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Leaf growth regulation in *Lolium perenne* under nutrient stress

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ABSTRACT

Aims: Plants respond to unfavourable nutritional conditions by reducing their growth rate. As yet, the cellular processes underlying the reduction in leaf growth under phosphorus and nitrogen deficiency are not fully understood. The studies summarised here aimed to (i) assess the contribution of nutrient status- and size- related factors at determining the rate of leaf growth, and (ii) analyse the effects of nitrogen and phosphorus status on cell division and cell growth in expanding leaves.

Materials & Methods: A kinematic analysis of leaf elongation was conducted on the grass *Lolium perenne* growing under a range of nitrogen and phosphorus availabilities. This was done by supplying different concentrations of nitrate and phosphate in the nutrient solution and, independently, inoculating plants with the arbuscular mycorrhizal fungus *Glomus hoi* in the case of phosphorus. First, the role of size-related nutrient treatment effects on the leaf growth rate was determined. Next, the relationship between the phosphorus status of the growth zone, and leaf elongation rate and its spatial components was analysed. Second, using even-sized tillers to ensure that only direct nutrient status (and not size-related factors) were evaluated, parameters of cell division and cell growth underlying the reduction of leaf growth were determined in phosphorus and nitrogen deficient plants.

Results & Discussion: The leaf elongation rate of plants growing at different levels of soluble phosphorus supply or in symbiosis with the arbuscular mycorrhizal fungus was correlated with the phosphorus status of the leaf growth zone. The spatial analysis revealed that the average relative elemental growth rate was strictly related to the phosphorus status of the growth zone. Conversely, the length of the growth zone was independent of the phosphorus status, and strongly correlated with the length of the enclosing sheath tube (*i.e.* a function of tiller size). The cellular analysis revealed that under nitrogen and phosphorus deficiency, the reduction in leaf growth arose from a reduction in the rates of cell division and mitotic elongation (decreasing thus the cell flux out of the division zone) and postmitotic cell elongation (decreasing thus the final cell length). Nutrient deficiency did not affect the positions where cell division and postmitotic elongation stopped. The slower division and elongation rates led to a longer cell residence time in both the division and elongation-only zone, suggesting that cell proliferation and elongation were not temporally controlled. The coordination between cell division and cell growth rate was maintained under nutrient stress, as illustrated by the constant meristematic cell length. Notably, the number of meristematic cells was also not affected by nutrient deficiency, implying that the maintenance of proliferative activity was irresponsive to nutrient status.

Conclusions: The studies reported here identified the cellular and kinematic parameters controlling the response of leaf elongation rate to nutritional signals, and as importantly, the parameters that did not respond. This constitutes a step towards the elucidation of the molecular regulation of the cellular processes of leaf growth, integrating both developmental and environmental signals.

ZUSAMMENFASSUNG

Zielsetzung: Pflanzen reagieren auf Nährstoffmangel mit der Reduktion des Wachstums. Es ist bisher nur unvollständig bekannt, welche zellulären Prozesse des Blattwachstums von Phosphor- und Stickstoffmangel betroffen werden. Die vorliegenden Untersuchungen verfolgten das Ziel, (*i*) die direkten (d.h. nährstoffabhängigen) und indirekten (d.h. größenabhängigen) Wirkungen des Nährstoffmangels auf das Blattwachstum zu charakterisieren, und (*ii*) die Effekte des Stickstoff- und Phosphorstatus auf Zellteilung und Zellstreckung in wachsenden Blättern zu quantifizieren.

Material und Methoden: Deutsches Weidelgras (*Lolium perenne*) wurde unter kontrollierten Bedingungen mit Nährlösungen unterschiedlicher Nitrat- und Phosphorkonzentration angezogen. Zusätzlich wurde die Phosphorernährung durch Inokulation von Pflanzen mit dem arbuskulären Mykorrhizapilz *Glomus hoi* modifiziert. An Pflanzen gleichen Alters und Pflanzen gleicher Größe wurden sodann die direkten und indirekten Wirkungen der Nährstoffversorgung auf das Blattwachstum ermittelt. Es folgten Untersuchungen zur Beziehung zwischen dem Phosphorstatus der Blattwachstumszone und der Blattwachstumsgeschwindigkeit sowie ihrer räumlichen Komponenten. Schließlich wurde an Trieben gleicher Größe die Wirkungen des Phosphor- und Stickstoffstatus auf die Parameter der Zellteilung und Zellstreckung untersucht.

Ergebnisse und Diskussion: Die Anzucht bei unterschiedlicher Phosphorkonzentration in der Nährlösung und die Symbiose mit dem Mykorrhizapilz *G. hoi* beeinflusste den Phosphorstatus der Blattwachstumszonen und die Wachstumsgeschwindigkeit der Blätter. Die Untersuchung der räumlichen Verteilung des Wachstums in der Blattwachstumszone zeigte eine direkte Wirkung des Phosphorstatus auf die relative Streckungsrate. Dagegen war die Länge der Blattwachstumszone unabhängig vom Phosphorstatus. Diese wurde direkt von der Länge der umhüllenden Blattscheide bzw. von der Triebgröße bestimmt. Die Verlangsamung des Blattwachstums durch Stickstoff- und Phosphormangel beruhte auf der Minderung der Zellteilungsrate und der mitotischen Streckungsrate (womit der Zellfluss aus der Zellteilungszone herabgesetzt wurde) sowie einer Reduktion der post-mitotischen Zellstreckungsgeschwindigkeit (welche eine kürzere Endlänge der Zellen nach sich zog). Nährstoffmangel hatte keine direkte Auswirkung auf die Länge der Zellteilungszone und der Zellstreckungszone. Verlangsamte Zellteilung und -streckung führte zu verlängerter Verweilzeit in der Zellteilungs und -streckungszone. Die Zellteilung und -streckung unterliegt offenbar keiner zeitlichen Kontrolle. Die Koordination von Zellteilung und mitotischer -streckung wurde durch Nährstoffmangel nicht verändert, sodass die Länge der Zellen in der Zellteilungszone vom Nährstoffmangel unbeeinflusst blieb. Der Nährstoffstatus hatte keinen Einfluss auf die Zahl meristematischer Zellen und die Aufrechterhaltung der meristematischen Aktivität.

Schlussfolgerungen: Mit den vorliegenden Untersuchungen konnten die Stellgrößen identifiziert werden, welche die Reduktion des Blattwachstums bei Nährstoffmangel kontrollieren. Gleichzeitig

wurden die Wachstumsparameter identifiziert, welche von Nährstoffmangel unbeeinflusst sind. Diese Erkenntnisse liefern eine wichtige Grundlage für die weitere Aufklärung der molekularen Regulation der zellulären Prozesse des Blattwachstums und ihrer Kontrolle durch entwicklungs- und umweltabhängige Signale.

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Chapter I. GENERAL INTRODUCTION

STATE OF THE ART

Leaf growth and development in grasses

The grass leaf represents a suitable model for unravelling the cellular processes of leaf growth, because cell proliferation and elongation are separated spatially along the longitudinal axis, but not temporally. Thus, in contrast to dicots (Donnelly *et al.* 1999), these processes can be analysed simultaneously on the same leaf. Leaves are initiated at the flanks of the shoot apical meristem. The development of a grass leaf begins with the appearance of a small bulge at the flank of the apical meristem that develops into a leaf primordium encircling the apex (Sharman 1942). A procambial strand running into the primordium marks the site of the future midrib. The plastochron (the time interval between the initiation of successive leaf primordia) generally ranges from three to nine days (Nelson 1996). Leaf primordia grow during the initial phase by cell proliferation, resulting in a relatively slow elongation of the primordium (Skinner & Nelson 1994). Shoot apical meristems of cool-season grasses usually have three or more leaf primordia shorter than 1 mm. After approximately four plastochrons, the oldest primordium assumes rapid growth (Nelson 1996). In this stage, the primordium, consisting of unexpanded cells, develops an intercalary meristem giving rise to the leaf blade. The ligule appears as a group of thick-walled cells that do not elongate, and separates meristematic cells of the growing blade from the cells of the primordium that will eventually form the sheath (Nelson 1996). A pattern of zones of cell division, elongation and maturation is established, and the leaf is expanding on a steady rate during several days (Fournier *et al.* 2005). During this phase of leaf growth, a clearly defined spatial pattern of cell development exists along the longitudinal axis, generating a basal *division zone*, where meristematic cells elongate and divide, and an *elongation-only* zone where cells undergo postmitotic elongation. Together the two zones constitute the *leaf growth zone* (Davidson & Milthorpe 1966; Kemp 1980b). Later, a transition between the activity of the blade to sheath division zone occurs. As a result, the growth and development finishes first in the blade and later in the sheath (Schnyder *et al.* 1990). The studies summarized in this thesis concentrate on the growth of leaf blades.

Kinematic methods used to analyse leaf growth components

Two different approaches to analyse leaf elongation rate and its components have been used in this thesis. First, I used a spatial or Eulerian description of growth, which is based on the growth activity at given distances along the growth zone (*cf.* Fig. II.1; Erickson & Sax 1956; Silk & Erickson 1979; Silk 1984). Relative elemental growth rate (*REGR*, mm mm^{-1} h^{-1}) is a measure of the magnitude of tissue elongation per unit existing tissue at a certain distance from the leaf base. The location where *REGR* decreases to zero marks the distal end of the leaf growth zone (mm). Leaf elongation rate (mm h^{-1}) is then the definite integral of *REGR* from the beginning to the end of the leaf growth zone.

Second, I used a material or Lagrangian description of growth, which analyses material elements to infer organ development from its elements' development (*cf*. Fig. III.1; Silk & Erickson 1979; Gandar 1983). This approach is particularly useful during the steady-state growth period, when neither the dimensions of the different zones nor the cell numbers change in time. Kinematic analysis then provides the analytical tools to derive from the spatial profiles of cell length and displacement velocity, rates and durations of cell division and elongation (Green 1976; Silk & Erickson 1979; Silk 1992; Ivanov & Dubrovsky 1997). Leaf elongation rate (mm h^{-1}), the flux of leaf tissue out of the growth zone, can be analysed in terms of changes in cell production rate (cell h^{-1}) and final cell length (mm $cell^{-1}$; Volenec & Nelson 1981). The number of meristematic cells (related to the average number of division cycles of the progeny of the initial cell at the meristem base) and their division rate (cell $cell^{-1}$ h^{-1}) determine the flow of cells out of the division zone, i.e. the cell production rate. On the other hand, the length of cells leaving the division zone (which depends on the balance between cell division rate and relative growth rate of meristematic cells; mm $cell^{-1}$), their relative postmitotic elongation rate (mm mm^{-1} h^{-1}) and the duration of elongation (h) determine the final cell length.

Effects of nitrogen and phosphorus deficiency on plant and leaf growth

Nitrogen and phosphorus are indispensable building blocks of many types of biomolecules, including nucleic acids, ATP and membrane phospholipids (phosphorus), and amino acids, proteins, nucleic acids and many secondary metabolites (nitrogen). Therefore, plants require large amounts of these two macronutrients for their growth and development (Marschner 1995). This section will briefly discuss the ways how phosphorus and nitrogen availability affects plant and leaf growth.

Initially, shoot growth is reduced to a greater extent than root growth under nitrogen or phosphorus deficiency, but usually both shoot and root growth rates are eventually decreased (Marschner 1995; Plaxton & Carswell 1999). Little information exists on the effects of nitrogen or phosphorus deficiency on the functioning and nutritional status of the shoot apical meristems. In soybean, the rate of leaf primordia initiation is decreased under phosphorus deficiency. However, the structural integrity of the shoot apical meristem is not affected (Chiera, Thomas & Rufty 2002).

Nitrogen and phosphorus deficiency have been reported to cause a substantial decrease in the mature leaf length and leaf area due to a decreased rate of leaf elongation rather than to a decrease in the duration of leaf expansion (Longnecker 1994, and references therein). Leaf growth reduction under phosphorus (Radin & Eidenbock 1984; Chiera *et al.* 2002; Assuero, Mollier & Pellerin 2004; Kavanová *et al.* 2006a) and nitrogen (Volenec & Nelson 1983; Thomas 1983; Gastal & Nelson 1994; Fricke, McDonald & Mattson-Djos 1997) deficiency is well documented. Ultimately, this growth reduction must be due to an alteration of cell division or cell elongation parameters.

The contribution of the different cellular parameters to leaf growth reduction under nutrient stress is, however, not well understood. Few studies assessed the effects of phosphorus and nitrogen deficiency on the cellular parameters of leaf growth, and gave diverse results. In cotton, Radin & Eidenbock

(1984) concluded that reduced cell expansion was the cause of the reduced leaf size under phosphorus deficiency, whereas in soybean Chiera *et al.* (2002) concluded that reduced cell division was the major factor. Although this disagreement might be related to a differences between species, it may also arise from the fact that neither of the studies directly measured these parameters. Instead, the role of cell expansion was inferred from smaller leaf cells, and the role of cell division from a reduced cell number. In maize, Assuero *et al.* (2004) attributed the reduction in leaf growth under phosphorus stress to a decreased cell production rate. Similarly, several studies ascribed the decrease in grass leaf elongation rate under nitrogen deficiency to a lower cell production (Volenec & Nelson 1983; Gastal & Nelson 1994; Fricke *et al.* 1997), with a minor or no effect on the final cell length, although the postmitotic relative elongation rate was reduced. In the dicot castor bean, the reduction in the final leaf size was associated with a reduction in the final cell size and in the number of produced cells, depending on the developmental stage of the leaf at the time of nitrogen deprivation (Roggatz *et al.* 1999). None of the studies reviewed above, however, analysed all parameters that would provide a full picture of the cytological phenomena of leaf growth under nutrient deficiency. Before a step toward an elucidation of the molecular regulation of the cellular processes under nutrient stress can be undertaken, it is essential to understand which of the many processes are regulated in response to changes in the nutrient status. Particularly, it is necessary to evaluate which of the parameters determining the number of produced cells and their final length are responsive to changes in nutrient status. This includes (*i*) the number and size of meristematic cells together with their division and elongation rate, and (*ii*) the duration and rate of postmitotic cell elongation.

AIMS AND OUTLINE OF THE THESIS

The main aim of this thesis was to provide a comprehensive kinematic analysis of the cellular processes underlying the reduction of leaf elongation rate under phosphorus and nitrogen deficiency in perennial ryegrass (*Lolium perenne* L.). The second objective was to assess the roles of the nutritional status and size-related effects in determining the leaf growth rate.

Chapter II explores the relationship between the phosphorus status of the growth zone, and leaf elongation rate and its spatial components. The contribution of the nutritional and size-dependent treatment effects to changes in leaf growth rate is evaluated. Finally, the effect of the symbiosis with arbuscular mycorrhizal fungus *Glomus hoi* on the leaf elongation rate is assessed.

Chapter III determines which parameters of cell division and cell growth are underlying the reduction of leaf elongation rate in phosphorus deficient plants.

Chapter IV analyses the cellular parameters underlying the reduction of leaf elongation rate in nitrogen deficient plants.

Chapter V comprises a general discussion of the major findings.

Chapter II. PHOSPHORUS NUTRITION AND MYCORRHIZA EFFECTS ON GRASS LEAF GROWTH. P STATUS- AND SIZE-MEDIATED EFFECTS ON GROWTH ZONE KINEMATICS [1]

ABSTRACT

This study tested whether leaf elongation rate (*LER*, mm h^{-1}) and its components: average relative elemental growth rate ($REGR_{avg}$, mm mm^{-1} h^{-1}) and leaf growth zone length (L_{LGZ}, mm) are related to phosphorus concentration in the growth zone (P_{LGZ}, mg P g^{-1} tissue water) of *Lolium perenne* L., and whether such relationships are modified by the arbuscular mycorrhizal fungus (AMF) *Glomus hoi*. Mycorrhizal and non-mycorrhizal plants were grown at a range of phosphors supply rates and analysed at either the same plant age or the same tiller size (defined by the length of the sheath of the youngest fully expanded leaf). Both improved phosphorus supply (up to 95%) and AMF (up to 21%) strongly increased *LER*. In tillers of even-aged plants, this was due to increased $REGR_{avg}$ and L_{LGZ}. In even-sized tillers, it was exclusively due to increased $REGR_{avg}$. $REGR_{avg}$ was strictly related to P_{LGZ} (r^2=0.95), and independent of tiller size. Conversely, L_{LGZ} strictly depended on tiller size (r^2=0.88), and not on P_{LGZ}. Hence, phosphorus status affected leaf growth directly only through effects on relative tissue expansion rates. Symbiosis with AMF did not modify these relationships. Thus, no evidence for phosphorus status-independent effects of AMF on *LER* was found.

[1] Kavanová M., Grimoldi A.A., Lattanzi F.A. & Schnyder H. (2006) *Plant, Cell & Environment* **29,** 511-520.

INTRODUCTION

Phosphorus is an essential macronutrient required for plant growth and development, but plants have to cope with limiting soil phosphorus availability in many terrestrial ecosystems (Schachtman, Reid & Ayling 1998). Low phosphorus availability activates a series of responses that maximize phosphorus acquisition or are directed to maintain internal phosphorus homeostasis of the plant (Raghothama 1999; Ticconi & Abel 2004). An early response to phosphorus deficiency is also the reduction of the leaf growth rate, usually more pronounced than the reduction of root growth rate (Plaxton & Carswell 1999).

A widespread adaptive evolutionary response to low soil phosphorus availability is the symbiosis with arbuscular mycorrhizal fungi (AMF), whose beneficial effects are usually manifest as improved phosphorus uptake and increased plant growth rate (Smith & Read 1997). As yet, the physiological bases of the AMF effect on plant growth are not fully understood. A still unsolved question is whether AMF affect the leaf growth directly by improving the phosphorus status of leaf meristems and growing leaves, or if other (additional or alternative) factors are involved.

