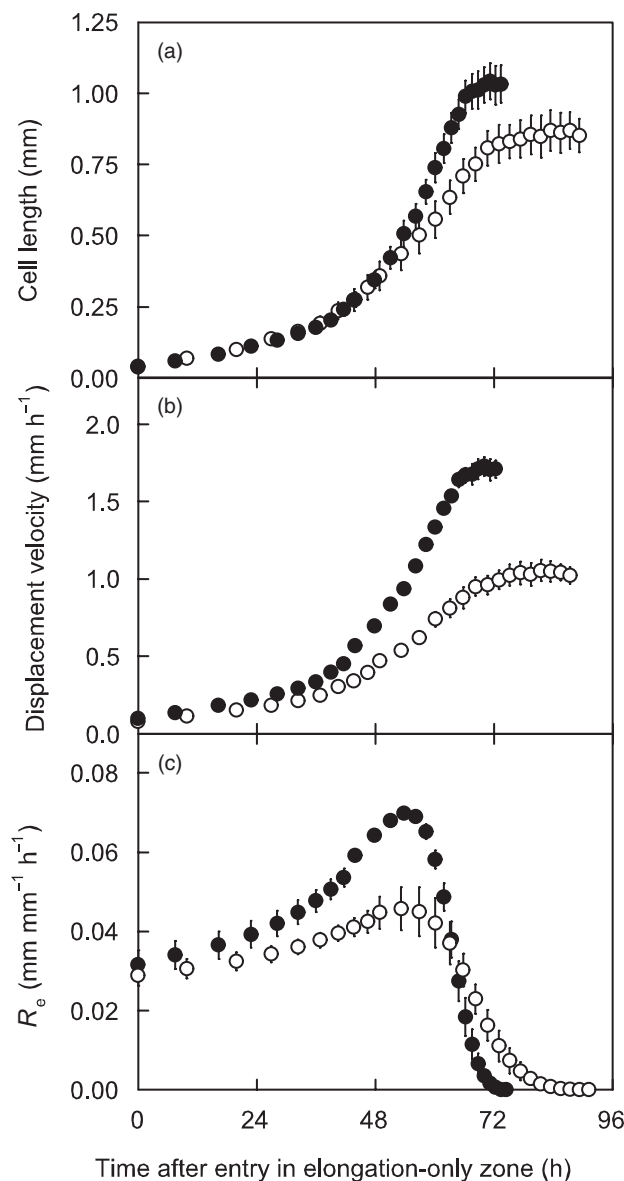


Figure 4. Temporal analysis of the nitrogen supply effect on the cell length (a), displacement velocity (b) and relative elongation rate (R_e ; c) of an epidermal cell from the time it enters the elongation-only zone. *Lolium perenne* plants were grown for 30 d at high (7.5 mm, ●) and 58 d at low (1 mm, ○) nitrogen supply. Data are means of five plants (\pm SE).

turn, was affected by the nitrogen supply. But, remarkably, the coordination between cell growth and division was maintained in nitrogen-deficient cells, as illustrated by the unchanged profile of meristematic cell lengths (Fig. 2). The causal relationship between the reduction in the division rate and mitotic growth rate under nitrogen deficiency still needs to be unravelled. Indeed, how the coordination between cell division and cytoplasmic growth rate is achieved is largely unknown in higher plants, even under optimal conditions (Ingram & Waites 2006). Three different coordination mechanisms have been envisaged (for discussion, see Neufeld & Edgar 1998; Beemster *et al.* 2006;

Kavanová *et al.* 2006b): (1) the rate of mitotic growth may affect the cell cycle duration; conversely, (2) the rate of cell division may limit the rate of mitotic growth; or (3) the rates of cell division and mitotic growth may concertedly be regulated by a common signal. Most of the available evidence supports the first and third scenarios. Direct evidence for the second scenario is missing.