Grasses offer a convenient system for studying the effects of phosphorus nutrition and AMF on the leaf growth processes. This is because the complexity of two-dimensional leaf area expansion rate of dicotyledons is reduced to predominantly one-dimensional elongation. In grasses, leaf growth is confined to a short region which is located at its base, close to the point of attachment of the leaf to the stem base (Davidson & Milthorpe 1966; Kemp 1980b). This region was named 'leaf growth zone' and includes a short meristematic region that continuously produces cells that enter into the subsequent zone of cell expansion (Fig. II.1).

Nutrient deficiencies result in decreased grass tiller size, since *LER* and final leaf length are generally correlated (Fournier *et al.* 2005 and references therein). Such a treatment-related change in tiller size might subsequently affect plant growth independently of the nutrient status (Niklas 1994). It is presently unknown if the decrease in *LER* under phosphorus deficiency is related exclusively to a decreased plant phosphorus status, or whether it is also partly related to effects mediated by the reduced tiller size. Interestingly, an allometric relationship between the length of the sheath enclosing the growing leaf and one of the *LER* components, L_{LGZ}, has been observed in a study of successive leaves in wheat (Kemp 1980a) and in eight grasses compared at different sheath lengths (Arredondo & Schnyder 2003). Further, Casey *et al.* (1999) showed that artificial shortening of sheaths reduced *LER* by decreasing L_{LGZ}. Usually, studies of the effects of environmental or nutritional factors on leaf growth have not distinguished direct from size-dependent treatment effects. However, such a distinction is essential for understanding the mechanisms controlling leaf growth.

The aims of this study were to (*i*) assess the effects of phosphorus supply on *LER* and its components, $REGR_{avg}$ and L_{LGZ}, (*ii*) verify if these parameters are related to growth zone phosphorus status, and (*iii*) investigate if these relationships are modified by AMF. To this end, *LER*, and its components were determined in perennial ryegrass (*Lolium perenne* L.) grown over a wide range of phosphorus

supplies, with and without AMF (*Glomus hoi*). In order to account for both phosphorus status- and tiller size-mediated effects, comparisons were made between responses of even-aged plants differing in tiller size, and plants with even-sized tillers.

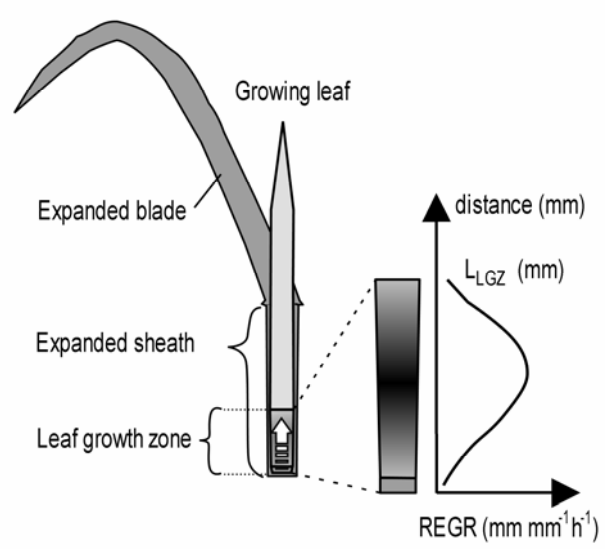

Figure II.1. Schematic representation of the leaf growth zone in a grass tiller. Growth is limited to the basal part of the growing leaf -leaf growth zone- surrounded by the enclosing sheaths of expanded leaves. Cells are produced by an intercalary meristem and then enter the zone of cell elongation. Relative elemental growth rate (*REGR*, mm mm^{-1} h^{-1}) is a measure of the magnitude of tissue expansion per unit existing tissue. The location where *REGR* decreases to zero marks the end of leaf growth zone (L_{LGZ}, mm). Leaf elongation rate (*LER*, mm h^{-1}) is the definite integral of *REGR* from the beginning of elongation zone to L_{LGZ}, or more simply, the product of L_{LGZ} times average *REGR* ($REGR_{avg}$).

MATERIALS AND METHODS

Plant culture, AMF inoculation and growth conditions

Surface sterilized seeds of perennial ryegrass (*Lolium perenne* L. cv. Condesa) were sown in plastic pots (diameter 5 cm, height 35 cm) on a mixture of quartz sand supplemented with fine powdered Hyperphos (63 mg P per pot), providing a source of phosphorus with low availability for the plants. Each pot contained one plant. Half of the pots were inoculated with AM fungus *Glomus hoi* (15 mL inoculum per pot). The inoculum consisted of a mixture of sand and roots originating from single spore pot culture of G*lomus hoi* BEG104 propagated on *Plantago lanceolata* L. Pots with and without inoculation were placed in separate containers (76×56×37 cm) in growth chambers. Two independent experiments were conducted.

Experiment 1: comparison at even age

The aim of this experiment was to analyse the response of *LER* and its components to phosphorus supply and AMF in even-aged tillers. Thus, plants were grown on different levels of soluble

phosphorus supply for the same time period (61 to 63 days after sowing, DAS). Growth room (VKZPH 005-120-S, Heraeus Vötsch, Balingen, Germany) conditions were 20/15 °C (day/night), 70% relative air humidity, and 425 µmol m^{-2} s^{-1} photosynthetically active photon flux density (PPFD) at plant height for 16 h day^{-1}. In order to promote AMF colonization, all plants were initially (first 34 DAS) irrigated four times a day with 25 mL of modified P-free half-strength Hoagland's solution (2.5 mM KNO_3, 2.5 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM KCl, 0.5 mM NaCl, 0.125 mM Fe-EDTA, 23 µM H_3BO_3, 4.5 µM $MnSO_4$, 0.38 µM $ZnSO_4$, 0.16 µM $CuSO_4$ and 0.05 µM Na_2MoO_4). Thereafter and till the end of experiment, four concentrations of soluble phosphorus (0, 0.02, 0.1 and 0.5 mM) in the form of KH_2PO_4 were supplied to both mycorrhizal and non-mycorrhizal plants.

Experiment 2: comparison at even size

The aim of this experiment was to analyse the response of *LER* and its components to phosphorus supply and AMF of plants with even-sized tillers, *i.e.* with similar sheath length. To this end, seeds for low phosphorus plants were germinated 14 days in advance of seeds for high phosphorus plants. Measurements were performed at 60 to 61 DAS in low phosphorus plants, and at 46 to 47 DAS in high phosphorus plants, when plants in the different treatments had mature tillers of similar size (sheath length of the youngest expanded leaf: 100±11 mm). Plants were grown in four growth chambers (E15, Conviron, Winnipeg, Canada), with 20/15 °C (day/night), 70 % relative air humidity and 525 µmol m^{-2} s^{-1} PPFD at plant height for 16 h day^{-1}. All plants were first irrigated for 21 DAS with the nutrient solution as described above, except that 0.02 mM KH_2PO_4 was included. Thereafter, two levels of soluble phosphorus supply were applied in both the mycorrhizal and non-mycorrhizal plants: 0.02 mM (low phosphorus) and 1 mM (high phosphorus).

AMF colonization

The root colonization by AMF was determined at 68 DAS in experiment 1, and at 63 DAS (low phosphorus treatments) and 49 DAS (high phosphorus treatments) in experiment 2. Colonization was estimated on Trypan Blue stained roots by the gridline intersect method (McGonigle *et al.* 1990). The root length colonized is represented by the percentage of total intercepts where hyphae were present.

Leaf elongation rate

Representative mature tillers (i.e. tillers having at least two fully expanded leaves) were selected for measurement of *LER* and leaf growth zone properties (see below). Leaf elongation rate (*LER*, mm h^{-1}) was measured on the most rapidly growing leaf on a tiller. The measured leaf was the youngest visible leaf during the phase of its maximal expansion when *LER* was near constant (Schnyder *et al.* 1990). *LER* was determined as the rate of change of the distance between the tip of the elongating blade and the ligule of the youngest fully expanded leaf, which was measured daily with a ruler. The length of the sheath of the youngest expanded leaf was recorded at every measurement. In experiment 1, *LER*

was measured on four plants per treatment at 61, 62 and 63 DAS. In experiment 2, five plants per treatment were measured at 60 and 61 DAS in the low phosphorus treatments, and at 46 and 47 DAS in the high phosphorus treatments. No difference in *LER* was observed between sampling dates, or growth chambers (P>0.1). Thus, data from different sampling times and chambers were pooled.

Components of leaf elongation rate: L_{LGZ} *and* REGR

LER components were estimated by determining the spatial distribution of growth within the leaf growth zone of mature tillers immediately after *LER* measurements on the same measured leaves using a pin-pricking method (Schnyder, Nelson & Coutts 1987). Briefly, two hours after the start of the light period, a series of holes 3 mm apart along the basal 40 to 60 mm of a tiller was pinned with a fine needle. Plants were returned to the growth chamber for 4 to 6 h. Thereafter, distances between the holes, both along the base of the growing leaf and along the non-growing surrounding sheath, were measured with 0.1 mm accuracy. Leaves with the ligule located further than 2 mm from the point of attachment were discarded, which assured only blade elongation was assessed.

Segmental elongation rate (SER_i, mm h^{-1}) was then calculated as:

$$SER_i = \frac{L_{i,t1} - L_{i,t0}}{\Delta t} \times \frac{LER_{control}}{LER_{pierced}}, \qquad \text{(Eq. II.1)}$$

where $L_{i,\,t1}$ is the length of a segment delimited by two neighbouring holes in the growing blade (measured Δt, h, after pinning), and $L_{i,\,t0}$ is the length of the corresponding segment measured in the surrounding non-growing leaf sheath. SER_i was corrected by the ratio between *LER* of a non-pierced leaf ($LER_{control}$, measured on the same leaf before pinning) and *LER* of the pierced leaf ($LER_{pierced}$, determined as the sum of all SER_i along the leaf), to account for effects of pinning on LER. It has repeatedly been shown that growth reductions caused by pinning do not modify the relative distribution of growth rates and the length of the leaf growth zone (Schnyder *et al.* 1987; Schnyder *et al.* 1990; Hu & Schmidhalter 2000), validating its use for the assessment of the spatial distribution of growth rates (*e.g.* Ben-Haj-Salah & Tardieu 1995; Fricke & Peters 2002; Assuero *et al.* 2004).

Velocity of displacement (V_i, mm h^{-1}) of a given segment i was calculated as the sum of elongation rates of all segments located more basally. The Richards function was fitted to each V_i profile (all fittings $r^2 \geq 0.99$; TableCurve 2D, v.5.01, Systat, USA):

$$V_i = a \frac{1}{(1 + \exp^{(b-c\,x)})^{\frac{1}{d}}}, \qquad \text{(Eq. II.2)}$$

where x is the distance from the leaf base, a is the asymptotic maximal V_i, and b, c and d are constants. Relative elemental growth rate (*REGR*, mm mm^{-1} h^{-1}) was estimated as the first derivative of the fitted Richards function at the midpoint of each 3 mm-long segment:

$$REGR = \frac{a\,c \exp^{(c\frac{x}{d}+b)} (\exp^{(c\,x)} + \exp^{b})^{-\frac{d+1}{d}}}{d}. \qquad \text{(Eq. II.3)}$$

The length of the leaf growth zone (L_{LGZ}, mm) was defined as the distance from the leaf base to the midpoint of the last segment where SER_i was positive. The average relative elemental growth rate ($REGR_{avg}$) was then determined as:

$$REGR_{avg} = \frac{LER_{control}}{L_{LGZ}} \quad . \qquad \text{(Eq. II.4)}$$

L_{LGZ} was alternatively calculated as the position where 95% of the a value (the asymptotic V_i predicted by the fitted Richards function) was reached:

$$L_{LGZ} = \frac{-\ln(20^d - 19^d)}{c} + \frac{d \ln 19}{c} + \frac{b}{c} \quad . \qquad \text{(Eq. II.5)}$$

The different estimations of L_{LGZ} gave near-identical results due to the high values of r^2 fittings of the Richards function (data not presented).

Sampling and chemical analyses

In experiment 1, five plants were sampled at the end of the dark period at 68, 74 and 83 DAS. A piece of tissue 1.7 times the length of the L_{LGZ} was dissected out from the base of elongating leaves of mature tillers similar to those used in *LER* measurements. In experiment 2, 12 plants were sampled at the end of the light period at 63 DAS in low phosphorus, and 49 DAS in high phosphorus treatments, and leaf growth zones (L_{LGZ} determined by the pin-pricking) were dissected out. The rest of the shoot tissue was pooled. In all cases, fresh weight was recorded, samples were immediately frozen in liquid N_2, freeze-dried for 72 h at -80°C, weighed, ground, and stored at -25°C before analyses.

Phosphorus concentration was determined on 10 to 20 mg pooled samples by a modified phosphovanado-molybdate colorimetric method following acid digestion (Hanson 1950). Concentrations of C and N were determined on 0.7 mg samples with an elemental analyser (NA1500, Carlo Erba Instruments, Milan, Italy). In experiment 1, total water soluble carbohydrates (WSC) were extracted from 2 to 10 mg of the sample and quantified as in Schnyder & de Visser (1999). In experiment 2, 20 mg of the sample were extracted as in Morvan-Bertrand *et al.* (2001), and WSC quantified using a refractometer following separation by HPLC on a cation exchange column (Sugar-PAK, Millipore Waters, Milford, MA, USA). All concentrations are expressed on a tissue water basis (mg g^{-1} tissue water).

Experimental design and statistical analysis

Both experiments were complete two-way factorials. The first experiment consisted of two levels of *AMF treatment* and four levels of *Phosphorus supply* arranged in a completely randomised design. The second experiment consisted of two levels of *AMF treatment* and two levels of *Phosphorus supply* arranged in a randomised complete block (*growth chambers*) design. ANOVA revealed no effect of *growth chamber* on *LER* (*P>0.1*). In both experiments, effects of phosphorus supply and AMF on the nutritional status and parameters of the kinematic analysis were then tested by two-way ANOVA, with

the main factors *Phosphorus supply* and *AMF treatment*. The relationships between P_{LGZ} and *LER* and its components were tested by linear regression analyses of treatment averages, and slopes and intercepts for AMF treatments were compared with *F*-test (Statistica 6.0, Statsoft, Tulsa, USA). Results are shown as means ± 1 SE.

RESULTS

Plant growth at different levels of phosphorus supply and AMF treatment

When sampled at the same age (experiment 1), plants grown at low phosphorus supply and/or in the absence of AMF had substantially lower plant biomass (Grimoldi *et al.* 2005) and mature tillers with shorter sheaths than plants grown at high phosphorus and/or in the presence of AMF (Fig. II.2a). In contrast, and as aimed for, tiller sheath length did not differ among treatments when plants were sampled at different dates in experiment 2 (60 and 46 DAS) (Fig. II.2b). Thus, the responses of *LER* and its components to phosphorus supply and AMF were analysed in plants where any possible tiller size-mediated effects could (experiment 1) or could not (experiment 2) interfere with phosphorus status-mediated effects.

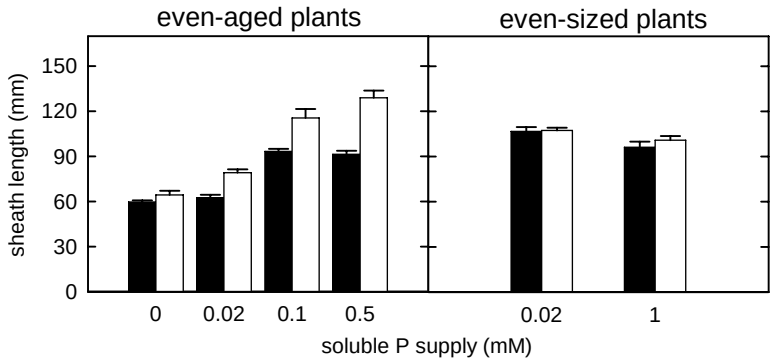

Figure II.2. Sheath length of the youngest expanded leaf enclosing the growing leaf. Perennial ryegrass was grown under different levels of soluble phosphorus supply for (a) 61 DAS (even age), or (b) 60 DAS (0.02 mM P, even size) and 46 DAS (1 mM P, even size) in the absence (■) or presence (□) of AMF. Bars indicate ±1 SE (n = 5-12).

Spatial distribution of growth along the leaf base

The profile of velocity of displacement along the leaf growth zones had the expected form in all treatments. Velocity increased with distance from the leaf base until the end of the leaf growth zone, where it became constant and equal to *LER*. Phosphorus deficiency consistently reduced the maximal displacement velocity, *i.e*. *LER*, by up to 60% in mature tillers of even-aged plants, and by 45% in even-sized mature tillers ($P<0.05$; Fig. II.3a-c).

On the other hand, phosphorus deficiency had contrasting effects on L_{LGZ} in the two experiments. It shortened L_{LGZ} by up to 46% when mature tillers of even-aged plants were compared ($P<0.05$). However, neither phosphorus supply nor AMF affected L_{LGZ} when even-sized tillers were compared ($P>0.05$; Fig. II.3c), even though they clearly differed in P_{LGZ} (see below). Likewise, mycorrhizal

plants had higher *LER* and longer L_{LGZ} in comparison to non-mycorrhizal plants in tillers of even-aged plants (P<0.05). Such an effect was not found when even-sized tillers were compared (P>0.05).

The spatial distribution of *REGR* within the growth zone had a common bell shape in all treatments, with a maximum near the centre of the growth zone (Fig. II.3d-f). Phosphorus deficiency lowered maximum *REGR*, an effect particularly evident in even-sized tillers.