The first scenario proposes that reaching a certain minimum cell size is required for the progression through the cell cycle. The frequency distribution of meristematic cell lengths in plant meristems shows that while average length is rather invariable, daughter cells differ, with a measurable regularity, in length (Ivanov 1971; Korn 1993, 2001). Because the relative elongation rate of adjacent cells does not differ, the cell cycle duration of daughter cells must be adjusted so as to produce similar mitotic cell length (Ivanov 1971). This scenario, in which the cell cycle duration would be rather indirectly determined by parameters controlling cell growth, has an appealing simplicity, and could easily account for the observed similarity in nitrogen and phosphorus effects (cf. this study and Kavanová *et al.* 2006b). Further, it predicts a similar outcome for any other factor that selectively reduces cell growth rate. However, this hypothesis has not been supported by molecular evidence yet. But notably, local induction of expansin expression did lead to the formation of normal leaf primordia, showing that increased expansion can drive cell division (Pien *et al.* 2001).

Although less conclusive, some data support the third scenario. In yeast, cell cycle progression and cell growth rate are coregulated by proteins involved in translation (Jorgensen & Tyers 2004). Similarly, the transcription factor *TCP20* has been proposed to link cell growth and division in higher plants (Li *et al.* 2005). Cytokinin signalling may provide a possible link, because the level and delivery rate of cytokinins in leaves is regulated by nitrogen availability (Yong *et al.* 2000; Rahayu *et al.* 2005; Dodd & Beveridge 2006), and affects both the cell cycle progression (del Pozo *et al.* 2005), and the expression of expansins (Downes, Steinbaker & Crowell 2001). Cytokinin levels respond to both phosphorus and nitrogen availability (Salama & Wareing 1979; Rahayu *et al.* 2005), thus providing a possible explanation of the similitude of nitrogen and phosphorus effects on cell division.

Nitrogen deficiency decreased both the mitotic and post-mitotic growth rates

The reduction in average relative elongation rates in low-nitrogen plants was of similar extent in meristematic cells and in cells situated in the elongation-only zone. Changes in the growth rate are generally analysed in terms of cell wall extensibility, tissue hydraulic conductivity and turgor pressure in excess of the yielding threshold (Fricke 2002). In barley, changes in turgor played only a minor role in leaf growth reduction under nitrogen deficiency (Fricke *et al.* 1997), and cell elongation was not limited by the lack of the osmotically active nitrate (Fricke & Flowers 1998). Nitrogen deficiency may thus either induce changes in cell wall

properties (Reidy, Nösberger & Fleming 2001), or in tissue hydraulic conductivity, or limit cell growth through a low rate of solute supply (Fricke & Flowers 1998). Interestingly, root hydraulic conductivity decreases rapidly in response to both low nitrogen and phosphorus availability, and this decrease is mediated by aquaporins (Carvajal, Cooke & Clarkson 1996), which provides a putative link between the similarity observed under nitrogen and phosphorus deficiency (cf. this study and Kavanová *et al.* 2006b). Clearly, the connection between nutrient deficiency and hydraulic conductivity in the growth zone merits further study, in particular the role of aquaporins highly expressed in meristematic and elongating cells (Chaumont *et al.* 1998).

If little is known about the biophysical controls of post-mitotic cell growth, still less is understood of the biophysical regulation of growth during cell proliferation. Meristematic cells grow by an increase in the volume of the cytoplasm and all organelles, and not by a preferential increase in the vacuolar volume. The similitude in the response of mitotic and post-mitotic growth rates to nitrogen deficiency is thus most intriguing, particularly when their contrasting demands for nitrogen are considered. Nitrogen deposition is to a great extent limited to a short basal zone where cell proliferation of epidermal and mesophyll cells occurs (Gastal & Nelson 1994). Growth (and division) rate of nitrogen-deficient meristematic cells may thus be, at least partly, supply limited by a low deposition rate of organic nitrogen (Gastal & Nelson 1994). Support for this view comes from a remarkable feature of nitrogen metabolism in grass leaf growth zones, namely its extensive recycling. Ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) represents 30–60% of soluble proteins in leaves of C₃ plants (Evans 1989), and is synthesized only in the distal part of the elongation-only zone, that is, where little net nitrogen deposition takes place neither in low- nor high-nitrogen plants (Gastal & Nelson 1994). Rather, Rubisco synthesis seems to rely chiefly on nitrogen released by proteolysis of the cell cycle machinery. Nitrogen recycling may thus underlie the substantial increase (up to the same values as in high-nitrogen cells) in the growth rate of nitrogen-deficient cells at the beginning of the post-mitotic phase (Fig. 3c). Notably, phosphorus would not be subject to such an intensive recycling because it mostly forms part of cellular structures far less recyclable than proteins, such as membrane phospholipids and DNA. Accordingly, the relative elongation rate of low-phosphorus cells remained lower compared with high-phosphorus cells along the whole growth zone (Kavanová *et al.* 2006b).

Nitrogen deficiency did not affect growth zone size, and increased durations of meristematic and growth activity

The sizes of the division and elongation-only zone were not affected by nitrogen deficiency. This reinforces the idea that nutrient status does not control the dimensions of both zones in grass leaves (Kavanová *et al.* 2006a,b). However, it

is also unlikely that the same regulatory mechanisms determine the beginning and end of both cell proliferation and elongation.

The size of the cell division zone is a function of the number and length of meristematic cells. In turn, the number of cells is determined by the number of mitotic cycles the progeny of the initial cell goes through, and cell length reflects the balance between the rates of division and growth. Nitrogen status affected none of these parameters. It is not clear whether this also occurred in other studies showing decreased epidermal cell production rates under nitrogen deficiency (Volenc & Nelson 1983; Gastal & Nelson 1994; Fricke *et al.* 1997; Rademacher & Nelson 2001; Tóth *et al.* 2002), because these did not report the number and size of meristematic cells. Certainly, it did occur in phosphorus-deficient plants (Kavanová *et al.* 2006b), suggesting that meristematic activity – expressed as the number of division cycles – is a constitutive parameter not affected by nutrient deficiency for epidermal cells in leaves of the same developmental stage. It is not clear whether the same holds true for other cell types, such as mesophyll cells (MacAdam, Volenc & Nelson 1989). Furthermore, our data do not support the view that cell's meristematic activity is temporally regulated: its duration increased both under phosphorus and nitrogen deficiency. Yet, the present data substantiate the option of a spatial regulation of proliferation (e.g. by a gradient of some growth regulator; cf. Fleming 2006; Li *et al.* 2005) and/or the possibility that the developmental programme of meristematic cells in leaf epidermis includes a defined number of division cycles.

The size of the leaf elongation-only zone can theoretically be regulated either spatially or temporally. Our data disagree with the view that post-mitotic cell elongation follows a temporal programme. This view was supported by the finding that the size of the elongation-only zone was proportional to the number of cells passing through it (e.g. Volenc & Nelson 1983; Gastal & Nelson 1994; Rademacher & Nelson 2001; Assuero, Mollier & Pellerin 2004), and may be interpreted as the result of a temporal programme of each cell's elongation (Beemster & Baskin 1998). Clearly, this was not the case in leaves of nitrogen- or phosphorus-deficient plants (cf. Kavanová *et al.* 2006b). Nitrogen and phosphorus deficiency decreased the flux of cells through the elongation-only zone, but did not significantly reduce its length, leading thus to a longer duration of post-mitotic elongation. This suggests that the cessation of cell elongation was either not time regulated, or that this control changed under nutrient deficiency. Instead, elongation-only zone length may be controlled spatially by changes in light quality reaching the different portions of the growth zone (see Kavanová *et al.* 2006a for discussion and references).

Other studies on nitrogen effects on leaf growth (Volenc & Nelson 1983; Gastal & Nelson 1994; Rademacher & Nelson 2001; Tóth *et al.* 2002) found differences in the elongation-only zone lengths in plants differing in their nitrogen status, which is in contrast to our data. This might be explained by a species difference. However, because the length of the growth zone closely correlates with tiller size

(Arredondo & Schnyder 2003; Kavanová *et al.* 2006a), the discrepancy might arise from not distinguishing between direct nutrient- and size-related effects in the previous studies.