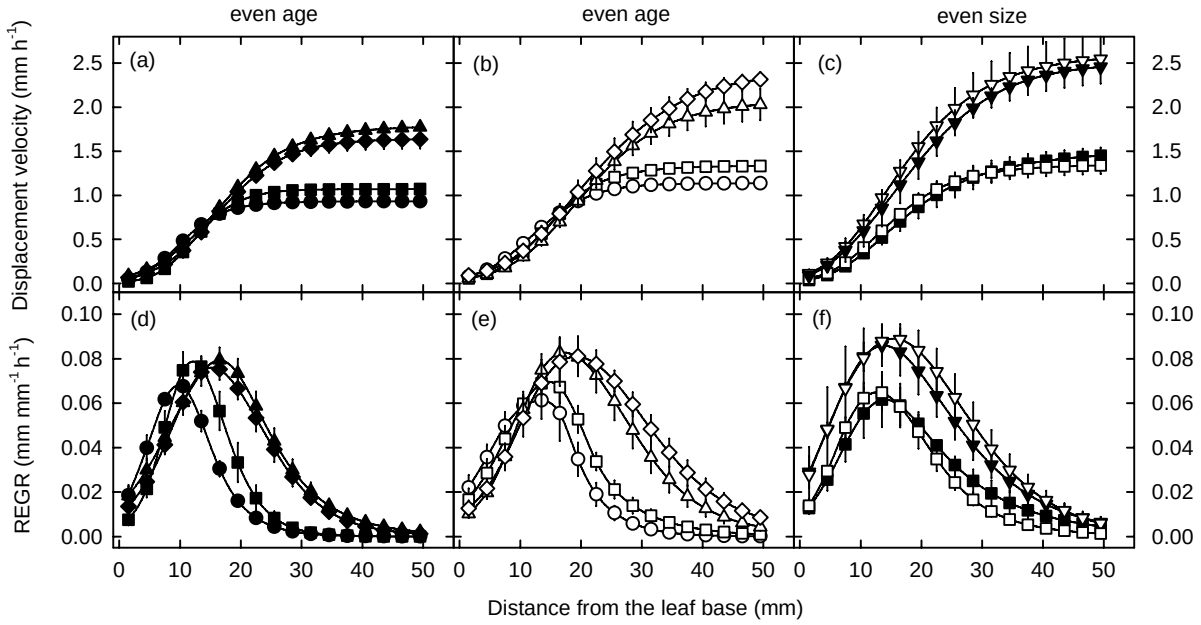

Figure II.3. Spatial distributions of (a-c) displacement velocity, and (d-f) relative elemental growth rate (*REGR*) along the leaf growth zone of the most rapidly growing leaf of perennial ryegrass tillers. Plants were grown with 0 mM (●○), 0.02 mM (■□), 0.1 mM (▲Δ), 0.5 mM (♦◇) or 1 mM (▼∇) soluble P supply in the absence (●■▲♦▼) or presence (○□Δ◇∇) of AMF. Bars indicate ±1 SE (n=5-12).

The effect of phosphorus supply and AMF on P, N and WSC concentrations

P_{LGZ} increased strongly with increasing phosphorus supplies in both experiments (P<0.01; Table II.1). AMF increased P_{LGZ} at the highest phosphorus supply level in the experiment comparing even-aged plants (P<0.05; Table II.1), although AMF colonization was low in comparison with the other treatments (Table II.2). In the other treatments AMF had no significant effect on P_{LGZ} (Table II.1; P>0.05).

Other components of leaf growth zone biomass were also affected by phosphorus supply. In both experiments, increasing phosphorus supplies led to 20 to 33% higher nitrogen (N) and 27 to 58% lower WSC concentrations in the leaf growth zone (Table II. 1). Since increasing phosphorus supplies affected phosphorus concentrations much more (by 70 to 134%), growth zone N : P and WSC : P ratios (w w^{-1}) were highest at the lowest phosphorus supplies. AMF colonization increased N and decreased WSC concentrations in the growth zone only when P_{LGZ} was also improved.

Table II.1. Nutrient status of perennial ryegrass plants. The effect of soluble phosphorus supply and presence (AMF+) or absence (AMF-) of the arbuscular mycorrhizal fungus *Glomus hoi* on the concentrations of P, N, C in the form of WSC, and total C in the leaf growth zone biomass (P_{LGZ}, N_{LGZ}, $C_{WSC-LGZ}$, C_{LGZ}, respectively). Values in brackets are SE (n = 2-10).

Experiment	AMF treatment	P supply (mM)	P_{LGZ}	N_{LGZ}	$C_{WSC-LGZ}$	C_{LGZ}
				mg g^{-1} tissue water		
even age	AMF-	0	0.42 (0.02)	4.77 (0.22)	23.4 (0.7)	73.0 (4.3)
		0.02	0.67 (0.04)	6.23 (0.29)	24.9 (2.3)	59.6 (2.8)
		0.1	0.90 (0.05)	6.85 (0.18)	18.9 (1.7)	57.3 (2.3)
		0.5	0.89 (0.08)	6.35 (0.40)	17.0 (1.9)	50.4 (4.4)
	AMF+	0	0.47 (0.08)	5.07 (0.07)	22.0 (1.7)	63.5 (3.6)
		0.02	0.66 (0.05)	5.53 (0.26)	21.9 (2.3)	56.4 (4.2)
		0.1	0.83 (0.07)	5.97 (0.17)	20.7 (2.4)	55.8 (3.3)
		0.5	1.10 (0.05)	6.06 (0.37)	15.1 (1.2)	50.4 (3.1)
even size	AMF-	0.02	0.71 (0.03)	6.97 (0.21)	46.0 (2.8)	71.8 (1.9)
		1.00	1.21 (0.11)	8.63 (0.24)	20.8 (4.3)	55.0 (1.5)
	AMF+	0.02	0.77 (0.10)	7.25 (0.28)	56.2 (2.6)	76.9 (3.1)
		1.00	1.47 (0.14)	8.82 (0.30)	23.8 (1.8)	59.1 (2.1)

Table II.2. Mycorrhizal colonization of perennial ryegrass roots. The effect of soluble phosphorus supply on the percentage of root length colonized by the AMF *Glomus hoi* was determined in even-aged plants at 68 DAS, and in even-sized plants at 63 DAS (0.02 mM P) and 49 DAS (1 mM P) on Trypan Blue stained roots by the gridline intersect method. Mycorrhizal colonization of the even-aged plants was reported before (Grimoldi *et al.* 2005). Non-mycorrhizal plants had null root colonization in both experiments. Values in brackets are SE (n = 4-9).

Experiment	P supply (mM)	Root length colonized (%)
even age	0	49.3 (3.8)
	0.02	48.1 (1.7)
	0.1	15.2 (1.6)
	0.5	8.3 (0.3)
even size	0.02	33.7 (3.5)
	1	2.4 (0.4)

Relationship between growth zone phosphorus status, and LER and its components

Both in even-aged and even-sized tillers, *LER* was linearly related to P_{LGZ} (P<0.05; Fig. II.4a & b, Table II.3). The slope and intercept of the regression tended to be higher in even-aged mycorrhizal plants, but this effect was not statistically significant (P>0.1; Table II.3).

Regression analysis also demonstrated a positive linear relationship between $REGR_{avg}$ and P_{LGZ} in both even-aged and even-sized tillers (P<0.05; Fig. II.4c & d, Table II.3). The slope of this relationship was somewhat higher in even-sized tillers, possibly due to a slightly different definition of the growth zone tissue sampled for phosphorus analysis (see Materials and Methods).

The relationship between L_{LGZ} and P_{LGZ} was more complex. When treatments differed in tiller sheath length (even-aged plants), L_{LGZ} was related linearly to P_{LGZ} (P<0.05; Fig. II.4e, Table II.3). Further, AMF colonization increased L_{LGZ} over the whole range of P_{LGZ} in even-aged plants (P>0.1; Table II.3). Notably, in this experiment AMF also increased the tiller size over the whole range of phosphorus supplies (Fig. II.2a). Conversely, none of these effects were observed when size-effects were avoided: P_{LGZ} and L_{LGZ} were unrelated, and AMF had no effect on L_{LGZ} when tillers of the same size were compared (P>0.1; Fig. II.4f, Table II.3), even though their P_{LGZ} were very different.

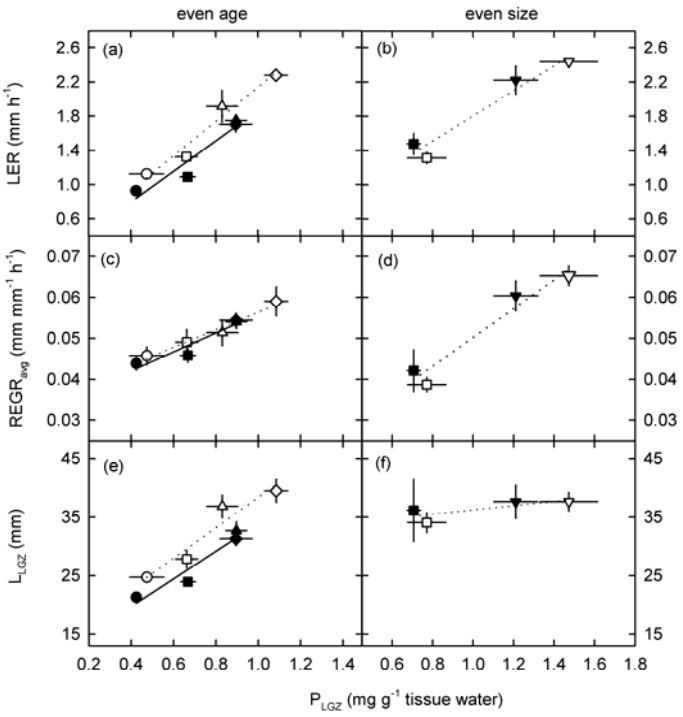

Figure II.4. (a & b) Leaf elongation rate (*LER*), (c & d) average relative elemental growth rate ($REGR_{avg}$), and (e & f) length of the leaf growth zone (L_{LGZ}), of the most rapidly growing leaf of perennial ryegrass tillers in relation to leaf growth zone phosphorus concentration (P_{LGZ}). Plants were grown with 0 mM (●○), 0.02 mM (■□), 0.1 mM (▲Δ), 0.5 mM (♦◇) or 1 mM (▼∇) soluble phosphorus supply in the absence (●■▲♦▼) or presence (○□Δ◇∇) of AMF. Bars indicate ±1 SE (n = 3-4 for P_{LGZ} and 5-12 for growth parameters). Lines are linear regressions (see Table II.3) for non-mycorrhizal (full) and mycorrhizal (dashed line) plants in the experiment with even-aged tillers, and for both AMF treatments in the experiment with even-sized tillers.

Table II.3. Linear regression analysis of the relationship between leaf growth variables and phosphorus status of the growth zone in non-mycorrhizal (AMF-) and mycorrhizal (AMF+) plants. Parameters of the linear regression of leaf elongation rate (*LER*) and its two components, average relative elemental growth rate ($REGR_{avg}$) and leaf growth zone length (L_{LGZ}) against P_{LGZ} are presented. In experiment with even-sized tillers, AMF- and AMF+ data were combined. Values in brackets are SE (n=4) for parameters different from zero ($P<0.05$); n.s., not significant at $P = 0.05$.

Experiment	Variable	AMF treatment	Slope	Intercept	r^2
even age	*LER*	AMF-	1.78 (0.39)	0.08 (n.s.)	0.91
		AMF+	2.01 (0.29)	0.13 (n.s.)	0.94
	REGRavg	AMF-	0.022 (0.005)	0.033 (0.004)	0.91
		AMF+	0.023 (0.003)	0.035 (0.002)	0.97
	L_{LGZ}	AMF-	23.6 (4.8)	10.3 (n.s.)	0.93
		AMF+	26.0 (5.6)	12.3 (n.s.)	0.92
even size	*LER*	AMF-/AMF+	1.47 (0.25)	0.33 (n.s.)	0.95
	$REGR_{avg}$	AMF-/AMF+	0.035 (0.006)	0.015 (n.s.)	0.95
	L_{LGZ}	AMF-/AMF+	3.65 (n.s.)	32.6 (2.2)	0.62

Accounting for sheath length effects on LER and its components

The data presented above revealed a strict allometric relationship between L_{LGZ} and the sheath length of the youngest expanded leaf through which the growing leaves emerged (Fig. II.5). This relationship was not modified by AMF, and did not differ between the two experiments.

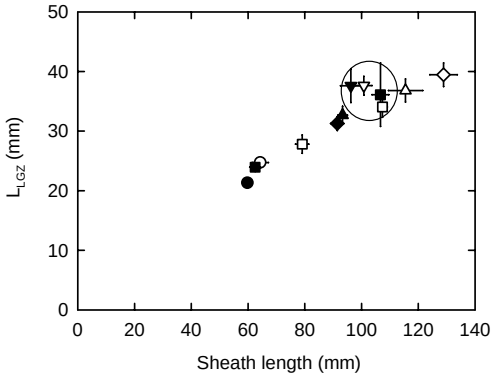

Figure II.5. Relationship between the sheath length of the youngest expanded leaf enclosing the growing leaf and the leaf growth zone length (L_{LGZ}) in perennial ryegrass. Plants grew with 0 mM (●○), 0.02 mM (■□), 0.1 mM (▲Δ) or 0.5 mM (♦◇) soluble phosphorus supply in the experiment with even-aged plants. Plants grew with 0.02 mM (■□) or 1 mM (▼∇) soluble phosphorus supply in the experiment with even-sized tillers (encircled data points). Plants were grown in the absence (●■▲♦▼) or presence (○□Δ◇∇) of AMF. Bars indicate ±1 SE (n = 5-12).

The correlation between L_{LGZ} and sheath length in Figure 5 includes data from different phosphorus supply treatments. We verified that the same relationship existed also in data subsets with uniform phosphorus status. Figure 6 shows one such subset including even-aged tillers with 0.83 to 0.90 mg P g^{-1} tissue water in the leaf growth zone. In these tillers, *LER* was positively correlated the with sheath length of the youngest expanded leaf (r^2=0.51, P<0.05; Fig. II.6a). The correlation was entirely due to the relationship between L_{LGZ} and sheath length (r^2=0.63, P<0.05; Fig 6c), since $REGR_{avg}$ was independent of sheath length (r^2=0.00, P>0.1; Fig 6b). These results were confirmed by regression analyses within each treatment in both experiments, and $REGR_{avg}$ never correlated with sheath length (data not shown).

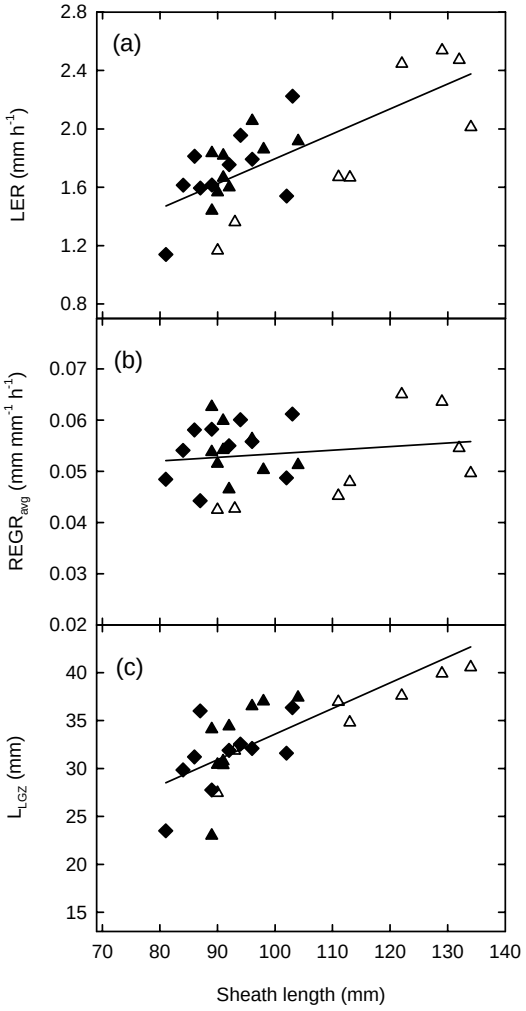

Figure II.6. Relationships between the sheath length of the youngest expanded leaf enclosing the growing leaf and (a) leaf elongation rate (*LER*), (b) average relative elemental growth rate ($REGR_{avg}$), and (c) leaf growth zone length (L_{LGZ}) in the experiment with even-aged tillers. Data correspond to a subset of plants with similar growth zone phosphorus concentration (0.83-0.90 mg g^{-1} tissue water), with symbols as for Fig. II.3. Linear regression equations are: y(*LER*) = 0.017x + 0.088, r^2 = 0.52 P < 0.001; y($REGR_{avg}$) = 0.000x + 0.046*, r^2 = 0.03 P = 0.41; y(L_{LGZ}) = 0.27x + 6.84, r^2 = 0.65 P < 0.001. Intercepts different from zero are marked with an asterisk (P < 0.05).