Several studies have suggested a coordinative mechanism maintaining constant final cell length under nutrient deficiency by extending the duration of cell elongation (Volenc & Nelson 1983; Gastal & Nelson 1994; Rademacher & Nelson 2001; Assuero *et al.* 2004). In contrast to meristematic cells, we found no indication of a full compensation under nitrogen (Table 1) nor under phosphorus deficiency (Kavanová *et al.* 2006b). The reason lies in the effect of nutrient deficiency on the shape of the curve of relative elongation rates. If the relative elongation rate had been decreased in the same proportion along the whole elongation-only zone, then a longer duration would have exactly compensated for the lower average relative elongation rates. But, both nitrogen and phosphorus deficiencies skewed the relative elongation rate profile slightly towards the base, and thus affected proportionally more the relative elongation rate at distal parts. Hence, the compensation was incomplete. Interestingly, over-compensation is theoretically possible if relative elongation rate profiles would be skewed to the distal part, although biophysical reasons make this response unlikely (Fricke 2002).

ACKNOWLEDGMENTS

This research was supported by the Deutsche Forschungsgemeinschaft (SFB 607). We thank Melanie Wild for growing the plants and measuring LER, and Milan Baláz (Department of Plant Physiology and Anatomy, Masaryk University, Brno, Czech Republic) and Stefan Raidl (Department of Systematical Botany and Mycology, Ludwig-Maximilians-Universität München, Germany) for their hospitality and access to microscopes.

REFERENCES

- Arredondo J.T. & Schnyder H. (2003) Components of leaf elongation rate and their relationship to specific leaf area in contrasting grasses. *New Phytologist* **158**, 305–314.
- Assuero S.G., Mollier A. & Pellerin S. (2004) The decrease in growth of phosphorus-deficient maize leaves is related to a lower cell production. *Plant, Cell & Environment* **27**, 887–895.
- Baskin T.I. (2000) On the constancy of cell division rate in the root meristem. *Plant Molecular Biology* **43**, 545–554.
- Beemster G.T.S. & Baskin T.I. (1998) Analysis of cell division and elongation underlying the developmental acceleration of root growth in *Arabidopsis thaliana*. *Plant Physiology* **116**, 1515–1526.
- Beemster G.T.S., Masle J., Williamson R.E. & Farquhar G.D. (1996) Effects of soil resistance to root penetration on leaf expansion in wheat (*Triticum aestivum* L.): kinematic analysis of leaf elongation. *Journal of Experimental Botany* **47**, 1663–1678.
- Beemster G.T.S., Vercauteren S., De Veylder L., Kuiper M. & Inzé D. (2006) The *Arabidopsis* leaf as a model system for investigating the role of cell cycle regulation in organ growth. *Journal of Plant Research* **119**, 43–50.
- Carvajal M., Cooke D.T. & Clarkson D.T. (1996) Responses of

- wheat plants to nutrient deprivation may involve the regulation of water-channel function. *Planta* **199**, 372–381.
- Chaumont F., Barriau F., Herman E.M. & Chrispeels M.J. (1998) Characterization of a maize tonoplast aquaporin expressed in zones of cell division and elongation. *Plant Physiology* **117**, 1143–1152.
- Crawford N.M. & Forde B.G. (2002) Molecular and developmental biology of inorganic nitrogen nutrition. In *The Arabidopsis Book* (eds C.R. Somerville & E.M. Meyerowitz), pp. 1–25. American Society of Plant Biologists, Rockville, MD, USA.
- Dodd I.C. & Beveridge C.A. (2006) Xylem-borne cytokinins: still in search of a role? *Journal of Experimental Botany* **57**, 1–4.
- Downes B.P., Steinbaker C.R. & Crowell D.N. (2001) Expression and processing of a hormonally regulated β -expansin from soybean. *Plant Physiology* **126**, 244–252.
- Evans J.R. (1989) Photosynthesis and nitrogen relationships in leaves of C_3 plants. *Oecologia* **78**, 9–19.
- Fiorani F. & Beemster G.T.S. (2006) Quantitative analyses of cell division in plants. *Plant Molecular Biology* **60**, 963–979.
- Fleming A.J. (2006) The integration of cell proliferation and growth in leaf morphogenesis. *Journal of Plant Research* **119**, 31–36.
- Fournier C., Durand J.L., Ljutovac S., Schäufele R., Gastal F. & Andrieu B. (2005) A functional–structural model of elongation of the grass leaf and its relationships with the phyllochron. *New Phytologist* **166**, 881–894.
- Fricke W. (2002) Biophysical limitation of cell elongation in cereal leaves. *Annals of Botany* **90**, 157–167.
- Fricke W. & Flowers T.J. (1998) Control of leaf cell elongation in barley. Generation rates of osmotic pressure and turgor, and growth-associated water potential gradients. *Planta* **206**, 53–65.
- Fricke W., McDonald A.J.S. & Mattson-Djos L. (1997) Why do leaves and leaf cells of N-limited barley elongate at reduced rates? *Planta* **202**, 522–530.
- Gastal F. & Nelson C.J. (1994) Nitrogen use within the growing leaf blade of tall fescue. *Plant Physiology* **105**, 191–197.
- González-Fernández A., López-Sáez J.F., Moreno P. & Giménez-Martin G. (1968) A model for dynamics of cell division cycle in onion roots. *Protoplasma* **65**, 263–276.
- Green P.B. (1976) Growth and cell pattern formation on an axis: critique of concepts, terminology, and modes of study. *Botanical Gazette* **137**, 187–202.
- Ingram G.C. & Waites R. (2006) Keeping it together: co-ordinating plant growth. *Current Opinion in Plant Biology* **9**, 12–20.
- Ivanov V.B. (1971) Critical size of the cell and its transition to division. *The Soviet Journal of Developmental Biology* **2**, 421–428.
- Ivanov V.B. & Dubrovsky J.G. (1997) Estimation of the cell-cycle duration in the root apical meristem: a model of linkage between cell-cycle duration, rate of cell production, and rate of root growth. *International Journal of Plant Sciences* **158**, 757–763.
- Ivanov V.B., Dobrochaev A.E. & Baskin T.I. (2002) What the distribution of cell lengths in the root meristem does and does not reveal about cell division. *Journal of Plant Growth Regulation* **21**, 60–67.
- Jorgensen P. & Tyers M. (2004) How cells coordinate growth and division. *Current Biology* **14**, R1014–R1027.
- Kavanová M., Grimoldi A.A., Lattanzi F.A. & Schnyder H. (2006a) Phosphorus nutrition and mycorrhiza effects on grass leaf growth. P status- and size-mediated effects on growth zone kinematics. *Plant, Cell & Environment* **29**, 511–520.
- Kavanová M., Lattanzi F.A., Grimoldi A.A. & Schnyder H. (2006b) Phosphorus deficiency decreases cell division and elongation in grass leaves. *Plant Physiology* **141**, 766–775.
- Kemp D.R. (1980) The location and size of the extension zone of emerging wheat leaves. *New Phytologist* **84**, 729–737.
- Korn R.W. (1993) The geometry of elongating plant cells. *Bulletin of Mathematical Biology* **55**, 345–364.
- Korn R.W. (2001) The geometry of proliferating dicot cells. *Cell Proliferation* **34**, 43–54.
- Lawlor D.W., Lemaire G. & Gastal F. (2001) Nitrogen, plant growth and crop yield. In *Plant Nitrogen* (eds P.J. Lea & J.F. Morot-Gaudry), pp. 343–367. Springer Verlag, Berlin, Germany.
- Li C.X., Potuschak T., Colón-Carmona A., Gutiérrez R.A. & Doerner P. (2005) *Arabidopsis* TCP20 links regulation of growth and cell division control pathways. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 12978–12983.
- Longnecker N. (1994) Nutrient deficiencies and vegetative growth. In *Mechanisms of Plant Growth and Improved Productivity: Modern Approaches* (ed. A.S. Basra), pp. 137–172. Marcel Dekker, New York, NY, USA.
- MacAdam J.W., Volenec J.J. & Nelson C.J. (1989) Effects of nitrogen on mesophyll cell division and epidermal cell elongation in tall fescue leaf blades. *Plant Physiology* **89**, 549–556.
- Marschner H. (1995) *Mineral Nutrition of Higher Plants*. Academic Press, London, UK.
- Morris A.K. & Silk W.K. (1992) Use of a flexible logistic function to describe axial growth of plants. *Bulletin of Mathematical Biology* **54**, 1069–1081.
- Neufeld T.P. & Edgar B.A. (1998) Connections between growth and the cell cycle. *Current Opinion in Cell Biology* **10**, 784–790.
- Pien S., Wyrzykowska J., McQueen-Mason S., Smart C. & Fleming A. (2001) Local expression of expansin induces the entire process of leaf development and modifies leaf shape. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 11812–11817.
- del Pozo J.C., Lopez-Matas M.A., Ramirez-Parra E. & Gutierrez C. (2005) Hormonal control of the plant cell cycle. *Physiologia Plantarum* **123**, 173–183.
- Rademacher I.F. & Nelson C.J. (2001) Nitrogen effects on leaf anatomy within the intercalary meristems of tall fescue leaf blades. *Annals of Botany* **88**, 893–903.
- Rahayu Y.S., Walch-Liu P., Neumann G., Römheld V., von Wirén N. & Bangerth F. (2005) Root-derived cytokinins as long-distance signals for NO_3^- -induced stimulation of leaf growth. *Journal of Experimental Botany* **56**, 1143–1152.
- Reidy B., Nösberger J. & Fleming A. (2001) Differential expression of XET-related genes in the leaf elongation zone of *F. pratensis*. *Journal of Experimental Botany* **52**, 1847–1856.
- Roggatz U., McDonald A.J.S., Stadenberg I. & Schurr U. (1999) Effects of nitrogen deprivation on cell division and expansion in leaves of *Ricinus communis* L. *Plant, Cell & Environment* **22**, 81–89.
- Salama A.M.S.E. & Wareing P.F. (1979) Effects of mineral nutrition on endogenous cytokinins in plants of sunflower (*Helianthus annuus* L.). *Journal of Experimental Botany* **30**, 971–981.
- Scheible W.R., Morcuende R., Czechowski T., Fritz C., Osuna D., Palacios-Rojas N., Schindelasch D., Thimm O., Udvardi M.K. & Stitt M. (2004) Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of *Arabidopsis* in response to nitrogen. *Plant Physiology* **136**, 2483–2499.
- Schnyder H. & Nelson C.J. (1988) Diurnal growth of tall fescue leaf blades. I. Spatial distribution of growth, deposition of water, and assimilate import in the elongation zone. *Plant Physiology* **86**, 1070–1076.
- Schnyder H., Nelson C.J. & Coutts J.H. (1987) Assessment of spatial distribution of growth in the elongation zone of grass leaf blades. *Plant Physiology* **85**, 290–293.
- Schnyder H., Seo S., Rademacher I.F. & Kühbauch W. (1990) Spatial distribution of growth rates and of epidermal cell lengths