DISCUSSION

AMF and phosphorus supply control leaf growth via the same phosphorus status- and size-dependent mechanisms

This is the first quantitative assessment of the relationship between the phosphorus status of leaf growth zones and the components of *LER*, and it revealed that AMF and phosphorus supply enhanced leaf growth *via* identical mechanisms. These included both phosphorus status-dependent and tiller size-mediated effects on *LER* (Fig. II.7). Remarkably, $REGR_{avg}$ was strictly related to the phosphorus status of the leaf growth zone (*i.e.* P_{LGZ}), and independent of tiller size and AMF. Conversely, L_{LGZ} was a function of the sheath length of the youngest expanded leaf through which the growing leaf emerged (*i.e.* tiller size), and independent of phosphorus status and AMF. Accordingly, in plants of similar tiller size, phosphorus supply and AMF affected *LER* only *via* the effect of phosphorus status on $REGR_{avg}$. Conversely, in plants of the same age, differences in tiller size contributed to treatment differences in *LER via* the effect of tiller size on L_{LGZ}. These results illustrate the need for a distinction of phosphorus status-dependent from size-mediated effects on *LER*.

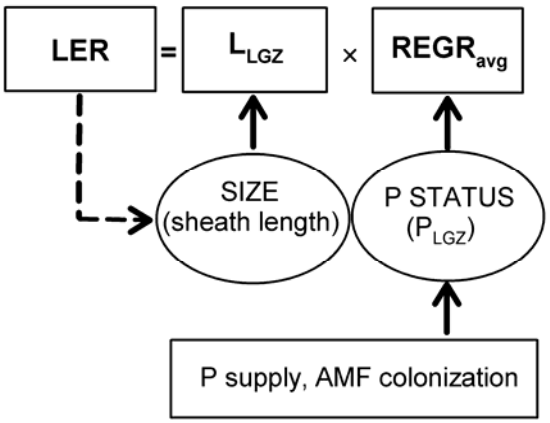

Figure II.7. Graphical summary of the effects of phosphorus supply and mycorrhizal colonization on *LER* and its components. Phosphorus supply and AMF treatment affect the leaf growth zone phosphorus status, which then directly affects *LER* through a change in $REGR_{avg}$. In turn, changes in the sheath length of the youngest expanded leaf (related to either the direct effect of phosphorus status on *LER* or to any other phosphorus -independent influence) will affect *LER* through an effect on L_{LGZ}.

Our results from the experiment with even-aged plants agree with those of the only other study on effects of phosphorus supply on leaf growth kinematics in grasses reported by Assuero *et al.* (2004). In their study with even-aged (but uneven-sized) plants, phosphorus deficiency caused a 63% reduction in *LER*, which resulted from a 56% shorter L_{LGZ}, and a 7% reduction in $REGR_{avg}$. Their work also demonstrated a higher cell production rate in the phosphorus sufficient tillers. The present data suggest that the responses observed by Assuero *et al.* (2004) resulted mainly from the size-mediated changes

of *LER*, and only marginally from the direct effect of growth zone phosphorus status on *LER*. Thus, it raises the question whether cell production rate is also related to tiller size. Clearly, the cellular mechanisms underlying the effects of phosphorus supply and AMF on leaf growth merit further study.

Our data also directly demonstrate that phosphorus deficiency did not limit leaf growth through a reduced C availability. In fact, growth zones of phosphorus deficient plants had a higher WSC concentration than growth zones of phosphorus sufficient plants, thus corroborating previous findings that phosphorus deficiency has a stronger effect on C utilization than on assimilation (Rao & Terry 1989; Rodriguez, Andrade & Goudriaan 2000). AMF have been reported to consume up to 20% of C fixed by the plant (Jakobsen & Rosendahl 1990). However, this cost was not reflected in WSC concentrations in the growth zone, suggesting that the presence of AMF did not negatively affect the amount of C available for leaf growth.

The uptake and subsequent metabolism of N are also known to be reduced under phosphorus deficiency (Rufty *et al.* 1993). In the present study, N concentration in leaf growth zone biomass decreased with phosphorus deficiency, but the N : P ratio of the growth zone biomass was highest in phosphorus deficient plants. This typical response of phosphorus limited (but not of nitrogen limited) plants (Agren 2004) indicates that the reduction of $REGR_{avg}$ under phosphorus deficiency was related exclusively to the low phosphorus status of the growth zone.

Tissue expansion rate is directly related to phosphorus status of the growth zone

This study demonstrated a direct and strong relationship between the leaf growth zone phosphorus status and the relative rate of tissue expansion ($REGR_{avg}$). Since *REGR* is a relative rate, it does not depend on the number of cells or cell length, and for a given position is equal to the relative rate of cell expansion (Schnyder *et al.* 1990; Ivanov, Dobrochaev & Baskin 2002). The actual mechanism by which expansion rate is affected by phosphorus status is not known. However, relative cell expansion rate is a function of cell wall extensibility and turgor pressure in excess of the yield threshold of the cell wall (Van Volkenburgh 1999). Arguably, phosphorus deficiency can affect both parameters, since it leads to low leaf ATP concentrations (Jacob & Lawlor 1993) and causes a variety of transcriptional and hormonal changes (Franco-Zorrilla *et al.* 2004). It also remains to be elucidated whether the growth reduction is mediated by unavailability of phosphorus as a substrate or by phosphorus as a signal (Ticconi & Abel 2004). Certainly, observed lower P_{LGZ} under phosphorus deficiency may result from decreased availability of phosphorus in xylem/phloem and directly limit the synthesis of phosphorus-containing cell components, though this can be partially compensated *e.g.* by galactolipids and sulfolipids partly replacing phospholipids in membranes (Dörmann & Benning 2002). Yet, decreased P_{LGZ} may result from lower phosphorus deposition due to cytokinin-mediated decreases in cell division/expansion as well (Werner *et al.* 2003).

What controls the length of the leaf growth zone?

The mechanism how the length of the leaf growth zone is actually regulated is so far unknown. Possible determinants include both positional and temporal controls of tissue expansion. We evaluated the possibility that cells require a fixed time interval to complete expansion. However, time-position trajectories (*cf.* Gandar & Hall 1988) generated from the present data suggested such a 'time-control' mechanism was unlikely, because phosphorus deficiency led to significantly longer residence times of tissue in the growth zone when size effects were taken into account (data not shown). The present – and also previous (Kemp 1980a; Casey *et al.* 1999; Arredondo & Schnyder 2003)– studies showed that L_{LGZ} is proportional to the sheath length of the youngest expanded leaf, suggesting a positional rather than temporal control. It is known that termination of cell expansion is associated with increased apoplastic peroxidase activity (Bacon, Thompson & Davies 1997; de Souza & MacAdam 1998). In turn, peroxidase activity in maize coleoptiles responded to changes in light quality, in a phytochrome-mediated response (Kim, Shinkle & Roux 1989). Thus, a coordination between L_{LGZ} and enclosing sheath mediated by morphogenic effects of light quality seems a reasonable hypothesis (*cf.* Skinner & Simmons 1993; Gautier & Varlet-Grancher 1996), albeit direct experimental evidence is still missing.
A review of kinematic studies evaluating the control of leaf elongation under different abiotic stresses indicates that when experimental designs produced no substantial difference between sheath length of control and treated plants, changes in *LER* were chiefly due to changes in $REGR_{avg}$, and not L_{LGZ} (e.g. light-dark cycles: Schnyder & Nelson 1988; salinity: Fricke & Peters 2002; N deficiency: Fricke *et al.* 1997; general nutrient deficiency: Snir & Neumann 1997; source limitation: Fricke 2002b; temperature: Ben-Haj-Salah & Tardieu 1995; ABA accumulation: Dodd & Davies 1996). Conversely, in studies where treatments altered sheath length substantially, both L_{LGZ} and $REGR_{avg}$ contributed to differences in *LER* (e.g. irradiance: Schnyder & Nelson 1989; salinity: Bernstein, Läuchli & Silk 1993; N deficiency: Gastal & Nelson 1994; Tóth *et al.* 2002; P deficiency: Assuero *et al.* 2004). Therefore, it seems these abiotic stresses initially affected *LER* solely by reducing *REGR*. Eventually, however, the treatment-related changes in tiller sheath length brought about an additional (indirect) effect: a change of L_{LGZ}.

In conclusion, this study showed that the effects of phosphorus supply and AMF on $REGR_{avg}$, and thus on *LER*, were closely and linearly related to their effects on P_{LGZ}. The other component of *LER*, L_{LGZ}, was strictly related to the sheath length of the youngest expanded leaf, independently of phosphorus supply or AMF treatment. Thus, tissue expansion rate was directly associated with phosphorus status, but the position at which expansion stopped was unrelated. AMF and phosphorus supply affected leaf growth through identical mechanisms.

Chapter III. PHOSPHORUS DEFICIENCY DECREASES CELL DIVISION AND ELONGATION IN GRASS LEAVES [2]

ABSTRACT

Leaf growth in monocotyledons results from a flux of newly born cells out of the division zone into the adjacent elongation-only zone, where cells reach their final length. We used a kinematic method to analyse the effect of phosphorus nutrition status on cell division and elongation parameters in the epidermis of *Lolium perenne* L. Phosphorus deficiency reduced leaf elongation rate by 39%, due to decreases in cell production rate (-19%) and final cell length (-20%). The former was solely due to a lower average cell division rate (0.028 *vs*. 0.046 cell cell^{-1} h^{-1}) and thus a lengthened average cell cycle duration (25 *vs*. 15 h). The number of division cycles of the initial cell progeny (5-6), and as a result the number of meristematic cells (32-64), and division zone length, were independent of phosphorus status. Accordingly, low phosphorus cells maintained meristematic activity longer. The lack of effect of phosphorus deficiency on meristematic cell length implies that lower division rate was matched to a lower elongation rate. Phosphorus deficiency did not affect the elongation-only zone length, leading thus to a longer cell elongation duration (99 h *vs*. 75 h). However, the substantially reduced post-mitotic average relative elongation rate (0.045 *vs*. 0.064 mm mm^{-1} h^{-1}) resulted in shorter mature cells. In summary, phosphorus deficiency did not affect the general controls of cell morphogenesis, but by slowing down the rates of cell division and expansion, it slowed down its pace.

[2] Kavanová M., Lattanzi F.A., Grimoldi A.A. & Schnyder H. (2006) *Plant Physiology* **141**: 766-775

INTRODUCTION

Although essential for plant growth and development, inorganic phosphorus is one of the least available nutrients in soils of many terrestrial ecosystems (Vance, Uhde-Stone & Allan 2003). Plants are profoundly affected by phosphorus deficiency, since phosphorus is an indispensable constituent of nucleic acids and membrane phospholipids. Moreover, phosphorus plays a pivotal role in energy transfer, as a regulator of enzyme activity, and in signal transduction. Thus, not surprisingly, low phosphorus availability activates a series of morphological and physiological responses that maximize phosphorus acquisition (Raghothama 1999), and are directed to maintain internal phosphorus homeostasis (Ticconi & Abel 2004). Leaf growth depression under phosphorus deficiency is well documented (Radin & Eidenbock 1984; Chiera *et al.* 2002; Assuero *et al.* 2004; Kavanová *et al.* 2006a). Ultimately, this growth reduction must be due to an alteration of cell division or cell elongation parameters.

We have chosen a grass leaf system to investigate the cellular bases of the growth reduction under phosphorus deficiency. In grasses, growth is confined to a short tissue segment located at the base of the developing leaf enclosed by older sheaths (Kemp 1980b). Here, meristematic cells proliferate, undergoing a number of cell cycles before entering a phase of elongation-only growth. This creates a clearly defined spatial pattern of cell development along the longitudinal axis, giving place to a basal *division zone*, where meristematic cells elongate and divide, and an *elongation-only* zone where cells undergo post-mitotic elongation. Together the two zones form the *leaf growth zone* (Fig. III.1).

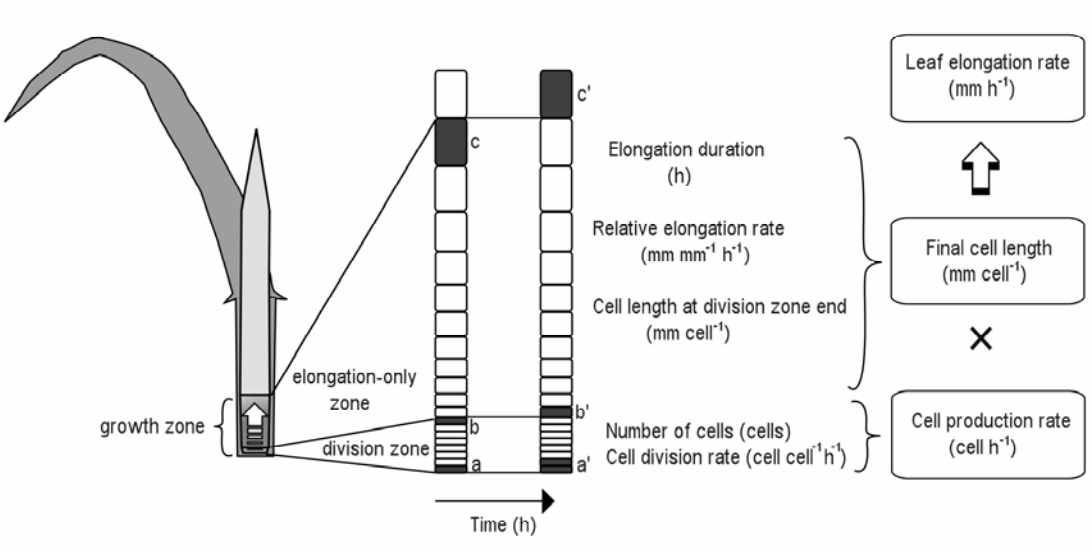

Figure III.1. Growth zone of a grass leaf. Growth is limited to the basal part of the growing leaf - the growth zone- which is enclosed by the sheaths of expanded leaves. Meristematic cells in the division zone elongate and divide simultaneously (a → a'). The progeny of the initial cell at the base of the meristem goes through a certain number of division cycles, thus determining the number of cells per meristematic file. Upon entering the elongation-only zone (b → b'), cells elongate without further divisions until they reach their final length at the distal end of the growth zone (c → c').

Kinematic analysis provides the appropriate analytical tools to translate back the spatial patterns into the time-history of an individual cell, making it possible to derive, from the spatial profiles of cell length and displacement velocity, rates and durations of cell division and elongation (Green 1976; Silk & Erickson 1979; Silk 1992). Leaf elongation rate (mm h^{-1}), the flux of leaf tissue out of the growth zone, can then be analysed in terms of cell production rate (cell h^{-1}) and final cell length (mm $cell^{-1}$) (Volenec & Nelson 1981). In turn, cell production rate is determined by the number of cells in the division zone and their division rate (cell $cell^{-1}$ h^{-1}), whereas final cell length is determined by the length of cells leaving the meristem (mm $cell^{-1}$), and their relative elongation rate (mm mm^{-1} h^{-1}) and elongation duration (h) (Fig. 1).

The contribution of the different cellular parameters to leaf growth reduction under nutrient stress is not well understood. Few studies addressed the effects of phosphorus deficiency, and gave different results. In cotton, Radin & Eidenbock (1984) concluded that reduced cell expansion underlay reduced leaf size, whereas in soybean Chiera *et al.* (2002) concluded that reduced cell division was the major cause. Although this divergence may be related to a different species response, it may also arise from the fact that neither of the studies directly measured these parameters. Instead, the role of cell expansion was inferred from smaller leaf cells, and the role of cell division from a reduced cell number. In maize, the first monocot studied, Assuero *et al.* (2004) attributed the reduction in leaf growth to a decreased cell production rate. In the only other kinematic study of phosphorus effects on leaf growth in the grass *Lolium perenne* L., low relative elongation rates along the elongation-only zone caused a severe reduction of leaf growth (Kavanová *et al.* 2006a).

This study provides a comprehensive analysis of the cellular responses underlying the reduction of leaf elongation rate in *L. perenne* leaves growing under phosphorus deficiency. Using a kinematic approach, we evaluated which of the parameters determining the number of produced cells and their final length responded to changes in phosphorus status and which did not. This included (*i*) the number of meristematic cells as controlled by a (constant) number of division cycles of the initial cell progeny, (*ii*) duration of cell elongation as determined by a spatially controlled elongation-only zone length, (*iii*) rate of cell division as determined by the growth rate of meristematic cells and a (constant) mitotic cell length, and (*iv*) rate of mitotic and post-mitotic elongation.

RESULTS

Leaf elongation rate

L. perenne plants grew at low (0.02 mM) or high (1 mM) phosphorus supply. Growth at low phosphorus supply caused a 42% reduction in the phosphorus concentration in the leaf growth zone ($P< 0.001$; Table III.1) and a 39% reduction in the leaf elongation rate ($P< 0.001$; Fig. III.2). In both treatments, leaves selected for measurement elongated at a steady rate over time (Fig. III.2).

The treatment effect on leaf elongation rate was entirely due to the different phosphorus nutrition status because selected tillers of low and high phosphorus plants did not differ in size or

developmental variables: Leaf blades and sheaths had similar lengths, and the tillers held a similar number of leaves (Table III.1). Further, growing leaves were in the same developmental stage, indicated by the ratio of the growing blade length to blade length of the youngest expanded leaf (Table III.1). This ensured that effects of phosphorus status on growth were not confused with effects of size and development (Kavanová *et al.* 2006a).

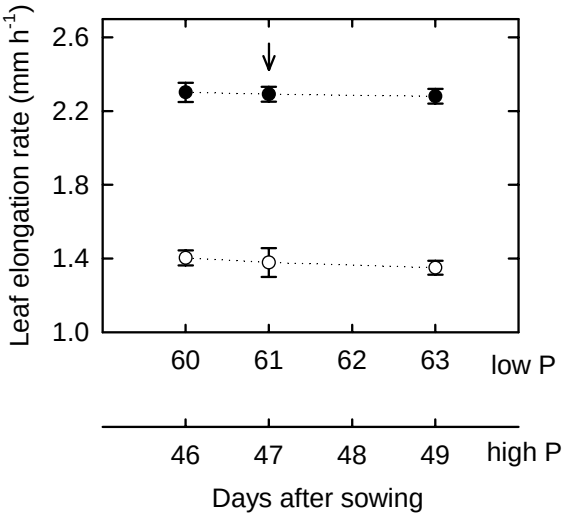

Figure III.2. Effect of phosphorus supply on leaf elongation rate. *Lolium perenne* plants were grown at high (1 mM, ●) and low (0.02 mM, ○) phosphorus supply. The arrow indicates the time when the kinematic analysis was performed. Data are means of five to six plants on each date (± SE).

Table III.1. Phosphorus status, tiller size, and leaf developmental stage of leaves selected for analysis of leaf growth and underlying cellular dynamics. *Lolium perenne* plants were grown for 47 d at high (1 mM) and 61 d at low (0.02 mM) phosphorus supply. Data are averages of six plants (± SE), along with the significance of the difference between phosphorus treatments based on a *t*-test (***, $P \leq 0.001$; NS, not significant, $P > 0.05$)

Parameter	High phosphorus	Low phosphorus	Significance
P in the growth zone (mg g^{-1} fresh weight)	1.07 ± 0.01	0.61 ± 0.01	***
Number of green leaves per tiller	4.8 ± 0.19	4.7 ± 0.24	NS
Sheath length of the youngest expanded leaf (mm)	90 ± 5	101 ± 5	NS
Blade length of the youngest expanded leaf (mm)	305 ± 14	342 ± 20	NS
Blade length of the growing leaf (mm)	206 ± 13	190 ± 24	NS
Blade length expanded : blade length growing leaf	0.68 ± 0.05	0.57 ± 0.08	NS

Cell proliferation

The reduction of leaf growth under low phosphorus originated partly from a decreased cell proliferation in the division zone. Phosphorus deficiency reduced cell production rate, *i.e.* the cell flux

out of the division zone estimated from leaf elongation rate and final cell length (Eq. III.2), by 19% (P<0.01; Table III.2). This difference arose entirely from a different average cell division rate, that is the number of cells produced per cell present in the division zone per unit time. Meristematic cells divided at a 39% lower rate in low phosphorus plants (P<0.01; Table III.2). As a result, the average cell cycle duration (Eq. III.8), was 10 h longer in low phosphorus plants (Table III.2).

Table III.2. Effect of phosphorus deficiency on kinematic parameters. *Lolium perenne* plants were grown for 47 d at high (1 mM) and 61 d at low (0.02 mM) phosphorus supply. Data are averages for epidermal cells of six plants (± SE), along with the significance of the difference between phosphorus treatments based on *t*-test (*, P≤0.05; **, P≤0.01; ***, P≤0.001; NS, not significant, P>0.05)

Parameter	High phosphorus	Low phosphorus	Significance
Leaf elongation rate (mm h^{-1})	2.30 ± 0.05	1.41 ± 0.05	***
Cell production rate (cell h^{-1})	1.91 ± 0.10	1.52 ± 0.10	**
Average cell division rate (cell $cell^{-1}$ h^{-1})	0.046 ± 0.008	0.028 ± 0.005	**
Average cell cycle duration (h)	15.1 ± 3.1	24.9 ± 2.0	***
Number of cells per meristematic cell file	41 ± 7	55 ± 9	NS
Number of division cycles	5.4 ± 2.7	5.8 ± 2.3	NS
Final cell length (mm)	1.208 ± 0.057	0.929 ± 0.052	**
Cell length in the meristem (μm)	19.3 ± 3.2	18.8 ± 2.4	NS
Postmitotic relative elongation rate (mm mm^{-1} h^{-1})	0.064 ± 0.005	0.045 ± 0.005	*
Elongation duration (h)	75 ± 6	99 ± 9	*

Phosphorus deficiency did not affect the average number of cells in a meristematic cell file (P>0.1; Table III.2). Cell division was confined to the basal 0.9 ± 0.1 mm in low phosphorus plants, and to 0.6 ± 0.1 mm in high phosphorus plants, but this difference was not statistically significant (P=0.06). It is important to note that we did not derive the length of the division zone and the number of meristematic cells from cell deposition rates. Instead, we counted all cells present in individual meristematic cell files, from the leaf base till the position of the last recently formed perpendicular cell wall. A closer examination of these data revealed that the number of cells per meristematic cell file was a weighted average of two major groups of files: files with *ca.* 32 cells and files with *ca.* 64 cells. Low phosphorus and high phosphorus plants had a similar frequency distribution of these two groups (data not shown).

The number of division cycles necessary to displace a transversal cell wall from the basal to the distal boundary of the division zone (i.e. 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) can be derived from the number of cells in the division zone (Eq. III.10). In both phosphorus treatments, the number of division cycles was on average five to six (P>0.1; Table III.2). Frequency distribution of the number of division cycles in different meristematic cell files revealed distinct peaks around 4, 5 and 6, indicating that variability exists between cell files within one division zone (Fig. III.3). Whereas in high phosphorus plants cell files were equally distributed around 5 and 6 division cycles, low phosphorus plants tended to have a frequency distribution shifted towards 6 division cycles.

As a consequence of a similar number of division cycles but longer average cell cycle duration, the average residence time of a cell in the division zone tended to be longer under phosphorus deficiency (144 ± 55 h *vs*. 81 ± 37 in high phosphorus plants). Thus, cells in low phosphorus plants tended to maintain meristematic activity for a longer period of time.

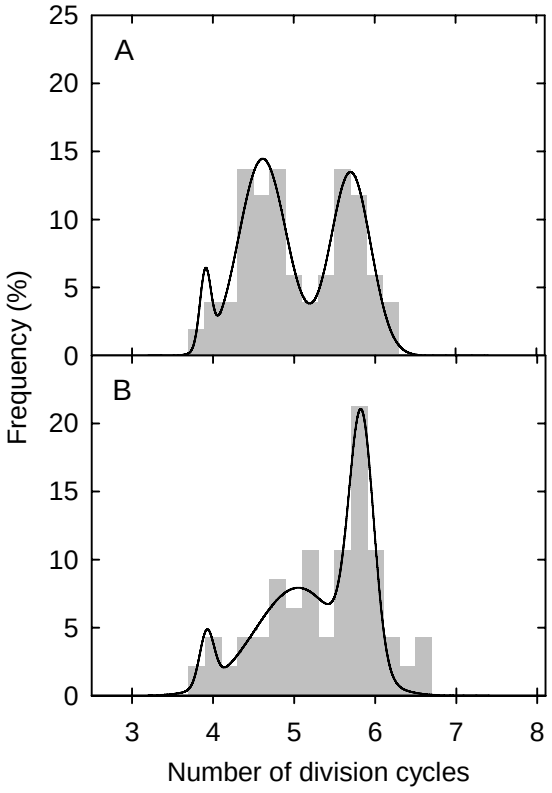

Figure III.3. Frequency distribution of cell files with different numbers of division cycles. *Lolium perenne* plants were grown for (A) 47 d at high (1 mM), and (B) 61 d at low (0.02 mM) phosphorus supply. For the analysis, data for eight to ten cell files of each of the six plants per treatment were combined. In every file, the number of division cycles of the progeny of the cell formed by the division of the initial cell at the base of the meristem was calculated as $\log_2$ (number of cells in the meristem) (Eq. III.10). Triple Gaussian normal distribution curves best fitted the frequency distributions (r^2=0.95 for high P, r^2=0.91 for low phosphorus plants), with peaks located at 3.9 ± 3.46, 4.6 ± 0.03 and 5.7 ± 0.03 divisions per cell (high phosphorus), and 3.9 ± 0.38, 5.1 ± 0.15 and 5.8 ± 0.02 divisions per cell (low phosphorus).

Relative elongation rate of meristematic cells

Cell length was typically constant within each individual meristematic cell file up to the position where division stopped (Fig. III.4, inset). When averaged over each treatment, average cell lengths were stable along the first half of the division zone, and phosphorus deficiency did not affect this pattern (Fig. III.4), but average cell length increased in the second half of the division zone, by 23% in low phosphorus, and 17% in high phosphorus plants. This increase was due to the fact that in some meristematic cell files the number of division cycles of the initial cell progeny was 5 (meristem length ~32 cells), whereas in others it was 6 (meristem length ~64 cells). Hence, in the second half of the meristem dividing cells (maintaining their average length unaltered) coexisted with non-dividing cells (that were increasing in length). This is also appreciated by comparing the stability of minimal cell lengths against the increase in maximal cell lengths

The stability of cell length along the division zone provides important information on the balance between relative rates of cell division and elongation (for discussion see Green 1976). It implies that the relative rates of meristematic cell elongation were very close to average cell division rates (0.028 ± 0.005 in low phosphorus *vs*. 0.046 ± 0.008 h^{-1} in high phosphorus plants). Phosphorus deficiency did not affect the size at which cells divided, or, consequently, their length at birth: The 39% higher average cell division rate of high phosphorus meristematic cells means they had 39% higher relative elongation rate.

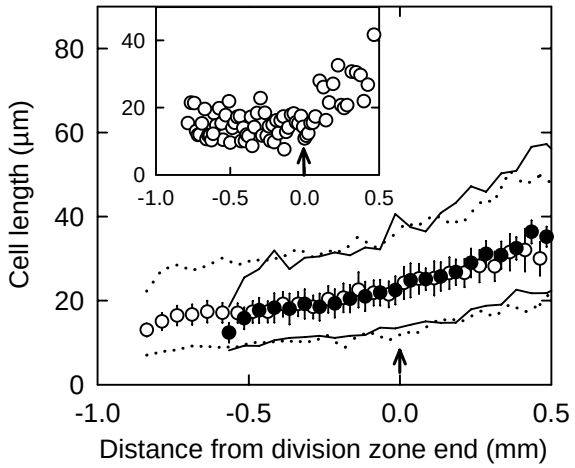

Figure III.4. Effect of phosphorus supply on epidermal cell length along the basal part of the leaf growth zone. *Lolium perenne* plants were grown for 47 d at high (1 mM, ●) and 61 d at low (0.02 mM, ○) phosphorus supply. The length of the shortest and the longest cell over 50 µm intervals is indicated by continuous lines (1 mM phosphorus) and dashed lines (0.02 mM phosphorus). Data are means of six plants (± SE). Arrows indicate the distal end of the division zone. Inset shows the raw cell length data for an individual cell file of a low phosphorus plant.

Final cell length

Whereas one-half of the leaf growth reduction under phosphorus stress was due to a reduced cell production rate, the other half originated from a decrease in final cell length. Mature epidermal cells were 20% shorter in low phosphorus plants (P<0.01, Table III.2 & Fig. III.5a). The final length of a cell depends on three factors: the length of the cell leaving the meristem (*i.e.* when it enters the elongation-only zone), and the relative rate and duration of the elongation-only phase. Phosphorus deficiency affected the latter two, but cell size at the position where elongation started was not different: 24.2 ± 3.8 µm in low *vs.* 22.5 ± 3.4 µm in high phosphorus plants (P>0.1; Fig. III.4).

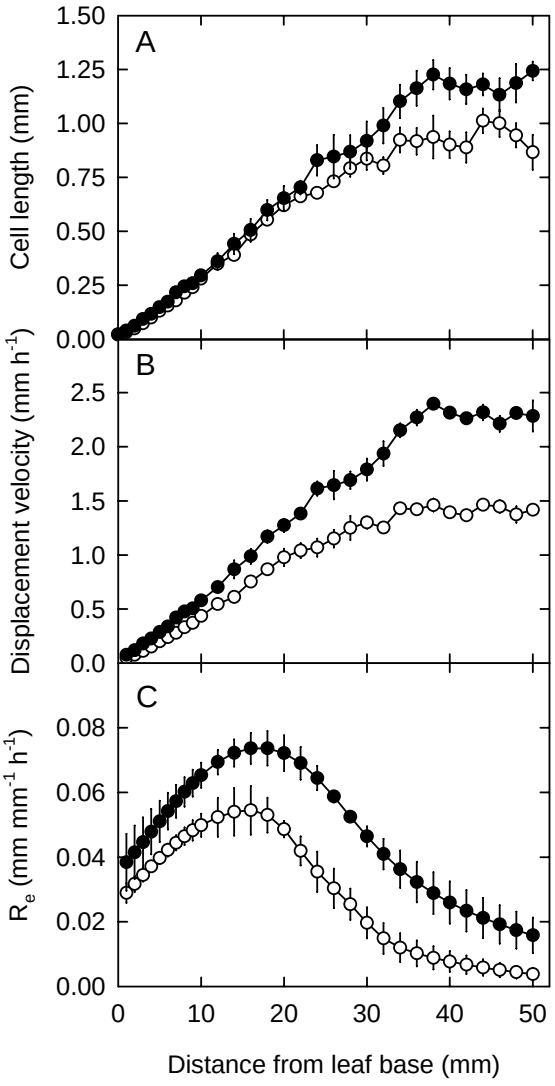

Figure III.5. Effect of phosphorus supply on spatial profiles of (A) epidermal cell length, (B) displacement velocity, and (C) relative elongation rate (R_e) along the base of the growing leaf. *Lolium perenne* plants were grown for 47 d at high (1 mM, ●) and 61 d at low (0.02 mM, ○) phosphorus supply. Data are means of six plants (± SE).

Spatial analysis of postmitotic elongation

Cell elongation was confined to the basal 31 to 36 mm of the growing leaf in low and high phosphorus plants, respectively (P>0.1, Fig. III.5A). The number of cells in the elongation-only zone was also not affected by phosphorus deficiency (136 ± 9 in low phosphorus, 117 ± 9 in high phosphorus plants; P>0.1). This confirms our previous observation (Kavanová *et al.* 2006a) that phosphorus deficiency has no (direct) effect on the length of the elongation-only zone.

Relative elongation rates along the elongation-only zone obtained by differentiating displacement velocity profiles (Fig. III.5B) were uniformly lower at all positions in low phosphorus plants (Fig. III.5C). Thus, phosphorus deficiency did not modify the spatial distribution of relative elongation rates, which were, on average, 30% lower in low phosphorus plants (P<0.05; Table III.2).

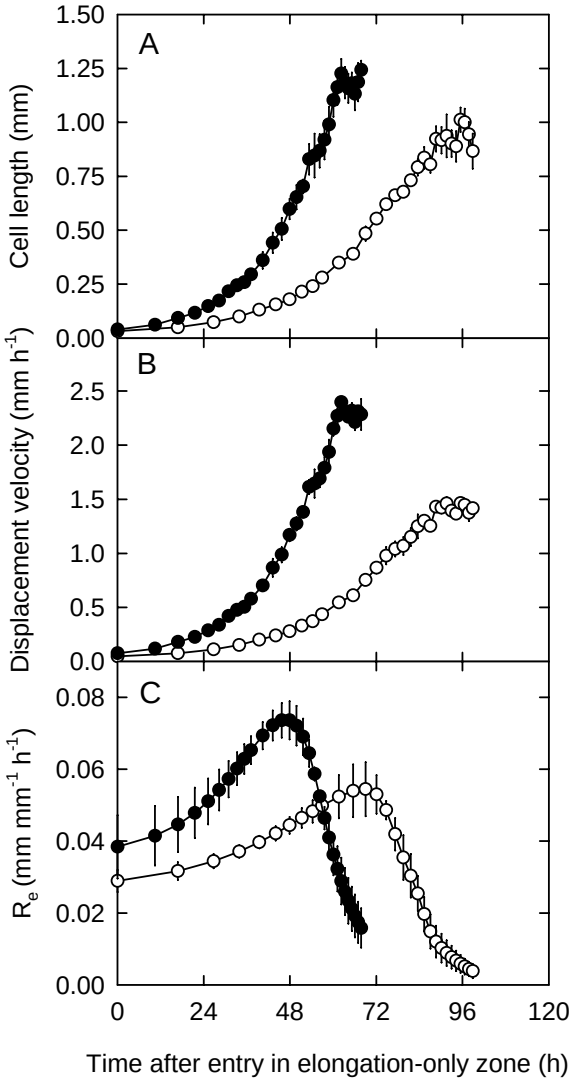

Figure III.6. Effect of phosphorus supply on temporal profiles of (A) cell length, (B) displacement velocity, and (C) relative elongation rate (R_e) of an individual epidermal cell from the time it enters the elongation-only zone. *Lolium perenne* plants were grown for 47 d at high (1 mM, ●) and 61 d at low (0.02 mM, ○) phosphorus supply. Data are means of six plants (± SE).

Temporal analysis of post-mitotic elongation

Furthermore, we carried out a temporal analysis of the elongation of an individual cell from the moment it enters the elongation-only zone. The spatial profiles of cell length, displacement velocity and relative elongation rate were transformed into time courses using the growth trajectory function, which relates spatial position of a cell to time coordinates (Eq. III.5). This analysis revealed that cells expanded for a substantially shorter period in high phosphorus plants ($P<0.05$; Table III.2), because they moved more rapidly through the elongation-only zone. Thus, the higher relative elongation rate of high phosphorus plants was partially offset by a shorter elongation duration (Fig. III.6).

DISCUSSION

Growth regulation constitutes a major field of interest in plant physiology. Yet, the cellular bases of the growth reduction under stress conditions are not fully understood. This kinematic study showed that, under phosphorus deficiency, the reduction of leaf growth in the grass *L. perenne* arose from inhibition of cell division and elongation rates, leading to reductions in both the cell production rate and the final cell length. The lengthened average cell cycle duration in low phosphorus plants was linked to a slower elongation rate so that meristematic cell length was not modified. Notably, other variables were unrelated to phosphorus status. In the division zone, phosphorus deficiency did not affect the number of division cycles (of the progeny of a cell formed by the division of the initial cell at the base of the meristem). In the elongation-only zone, phosphorus deficiency did not modify the position where post-mitotic elongation stopped. Hence, phosphorus deficiency did not affect the putative controls of the cell morphogenetic program, but, by slowing down the rates of cell division and elongation (and thus increasing the residence time in both zones), it slowed down the pace at which it was carried out.