- in the elongation zone during leaf development in *Lolium perenne* L. *Planta* **181**, 423–431.
- Silk W.K. (1992) Steady form from changing cells. *International Journal of Plant Sciences* **153**, S49–S58.
- Silk W.K. & Erickson R.O. (1979) Kinematics of plant growth. *Journal of Theoretical Biology* **76**, 481–501.
- Silk W.K., Lord E.M. & Eckard K.J. (1989) Growth patterns inferred from anatomical records – empirical tests using long-sections of roots of *Zea mays* L. *Plant Physiology* **90**, 708–713.
- Stitt M. (1999) Nitrate regulation of metabolism and growth. *Current Opinion in Plant Biology* **2**, 178–186.
- Tóth V.R., Mészáros I., Palmer S.J., Veres S. & Précseányi I. (2002) Nitrogen deprivation induces changes in the leaf elongation zone of maize seedlings. *Biologia Plantarum* **45**, 241–247.
- Volenc J.J. & Nelson C.J. (1983) Responses of tall fescue leaf meristems to N fertilization and harvest frequency. *Crop Science* **23**, 720–724.
- Wang R.C., Okamoto M., Xing X.J. & Crawford N.M. (2003) Microarray analysis of the nitrate response in *Arabidopsis* roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. *Plant Physiology* **132**, 556–567.
- Yong J.W., Wong S.C., Letham D.S., Hocart C.H. & Farquhar G.D. (2000) Effects of elevated [CO₂] and nitrogen nutrition on cytokinins in the xylem sap and leaves of cotton. *Plant Physiology* **124**, 767–780.

Received 6 August 2007; received in revised form 5 January 2008; accepted for publication 8 January 2008