Cell proliferation is modulated in response to phosphorus status

Coupling between cell elongation and cell division has been observed under undisturbed conditions in plant meristems, where cells double in size from birth until the next division (Cánovas *et al.* 1990; Korn 2001; Ivanov *et al.* 2002). However, only scarce knowledge exists on the links between cell growth and cell division (Li *et al.* 2005), and how cell size, nutrient status or other signals impinge upon the cell cycle progression in multicellular plants (De Veylder, Joubes & Inzé 2003).

Our study shows that phosphorus deficiency decreased the average division rate of meristematic cells. But, phosphorus deficiency did not affect meristematic cell length (Fig. III.4), implying that a decrease in division rate was accompanied by an equivalent reduction in their elongation rate. Therefore, phosphorus deficiency did not affect the close coordination between cell growth and cell division in the leaf meristem (see Fig. III.7, trajectory A).

What would be the result of uncoupling cell division from elongation in the meristem? Figure III.7 illustrates the possible outcomes. A factor that decreases cell division rate but does not affect

elongation will increase meristematic cell length (Fig. III.7, trajectory B). This would also increase the initial length at which cells start expanding, and result in longer mature cells, even though their elongation rate is not affected. The same will occur when a factor increases elongation rate but does not affect the division rate (Fig. III.7, trajectory C). This analysis also illustrates the risks of inferring changes in cell division or elongation rates based only on meristematic or mature cell length.

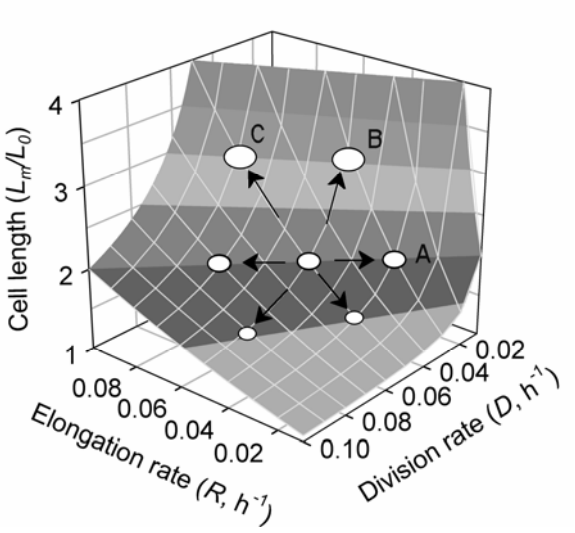

Figure III.7. Coordination between rates of cell division, elongation, and cell length. A cell growing exponentially at a relative rate R (h^{-1}) and dividing at relative rate D (h^{-1}) increases during one cell cycle its size from the initial length (L_0, mm) to its mitotic length (L_m, mm): $L_m = L_0 * exp (R_m * ln2/D)$. Solving this equation for the relative increase in length gives: $L_m/L_0 = 2\text{^}(R_m/D)$. The ratio of L_m/L_0 equal to 2 indicates that the cell progeny maintains constant size. Ratio L_m/L_0 higher than 2 indicates that R is higher than D, resulting in longer cells; and a ratio lower than 2 indicates that D is higher than R, resulting in shorter cells. Three cases are shown: A, Equal decrease in D and R does not affect meristematic cell length. B, Lower D with no change in R leads to longer meristematic cells. C, Faster R with no change in D results in longer meristematic cells.

The coupling of cell growth and division in proliferating cells may be achieved by alternative means: The cell division rate may affect the cell growth rate, or the cell growth rate may influence the cell division rate, or both processes may respond to a common signal. In the first scenario, phosphorus deficiency would have inhibited the cell cycle progression, and the reduced cell division rate would have decreased the cell elongation rate. Some authors have indeed suggested cell division might affect cell growth (Doerner *et al.* 1996; Cockcroft *et al.* 2000). However, several studies showed that cell cycle modulators, either accelerating or slowing cell division rates, decoupled cell division from cell growth. Overexpression of the cyclin-dependent kinase inhibitors KRP1 and KRP2 (De Veylder *et al.* 2001), and expression of a dominant negative allele of the Arabidopsis (*Arabidopsis thaliana*) *CDKA* gene in tobacco (*Nicotiana tabacum*; Hemerly *et al.* 1995) resulted in fewer, but bigger, meristematic cells. Similarly, meristematic and mature cells of plants overexpressing cyclin D3 were increased in number, but of smaller size due to accelerated progression through the G1 phase

(Dewitte *et al.* 2003). Thus, it seems improbable that a lower cell division rate drove reductions in elongation rate of meristematic cells under phosphorus stress.

The second scenario puts forth that a lower elongation rate of meristematic cells lengthened the average cell cycle duration. This view is supported by the fact that the probability of G1-to-S transition, a major cell size checkpoint, increased with increasing cell size (Cánovas *et al.* 1990). Further, Pien *et al.* (2001) showed that local induction of expansin expression led to the formation of normal leaf primordia, suggesting that increased cell expansion was driving cell division. In our study, the decrease in the elongation rate of meristematic cells under phosphorus deficiency would have prolonged the time needed to reach the critical length (see Fig. III.7, trajectory A), und thus would have lengthened the average cell cycle duration. Consequently, phosphorus deficiency may primarily affect cell elongation, and the effect on cell cycle duration may be a consequence of the reduction in growth rate. Nonetheless, a further study is needed to determine if phosphorus deficiency extends specifically the G1 phase or all cell cycle phases.

It is not clear which signal could regulate both cell growth and division rate in the third scenario. Cytokinins are a putative candidate, because phosphorus deficiency decreases their shoot levels (Horgan & Wareing 1980), and they affect both the cell cycle progression at the G1-to-S and G2-to-M transition (del Pozo *et al.* 2005), and have an effect on the expression of expansins and thereby on cell wall expansibility as well (Downes, Steinbaker & Crowell 2001).

Relative elongation rate is related to phosphorus status

A decrease in the relative elongation rate along the elongation-only zone led to shorter mature epidermal cells in phosphorus deficient plants and thus contributed to the decrease in the leaf elongation rate (Fig. III.5). Other parameters influencing final cell length were little affected by phosphorus status (the length of cells leaving the division zone, Fig. III.4) or even increased under phosphorus stress (elongation duration, Table III.2).

Proliferating cells grow primarily by an increase in the cytoplasmic volume, whereas cells in the postmitotic phase expand primarily through an increase in the vacuolar volume (Fagerberg 1984). Thus, it is telling that the reduction in the relative elongation rate was of similar magnitude in both the division and elongation-only zone (30-40%), raising the question of whether this was due to the same mechanism. The relative elongation rate depends on cell wall extensibility, tissue hydraulic conductance and turgor pressure in excess of the yield threshold of the cell wall (Fricke 2002a). No information exists concerning phosphorus effects on these processes. Changes in turgor have been found to play only a minor role in leaf growth responses to nitrogen and carbon stress and salinity (Fricke 2002a, and references therein). Thus, it is more likely that phosphorus status induced either changes in cell wall properties (mediated by *e.g.* expansins) or changes in tissue hydraulic conductivity (possibly mediated by aquaporins, which are highly expressed in dividing and elongating cells; Chaumont *et al.* 1998). We believe that understanding the effects of phosphorus deficiency on

leaf growth will progress little until the mechanism of the reduction in relative elongation rate is understood.

Phosphorus deficiency does not affect cell number, but increases residence time in the growth zone

Whereas division and elongation rates varied in response to phosphorus status, the size of the division and elongation-only zone remained unaffected. Two main models of growth zone regulation have been proposed for roots, and may also be valid for grass leaves. The first one proposes that a spatial gradient of growth regulators determines the developmental state of cells at any position along the growth zone (Barlow 1984). An alternative model claims that spatial patterns result from a certain developmental program followed by each cell (González-Fernández *et al.* 1968).

We evaluated if the length of the cell division zone could be determined by a temporally limited proliferation of meristematic cells. Under low phosphorus, cells were proliferative for a longer time than at high phosphorus, suggesting either that the termination of cell proliferation was not time regulated or that the temporal control changed. The spatial dimensions of the division zone might be related to the constant number of division cycle of the progeny of a cell formed by the division of the initial cell at the base of the meristem. Regardless of phosphorus status, the number of division cycles before cells entered into the elongation-only zone was four to six. There was more variation between cell files within a division zone than between plants of different phosphorus status, showing the importance of evaluating meristem parameters for individual cell files.

A review of the literature provided further support for the observed constancy. The length of the division zone in leaves of different C3 grass species has been reported to vary between 1 and 8 mm (Beemster *et al.* 1996; Fiorani *et al.* 2000; Masle 2000; Bultynck *et al.* 2003). The range of number of division cycles of the progeny of the initial cell is, however, narrower. We calculated from the published data that this number was six to eight. This suggests that the number of division cycles is a relatively conservative parameter.

Interestingly, this study indicated that the length of the elongation-only zone was not affected by phosphorus deficiency. As discussed previously, phosphorus deficiency decreased the flux of cells through this zone, but did not affect the elongation-only zone length, thus increasing the duration of an individual cell's elongation. This contradicts the view that the termination of cell elongation is time regulated. Support for the temporal regulation has been obtained by finding the opposite – namely, that the size of the elongation-only zone is proportional to the number of cells flowing through it (*i.e.* cell production rate) because each cell has a temporal program of elongation to execute (Beemster and Baskin, 1998). Following this reasoning, a change in cell flux should lead to a change in elongation-only zone length. This did not happen in our study, suggesting either that termination of cell elongation was either not time regulated, or that this control changed. Previously, we have shown that the elongation-only zone length correlates with tiller size (Kavanová *et al.* 2006a), and suggested that

morphogenic effects of light quality could provide a mechanism for the spatial control of its length (Barlow 1984).

In contrast to the only other kinematic study of phosphorus effects on leaf growth (Assuero *et al.* 2004), we found no difference in the division and elongation-only zone lengths in plants differing in their phosphorus status. This might be due to a species difference. However, the length of both zones varies during leaf development, and with changing tiller size (Durand, Schäufele & Gastal 1999; Kavanová *et al.* 2006a). Thus, the discrepancy might arise from size differences between phosphorus treatments in the study of Assuero *et al.* (2004). In the primary root of Arabidopsis, phosphorus deficiency did not affect meristem length, but decreased the length of the rapid elongation zone (Ma *et al.* 2003). Similar to leaves, regulation of the growth zone length in roots is not well understood. The comparison between phosphorus deficiency effects on leaf and root elongation suggests that root and leaf growth zones may differ in the cellular mechanisms underlying the growth response.

MATERIALS AND METHODS

Plant material and growth conditions

Surface sterilized seeds of *Lolium perenne* L. cv. Condesa were sown in pots (diameter 5 cm, height 35 cm) on a mixture of quartz sand with 63 mg phosphorus per pot in the form of finely ground Hyperphos (Deutsche HyperPhos-Gesellschaft, Budenheim, Germany) providing a source of phosphorus with low availability. Each pot contained one plant. Plants grew in a growth chamber (E15, Conviron, Winnipeg, Canada) with 20/15 °C (day/night), 70% relative air humidity and 525 µmol m^{-2} s^{-1} PPFD at plant height for 16 h day^{-1}. Plants were irrigated for 21 days after sowing four times a day with 25 mL of modified half-strength Hoagland's solution (0.02 mM KH_2PO_4, 2.5 mM KNO_3, 2.5 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM KCl, 0.5 mM NaCl, 0.125 mM Fe-EDTA, 23 µM H_3BO_3, 4.5 µM $MnSO_4$, 0.38 µM $ZnSO_4$, 0.16 µM $CuSO_4$ and 0.05 µM Na_2MoO_4). Thereafter, two levels of soluble phosphorus were applied: 0.02 mM KH_2PO_4 (low phosphorus) and 1 mM KH_2PO_4 (high phosphorus).

Leaf elongation rate

To avoid confounding phosphorus status with tiller size effects (see Kavanová *et al.* 2006a), the leaf elongation rate (mm h^{-1}) and its components were analysed in tillers with similar sheath length of the youngest fully expanded leaf (Table III.1). To this end, the leaf elongation rate was measured 46 to 49 days after sowing in high phosphorus plants, and 60 to 63 days after sowing in low phosphorus plants.

In five to six plants per treatment, representative tillers with at least three fully expanded leaves were selected at each date. The leaf elongation rate was determined on the youngest, most rapidly growing blade, during the phase of maximal growth, when leaf elongation rate was near constant. During this developmental stage, leaf growth is due exclusively to the activity of the blade growth zone, and cell division in the blade meristem and blade expansion are approximately steady (developmental stage

A→B in Schnyder *et al.* 1990). The leaf elongation rate 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, which was measured with a ruler every 24 h.

Sampling and phosphorus analysis

Twelve plants per treatment were sampled at the end of the light period 49 days after sowing in high phosphorus, and 63 days after sowing in low phosphorus plants. Leaf growth zones were dissected from leaves similar to those used for leaf elongation rate measurements. Fresh weight was recorded, samples were frozen in liquid N_2, freeze-dried, weighed, ground, and stored at –25°C. Phosphorus concentration was determined on 10 to 20 mg pooled samples as described by Kavanová et al. (2006).

Cell length measurement

The growing blade was carefully freed from surrounding older leaves in six plants per treatment 47 days after sowing in high phosphorus plants, and 61 days after sowing in low phosphorus plants. 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 polyvinylformaldehyd (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., Tokyo, Japan). 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 was contributing to leaf elongation (Schnyder *et al.* 1990). 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, USA). The mean epidermal cell length at each distance from the base was determined by measuring the length of 20 to 80 cells in cell files located midway between files containing stomata.

In addition, a sequence of overlapping images was taken along the basal 2 mm (starting from the ligule), and composite images were created. The length and distance from the leaf base of every cell in eight to 12 cell files located midway between files containing stomata were recorded in each leaf. Mean epidermal cell length over 50 μm intervals was determined for each plant, and then averaged over plants of the same treatment. Similarly, the length of the longest and shortest epidermal cell over 50 μm intervals was determined for each plant, and then averaged over plants of the same treatment. 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.

This is a novel method based on the same rationale as that introduced by Beemster *et al.* (1996) and used by Masle (2000). The latter method assessed newly formed perpendicular cell walls on cleared

fixed tissue instead of replicas of the leaf surface. We validated the new method by comparison with that of Beemster *et al.* (1996). To this end, the base of 12 growing leaves in different stages of development of *L. perenne* cv. Agenta was halved along the midrib. One half of the leaf was treated as in Beemster *et al.* (1996), from the other half a Formvar replica was taken. The position of the last newly formed perpendicular wall in epidermal cell files mid-way between files with stomata was measured in both sets of samples. The two methods yielded virtually identical results (Fig. III.8).

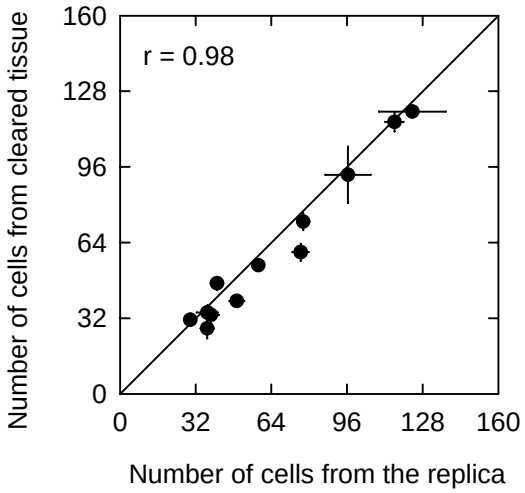

Figure III.8. Comparison of the number of cells in the division zone determined from the transparent Formvar replica versus the number of cells determined from fixed and cleared leaf tissue. Each leaf was cut in two equal pieces along the central midrib. One half was fixed and cleared following the procedure of Beemster et al. (1996). A Formvar replica was taken from the other half. The number of cells in the meristem was counted from the basal end (the ligule) to the position of the last recently formed (visually thinner) cell wall. Each data point represents the average of five to 10 cell files per leaf, along with its standard error. The line indicates the $y = x$ relationship.

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 (TableCurve 2D, SYSTAT, Point Richmond, USA):

$$y = e + a\frac{1}{(1+\exp^{(b-c\,x)})^{\frac{1}{d}}}, \qquad \text{(Eq. III.1)}$$

where y is the cell length, x is the 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. Since a reaches the maximal 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.

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 leaf elongation rate (LER) and final cell length (L_f):

$$F = \frac{LER}{L_f}. \quad \text{(Eq. III.2)}$$

Under steady-state growth, when the leaf elongation rate 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 leaf elongation rate. 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 (Silk 1992; Morris & Silk 1992):

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

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:

$$\overline{R_e} = \frac{v_e - v_d}{L_e}, \quad \text{(Eq. III.4)}$$

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):

$$t(x) = c \times N_e(x), \quad \text{(Eq. III.5)}$$

where c, the cellochron (h $cell^{-1}$), 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 $N_e(x)$ 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_e, h) was then calculated as:

$$T_e = \frac{N_e}{F}, \qquad \text{(Eq. III.6)}$$

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

Analysis of cell division

The average division rate of cells in the meristem can be determined by relating the cell production rate to the number of cells per meristematic cell file in the division zone (Ivanov & Dubrovsky 1997). This estimation assumes 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 *et al.* 2002, and references therein). Also, the 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 cell^{-1} h^{-1}) was calculated as:

$$D = \frac{F}{N_{div}}, \qquad \text{(Eq. III.7)}$$

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):

$$T_c = \frac{\ln(2)}{D}. \qquad \text{(Eq. III.8)}$$

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):

$$T_{div} = T_c \times \log_2(N_{div}) \qquad \text{(Eq. III.9)}$$

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):

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

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 (*e.g.* cell production and average cell division rate) were estimated by Gaussian error propagation. Results are shown as means ± SE.

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Chapter IV. NITROGEN DEFICIENCY EFFECTS ON CELL DIVISION AND CELL GROWTH IN GRASS LEAVES [3]

ABSTRACT

Leaf growth in monocots is the result of the interplay between two concerted processes. First, the proliferation of meristematic cells in the basal division zone produces a flux of new cells. Second, these cells attain their final length in the adjacent elongation-only zone. It is unclear which of these processes are causing the reduction in leaf elongation rate under nitrogen deficiency. A kinematic method was used to analyse the effect of nitrogen status on parameters of cell proliferation and growth in the epidermis of *Lolium perenne* L. Nitrogen deficiency reduced leaf elongation rate by 42%, due to decreases in cell flux (-28%) and final cell length (-20%). The former was solely due to a lower average cell division rate (0.023 *vs*. 0.032 cell cell^{-1} h^{-1}) and thus lengthened cell cycle duration (30 *vs*. 22 h). The number of division cycles of the initial cell progeny (5.7), and consequently the number of meristematic cells (53), were independent of nitrogen status. As a result, low nitrogen cells maintained meristematic activity for a longer time. Nitrogen deficiency did not affect the average meristematic cell length, implying that the lower division rate was matched to a lower elongation rate. Nitrogen stress also did not affect the elongation-only zone length, leading thus to a longer cell elongation duration (82 h *vs*. 68 h). However, the substantially reduced post-mitotic relative elongation rate (0.033 *vs*. 0.049 mm mm^{-1} h^{-1}) resulted in shorter mature cells. In conclusion, nitrogen deficiency decreased leaf growth by increasing the cell cycle duration and decreasing postmitotic relative elongation rates, delaying thus cell maturation.

[3] Kavanová M., Lattanzi F.A. & Schnyder H. *in preparation*

INTRODUCTION

Nitrogen is an essential constituent of amino acids, proteins, nucleic acids, and secondary metabolites (Marschner 1995). Due to its ubiquity, nitrogen deficiency affects most aspects of plant functioning, from metabolism to resource allocation, and development (Crawford 1995; Stitt 1999; Wang *et al.* 2003; Scheible *et al.* 2004). The availability of nitrogen is a crucial factor governing the leaf area expansion rate of plants. Yet, the understanding of the effects of nitrogen status on the cellular processes underlying the growth response is far from complete (Longnecker 1994; Lawlor, Lemaire & Gastal 2001).

The grass leaf is a suitable model for unravelling the cellular processes underlying the growth reduction. In grasses, growth is confined to a short tissue segment located at the base of the developing leaf, the *leaf growth zone* (Kemp 1980b). Here, meristematic cells proliferate, undergoing a number of cell cycles before entering a phase of postmitotic growth. This creates a clearly defined spatial pattern of cell development along the longitudinal axis, giving place to a basal *division zone*, where meristematic cells elongate and divide, and an *elongation-only* zone where cells undergo postmitotic elongation. Leaf elongation rate (mm h^{-1}), the flux of leaf tissue out of the growth zone, can be analysed in terms of changes in cell production rate (cells h^{-1}) and final cell length (mm $cell^{-1}$; Volenec & Nelson 1981). The number of meristematic cells (which is related to the average number of division cycles of the progeny of the initial cell at the meristem base) and their division rate (cells $cell^{-1}$ h^{-1}) determine the flow of cells out of the division zone, i.e. the cell production rate. On the other hand, the length of cells leaving the division zone (which depends on the balance between cell division rate and relative growth rate of meristematic cells; mm $cell^{-1}$), their relative postmitotic elongation rate (mm mm^{-1} h^{-1}) and the duration of elongation (h) determine the final cell length. Kinematic analysis provides the appropriate analytical tools to translate back the spatial patterns into the time-history of an individual cell, making possible to derive, from the spatial profiles of cell length and displacement velocity, rates and durations of cell division and elongation (Green 1976; Silk & Erickson 1979; Silk 1992; Ivanov & Dubrovsky 1997).

In grasses, several studies attributed the decrease in leaf elongation rate under nitrogen deficiency to a lower cell production (Volenec & Nelson 1983; Gastal & Nelson 1994; Fricke *et al.* 1997) with a minor or no effect on the final cell length, although the postmitotic relative elongation rate was reduced. None of these studies, however, measured all parameters that would allow the calculation of cell division parameters. Before a step toward an elucidation of the genetic and molecular regulation of the cellular processes under nutrient stress can be undertaken, it is essential to understand which of these processes respond to changes in nitrogen status.

This study provides a comprehensive analysis of the cellular processes underlying the reduction of leaf elongation rate in *Lolium perenne* leaves growing under nitrogen deficiency. Using a kinematic approach, we evaluated which of the parameters determining the number of produced cells and their final length responded to changes in nitrogen status and which did not. This included (*i*) the number of

meristematic cells as controlled by a (constant) number of division cycles of the initial cell progeny, (*ii*) the duration of cell elongation as determined by a spatially controlled elongation-only zone length, (*iii*) the rate of cell division as determined by the growth rate of meristematic cells and a (constant) mitotic cell length, and (*iv*) the rate of mitotic and postmitotic elongation.

RESULTS

Leaf elongation rate

L. 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 IV.I) and the leaf elongation rate by 46% ($P < 0.001$; Table IV.I & Fig. IV.1).

The treatment effect on the leaf elongation rate was entirely due to the different nitrogen nutrition status because selected tillers of low and high nitrogen plants did not differ in size or developmental parameters: Leaf sheath of the youngest expanded leaf had similar length (82 ± 2 mm for both treatments), and the tillers held a similar number of green leaves (4-5). Further, growing leaves were in the same developmental stage, when leaf elongation rate is approximately constant, and maximal: The blade length of the growing leaf was between one-third to one-half of the blade length of the youngest expanded leaf (Fig. IV.1). This ensured that effects of nitrogen status on growth were not confused with effects of tiller size and leaf developmental stage (Kavanová *et al.* 2006a).

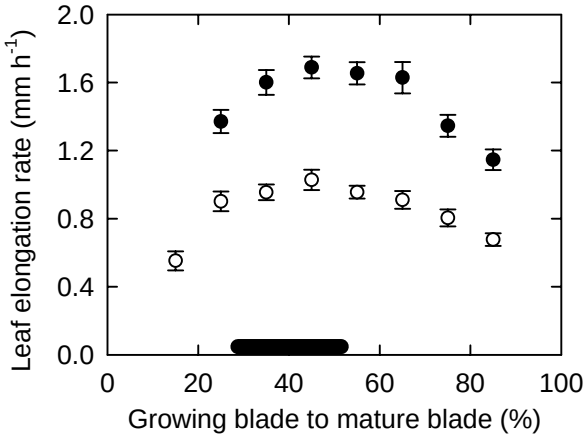

Figure IV.1. Effects of nitrogen supply and leaf developmental stage on the leaf elongation rate. *Lolium perenne* plants were grown at high (7.5 mM, ●) and low (1 mM, ○) nitrogen supply. Leaf developmental stage is expressed as the percentage of the growing blade length to the blade length of the youngest expanded leaf. The thick line indicates the stage when the kinematic analysis was performed. Data points are means of 2 to 26 plants (± SE).

Cell proliferation: number of meristematic cells and their division rate

Nitrogen deficiency decreased leaf elongation rate mainly through a reduction in the proliferation of meristematic cells in the division zone. Low nitrogen reduced the cell production rate, *i.e.* the cell flux

out of the division zone estimated from the leaf elongation rate and final cell length (Eq. 2), by 28% (P<0.01; Table IV.I).

Table IV.1.The effect of nitrogen deficiency on the nitrogen status of the growth zone and kinematic parameters of leaf growth. *Lolium perenne* plants were grown for 30 d at high (7.5 mM) and 58 d at low (1 mM) nitrogen supply. Data are averages for epidermal cells of five plants (± SE), along with the significance of the difference between treatments based on a *t*-test (*, P≤0.05; **, P≤0.01; ***, P≤0.001; NS, not significant, P>0.05)

Parameter	High nitrogen	Low nitrogen	Significance
Nitrogen in the growth zone (mg g^{-1} dry weight)	36.4 ± 1.2	21.9 ± 0.6	***
Leaf elongation rate (mm h^{-1})	1.70 ± 0.06	0.97 ± 0.06	***
Cell production rate (cell h^{-1})	1.67 ± 0.07	1.21 ± 0.03	**
Cell division rate (cell $cell^{-1}$ h^{-1})	0.032 ± 0.003	0.023 ± 0.004	**
Cell cycle duration (h)	22 ± 2	30 ± 3	**
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
Final cell length (mm)	1.03 ± 0.06	0.82 ± 0.06	*
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	***
Residence time in the elongation-only zone (h)	69 ± 3	82 ± 2	*

Nitrogen deficiency did not affect the length of the division zone (1.4 ± 0.1 mm in both low and high nitrogen plants; P>0.05) nor the average number of cells per meristematic cell file (53 ± 3 ; P>0.1; Table IV.I). The number of division cycles necessary to displace a transversal cell wall from the basal to the distal boundary of the division zone (i.e. the average number of division cycles of the progeny of a cell formed by the division of the initial cell at the meristem base) can be derived from the number of cells in the division zone (Eq. 10). In both nitrogen treatments, the average number of division cycles was 5.7 ± 2.0 (P>0.1; Table IV.I).

Since nitrogen deficiency did not affect the number of meristematic cells, the decrease in the cell production rate in low nitrogen plants was only due to a lower average cell division rate, that is the number of cells produced per cell in the division zone per unit time (Ivanov 1997). Meristematic cells divided at a 28% lower rate in low nitrogen plants (P<0.01; Table IV.I). Therefore, the average cell cycle duration was 8 h longer in low nitrogen plants (Table IV.I). As a consequence of an unaltered number of division cycles, but a longer cell cycle duration, the average cell residence time in the division zone was increased under nitrogen stress (174 ± 70 h *vs*. 124 ± 39 in high nitrogen plants). Thus, nitrogen-deficient cells maintained proliferative competence for a longer period of time.

Cell proliferation: relative elongation rate of meristematic cells

Cell length was typically constant along each individual meristematic cell file till the position where division stopped, or slightly increased in the distal half of the cell file (Fig. IV.2, inset). Cells situated in the distal half of the division zone were 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). Similarly, average cell lengths were stable along the basal half of the division zone and nitrogen deficiency did not affect this pattern (Fig. IV.2). Yet, the average cell length increased during the last division cycle by 30% in low nitrogen, and by 38% in high nitrogen plants. The increase in the cell length indicates that meristematic cells undergoing the last division had either a higher rate of growth than of division, or that some cells underwent the last division (maintaining thus their average length unaltered) and some did not (and were thus increasing in length).

The stability of cell length during the major part of the proliferative phase provides information on the balance between relative rates of cell division and elongation (Green 1976). It implies that the relative rates of meristematic cell elongation were very close to average cell division rates (0.023 ± 0.004 in low *vs*. 0.032 ± 0.003 h^{-1} in high nitrogen plants). Nitrogen deficiency did not affect the size at which cells divided, neither, consequently, their length at birth. Thus, the 28% higher average cell division rate of nitrogen-sufficient meristematic cells means that they were growing at 28% higher rate.

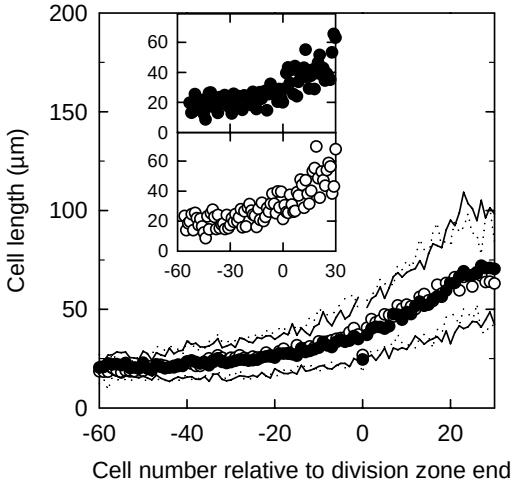

Figure IV.2. Effect of nitrogen supply on the average epidermal cell length along the basal part of the leaf growth zone. *Lolium perenne* plants were grown for 30 d at high (7.5 mM, ●) and 58 d at low (1 mM, ○) nitrogen supply. The average length of the shortest and the longest cell at each cell rank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

Final cell length

The leaf growth reduction under nitrogen stress was also partly due to a decrease in the final cell length. Mature epidermal cells were 20% shorter in low nitrogen plants (P<0.05, Table IV.I & Fig.

IV.3a). The final length of a cell depends on three factors: the length of the cell leaving the meristem (*i.e.* when it enters the elongation-only zone), and the relative rate and duration of the elongation-only phase. Nitrogen deficiency affected the latter two, but cell size at the position where elongation started was not different: 26 ± 5 µm in low nitrogen *vs.* 25 ± 1 µm in high nitrogen plants (P>0.1; Fig. IV.2).

Spatial analysis of postmitotic elongation

Cell elongation was confined to the basal 31 to 34 mm of the growing leaf in low and high nitrogen plants, respectively (P>0.1, Fig. IV.3). The number of cells in the elongation-only zone was also not affected by nitrogen deficiency (101 ± 3 in low nitrogen, 116 ± 8 in high nitrogen plants; P>0.1). This confirms our previous finding (Kavanová *et al.* 2006a) that nutrient deficiency does not (directly) affect the length of the elongation-only zone.

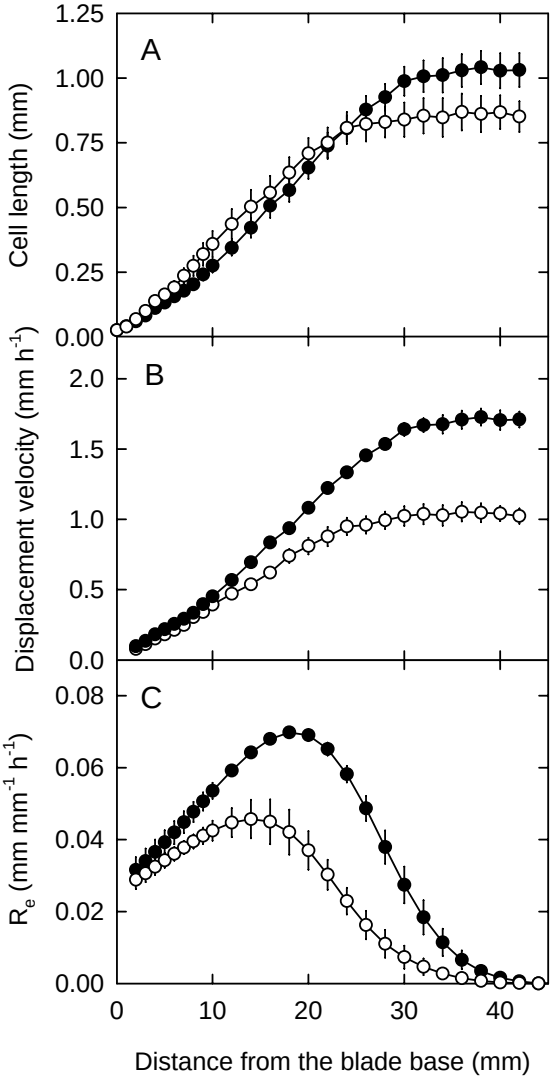

Figure IV.3. Effect of nitrogen supply on spatial profiles of epidermal cell length (A), displacement velocity (B), and relative elongation rate (R_e; C) along the base of the growing leaf. *Lolium perenne* plants were grown for 30 d at high (7.5 mM, ●) and 58 d at low (1 mM, ○) nitrogen supply. Data are means of five plants (± SE).

Relative elongation rates along the elongation-only zone, obtained by differentiating displacement velocity profiles (Fig. IV.3B), did not differ in the first 7 mm, but were lower at all more distal positions in low nitrogen plants ($P<0.05$; Fig. IV.3C). On average, relative elongation rate was 33% lower in low nitrogen plants ($P<0.05$; Table IV.I).

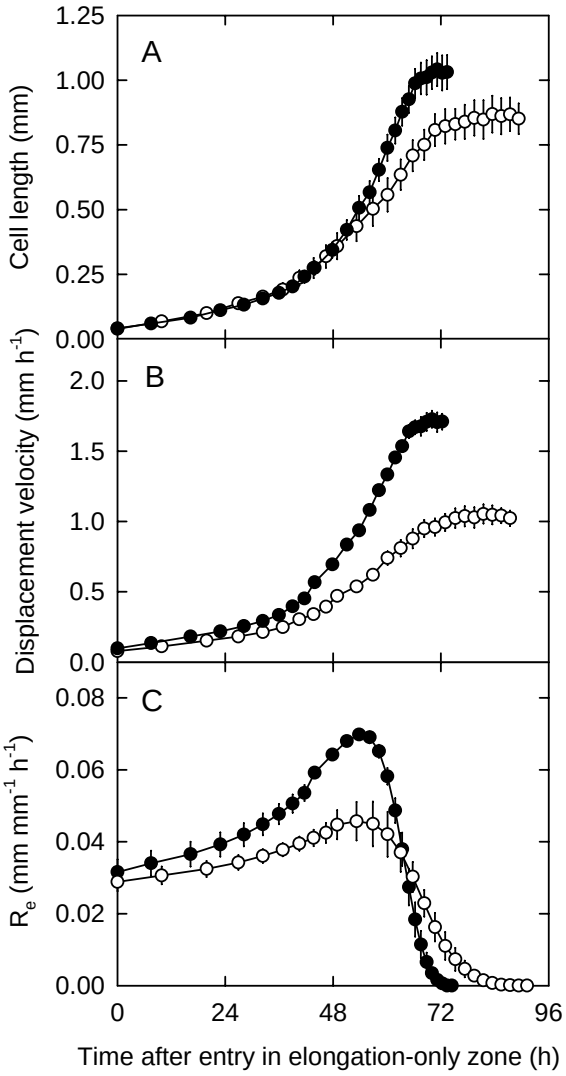

Figure IV.4. Effect of nitrogen supply on temporal profiles of cell length (A), displacement velocity (B), and relative elongation rate (R_e; C) of an individual epidermal cell from the time it enters the elongation-only zone. *Lolium perenne* plants were grown for 30 d at high (7.5 mM, ●) and 58 d at low (1 mM, ○) nitrogen supply. Data are means of five plants (± SE).

Temporal analysis of postmitotic elongation

Further, we carried out a temporal analysis of the elongation of an individual cell from the time it enters the elongation-only zone. The spatial profiles of cell length, displacement velocity and relative elongation rate were transformed into time profiles using the growth trajectory function, which relates spatial position of a cell to time coordinates (Eq. IV.5). This analysis revealed that cells expanded for 14 hours less under high nitrogen ($P<0.05$; Table IV.I), because they moved more rapidly through the

elongation-only zone. Thus, the higher relative elongation rate of high nitrogen plants was partially offset by a shorter elongation duration (Fig. IV.4).

DISCUSSION

Despite the wide knowledge of the effects of nitrogen deficiency on the whole plant growth (for review see Longnecker 1994; Lawlor *et al.* 2001), the cellular bases of the growth reduction are not well understood. This kinematic study analysed the causes of the reduction in the leaf growth of the grass *L. perenne*. Under nitrogen deficiency, the reduction of leaf growth was due to a reduction of both cell division and mitotic elongation rates (leading to a reduction in the cell flux) and postmitotic elongation rates (leading to a reduction in the final cell length). A tight coordination between the rates of cell division and mitotic elongation was maintained in nitrogen-deficient cells, as illustrated by the constancy of the average meristematic cell length. Notably, this study revealed that some processes were also irresponsive to the nitrogen status. Nitrogen deficiency did not alter the number of meristematic cells in the division zone nor the position where postmitotic elongation stopped. As a result of slowing down the rates of division and elongation, nitrogen deficiency prolonged the residence time of a cell in the growth zone and delayed its maturation.

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

This study showed that the division rate of meristematic cells was positively correlated with the nitrogen status of the growth zone. Remarkably, the length of meristematic cells in the division zone was not affected by the nitrogen status, demonstrating that the coordination between cell division and cytoplasmic growth rates was not perturbed. Similar coordination between the rates of growth and of division in meristematic cells seems logical, and has been reported in plant meristems under undisturbed conditions (Cánovas *et al.* 1990; Korn 2001; Ivanov *et al.* 2002), and under conditions of phosphorus deficiency (Kavanová *et al.* 2006b), but the mechanisms that would explain this phenomenon in higher plants are largely unidentified (Ingram & Waites 2006).

The causality between the reduction in the division and cytoplasmic growth rate under nitrogen deficiency could not be conclusively established in this study. This coordination may be achieved in different ways (*cf.* Kavanová *et al.* 2006b). First, the rate of cell elongation may be limiting for the rate of division. This is the way how in the unicellular *Chlamydomonas* the size homeostasis is achieved. A critical size checkpoint in the G1 phase of the cell cycle ensures that a cell does not complete the division before reaching an appropriate size (John, Zhang & Dong 1993). In higher plants, the probability of G1-to-S transition also increases for bigger cells (Cánovas *et al.* 1990). Pien *et al.* (2001) showed that local induction of expansin expression led to the formation of normal leaf primordia, suggesting that increased cell expansion was driving cell division. In this study, a decrease in the growth rate of nitrogen-deficient meristematic cells would have prolonged the time needed to

reach the critical length, und thus would have lengthened the average cell cycle duration. Accordingly, nitrogen deficiency may primarily affect cell growth, and the effect on the cell cycle may be a consequence of the reduction in growth rate.

Second, the rate of cell division may be limiting for the rate of elongation. However, an evidence for this scenario is missing. Third, both the rate of cell division and elongation may be concertedly regulated by some signal. In yeast, the progression through the cell cycle and cell growth rate are co-regulated by proteins involved in the translation (Jorgensen & Tyers 2004). Similar mechanism, involving the transcription factor *TCP20*, has been proposed to link cell growth and division in higher plants (Li *et al.* 2005). Cytokinins are another putative candidate, because their levels are regulated by nitrogen availability (Rahayu *et al.* 2005; Dodd & Beveridge 2006), and they affect both the cell cycle progression from G1-to-S and G2-to-M phase (del Pozo *et al.* 2005), and have an effect on the expression of expansins and thereby on cell wall properties (Downes *et al.* 2001).

Under nitrogen deficiency, the decrease in the cell production rate was not related to a change in the number of meristematic cells, but to their lower division rate. Whether this also occurred in other studies reporting a decrease in cell production rate under nitrogen deficiency (Volenec & Nelson 1983; Gastal & Nelson 1994; Fricke *et al.* 1997; Rademacher & Nelson 2001) can not be conclusively established, because these studies did not determine the number of meristematic cells. It is also necessary to stress here that a change in cell production rate associated to a change in cell division rate does not necessarily result from cell cycle regulation, because the causality between changes in cell division rate and mitotic cell growth rate is not clear.

Nitrogen deficiency decreased both the cytoplasmic and vacuolar growth rates

Intriguingly, the relative rates of elongation were reduced to a similar extent in meristematic cells and in cells situated in the elongation-only zone. Despite the similitude in the response of the cytoplasmic and vacuolar growth rate to nitrogen deficiency, these two processes might have been controlled differently. During the mitotic phase, cells grow predominantly by an increase in the volume of the cytoplasm and organelles. During the postmitotic phase, cell growth is largely based on an increase in the vacuolar volume, accompanied by the uptake of water and solutes, and by a deposition of new cell wall material in the surrounding cell wall. The relative growth rate of a cell depends on cell wall extensibility, tissue hydraulic conductance and turgor pressure in excess of the yield threshold of the cell wall (Fricke 2002a). Changes in turgor have been found to play only a minor role in leaf growth responses to nitrogen deficiency (Fricke *et al.* 1997). Thus, low nitrogen status either induced changes in cell wall properties (mediated by *e.g.* expansins) or in tissue hydraulic conductivity (possibly mediated by aquaporins, highly expressed in dividing and elongating cells; Chaumont *et al.* 1998).

There is a high requirement for nitrogen in the meristematic cells, because up to 50% of their total dry weight is in the form of nucleic acids and proteins (Gastal & Nelson 1994). This requirement is also reflected in the deposition pattern, which is limited to the basal segment where cell proliferation of

epidermal and mesophyll cells occurs. The reduction in the division and growth rate of meristematic cells might then be directly related to the low deposition rate of nitrogen compounds in the zone of cell division (Gastal & Nelson 1994). An extensive recycling of nitrogen is later taking place in the elongation-only and maturation zone. This can be seen in the example of Rubisco, a protein that represents up to 30 to 60% of soluble proteins in leaves of C3 plants (Sage, Pearcy & Seemann 1987; Evans 1989). Rubisco is synthesized in the distal part of the elongation-only zone where no net nitrogen deposition takes place (Gastal & Nelson 1994), supporting an intensive recycling of nitrogen contained in cell cycle machinery. The recycling of nitrogen may explain why the relative elongation rate was not significantly reduced in the first millimetres of the elongation-only zone in nitrogen-deficient plants.

Nitrogen deficiency did not affect cell numbers, and increased residence times in the growth zone

The size of the division and elongation-only zone were not affected by nitrogen deficiency, suggesting that the control of the dimensions of these zones was not related to nutrient status. Two main models of growth zone regulation have been proposed for roots, and these may also be valid for grass leaves. The first one proposes that a spatial gradient of growth regulators determines the developmental state of a cell at any position (Barlow 1984). An alternative model puts forward that spatial patterns result from a certain developmental program followed by each cell (González-Fernández *et al.* 1968).

First, we evaluated if the length of the cell division and elongation-only zone could be determined by a temporally limited cell proliferation and elongation. But, we found that nitrogen-deficient cells maintained proliferative competence for a longer time interval than nitrogen-sufficient cells. And, although nitrogen deficiency decreased the flux of cells through the elongation-only zone, it did not affect its length, increasing thus the duration of an individual cell elongation. This contradicts the view that the termination of cell elongation is time regulated. The support for the temporal regulation has been obtained by finding the opposite, namely that the size of the elongation-only zone is proportional to the number of cells flowing through it, because each cell has a temporal program of elongation to execute (Beemster & Baskin 1998). Following this reasoning, a change in cell flux should lead to a change in elongation-only zone length. Clearly, this did not happen in this study on nitrogen. The same finding has been reported for phosphorus-deficient plants (Kavanová *et al.* 2006b), suggesting that the termination of cell proliferation and elongation is either not time regulated, or that the temporal control changes under nutrient deficiency.

Second, the possibility that the division and elongation-only zone were determined spatially was evaluated. It has been proposed that the dimensions of the division zone might be regulated spatially by a gradient of some growth regulator (*cf.* Li *et al.* 2005; Fleming 2006). Alternatively, the spatial dimensions of the division zone might be related to a constant number of division cycles of the initial cell's progeny. The number of division cycles before a cell entered into the elongation-only zone was

five to six, independently of the nitrogen or phosphorus status (Kavanová *et al.* 2006b), implying that the maintenance of proliferative capacity, expressed as the number of division cycles of the initial cell's progeny, is not affected by nutrient status. Previously, we have shown that the elongation-only zone length correlates with tiller size (Kavanová *et al.* 2006a), and suggested that morphogenic effects of light quality could provide a mechanism for the spatial control of its length (Barlow 1984).

In contrast to other kinematic studies of nitrogen effects on leaf growth (Volenec & Nelson 1983; Gastal & Nelson 1994; Rademacher & Nelson 2001), we found no difference in the elongation-only zone lengths in plants differing in their nitrogen status. This might be due to a species difference. However, the length of the growth zone varies during leaf development, and with changing tiller size (Durand *et al.* 1999; Kavanová *et al.* 2006a). Thus, the discrepancy might arise from size differences between nitrogen treatments in the previous studies.

MATERIALS AND METHODS

Plant material and growth conditions

Surface sterilized seeds of *Lolium 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, 85 % relative air humidity and 275 µmol m^{-2} s^{-1} PPFD at plant height for 24 h day^{-1}. The chamber was equipped with an automatic irrigation system that flooded 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: (*i*) High nitrogen: macronutrients, 2.5 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, 1 mM $MgSO_4$, 0.18 mM KH_2PO_4, 21 mM K_2HPO_4, 0.5 mM NaCl, 0.4 mM KCl, 0.4 mM $CaCl_2$; micronutrients: 0.125 µM Fe-EDTA, 46µM H_3BO_3, 9 µM $MnSO_4$, 1 µM $ZnSO_4$, 0.3 µM $CuSO_4$, 0.1 µM Na_2MoO_4. (*ii*) 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

To avoid confounding nitrogen status with tiller size effects (see Kavanová *et al.* 2006a), the leaf elongation rate (mm h^{-1}) and its components were analysed in tillers with similar sheath length of the youngest fully expanded leaf (82 ± 2 mm). To this end, the leaf elongation rate was measured 30 days after sowing in high nitrogen plants, and 58 days after sowing in low nitrogen plants.

In five plants per treatment representative tillers with at least three fully expanded leaves were selected. The leaf elongation rate was determined on the youngest, most rapidly growing blade, during the phase of maximal growth, when leaf elongation rate was near constant. During this developmental stage, leaf growth is due exclusively to the activity of the blade growth zone, and cell division in the blade meristem and blade expansion are approximately steady (developmental stage A→B in Schnyder *et al.* 1990). The leaf elongation rate 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, which was measured with a ruler every 24 h.

Sampling and nitrogen analysis

Fourteen plants per treatment were sampled 34 days after sowing in high nitrogen, and 57 days after sowing in low nitrogen plants. Leaf growth zones were dissected from leaves similar to those used for leaf elongation rate 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 blade was carefully freed from surrounding older leaves in five plants per treatment 30 days after sowing in high nitrogen plants, and 58 days after sowing in low nitrogen plants. 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 polyvinylformaldehyd (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., Tokyo, Japan). 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 was contributing to leaf elongation (Schnyder *et al.* 1990). 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, USA). The mean epidermal cell length at each distance from the base was determined by measuring the length of 20 to 80 cells in cell files located midway between files containing stomata.

In addition, a sequence of overlapping images was taken along the basal 2 to 3 mm (starting from the ligule), and composite images were created. The length and distance from the blade base of every cell in eight to 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).

Analysis of cell elongation and cell division

Parameters of cell elongation and cell division were calculated from the measured data following the same procedure as in Chapter III (Kavanová *et al.* 2006b).

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 were estimated by Gaussian error propagation. Results are shown as means ± SE.

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Chapter V. General and Summarizing Discussion

The regulation of leaf growth constitutes a field of paramount interest in plant physiology. Yet, our understanding of the cytological phenomena underlying changes in the leaf growth rate is only limited. In particular, the role of cell division and expansion in the response of leaf growth to environmental conditions remains to be elucidated. Previous studies stressed out the importance of cell production rate and mature cell size as parameters responsible for changes in leaf elongation rate under unfavourable nutritional conditions. The kinematic studies summarized in this thesis analysed the cellular mechanisms underlying these two phenomena, and helped to clear previous contradictory results regarding the respective roles of cell production rate and mature cell size in explaining reductions in leaf growth.

Phosphorus and nitrogen deficiency have similar effects on leaf growth

Phosphorus (Chapter II & III) and nitrogen deficiency (Chapter IV) substantially reduced leaf elongation rate in *Lolium perenne*. This response of leaf growth to nutrient stress has been commonly observed in the past (Volenec & Nelson 1983; Thomas 1983; Gastal & Nelson 1994; Fricke *et al.* 1997; Tóth *et al.* 2002; Chiera *et al.* 2002; Assuero *et al.* 2004). The novelty of the present kinematic studies resides in the comprehensive analysis of the cellular processes underlying the reduction of leaf growth. Chapter III and IV show that phosphorus and nitrogen deficiency had similar negative effects on the rates of cell division and mitotic elongation (leading thus to a lower cell production rate), and postmitotic elongation (leading thus to a shorter final cell length). The similitude might be related to the fact that both macronutrients are required in large quantities, and constitute building blocks of many indispensable macromolecules. Also, several regulatory systems, such as the cytokinin signalling pathway, respond to both phosphorus (Horgan & Wareing 1980; Karthikeyan *et al.* 2002) and nitrogen (Rahayu *et al.* 2005) status. Before discussing the particular cellular parameters of leaf growth, it is necessary to consider tiller size effects on leaf growth in grasses.

Size- *vs*. nutritional status- effects of nutrient deficiency on leaf growth

An important advance of the present studies is that size effects on the leaf growth rate were effectively distinguished from the responses to the nutritional status. Grasses growing under nutrient deficiency have smaller tiller size, because leaf elongation rate and final leaf length are positively correlated (Fournier *et al.* 2005). Size has effects upon many physiological processes (Brown *et al.* 2004; Reich *et al.* 2006), and should therefore be accounted for by the experimental design. Yet, studies on the effects of nutrients on leaf growth have usually not distinguished between direct nutritional and size-dependent treatment effects. Chapter II shows that such a distinction is essential for understanding the

mechanisms controlling leaf growth: the decrease in leaf elongation rate under phosphorus deficiency was related exclusively to the effect of low nutrient status on tissue expansion when effects of tiller size were excluded, but it was partly due to the effects of reduced tiller size on the leaf growth zone length when the size effect was not accounted for (Fig. II.4 & II.7).

This finding was also supported by a review of kinematic studies. In cases where experimental designs did not produce substantial differences in size, changes in leaf elongation rate were mainly due to relative elongation rate (e.g. nitrogen deficiency: Fricke *et al.* 1997). Conversely, in studies where sheath lengths were substantially influenced, both leaf growth zone length and relative elongation rates contributed to differences in leaf elongation rate (e.g. nitrogen deficiency: Gastal & Nelson 1994; Tóth *et al.* 2002; phosphorus deficiency: Assuero *et al.* 2004). In the present studies (Chapter III & IV), accounting for size effects revealed that nutrient deficiency did not change the position where cell elongation stopped, thus increasing the residence time of a cell in the elongation-only zone. This led to the conclusion that the existence of a temporal control of cell elongation is unlikely. All this underscores the importance of evaluating parameters of leaf growth on tillers that have a similar sheath length of the last expanded leaf. This ensures that direct treatment, and not size-related effects are being evaluated.

The control of cell division and elongation-only zone sizes

Cell division zone

In *L. perenne*, the number of meristematic cells, and subsequently the division zone length, were not affected by phosphorus or nitrogen deficiency. This shows that nutrient deficiency did not influence the proliferative competence of meristematic cells, expressed as the number of division cycles of the initial cell progeny. The maintenance of the meristematic activity was most likely not temporally regulated, because its duration increased under phosphorus or nitrogen deficiency (Table III.2 & IV.1). The number of meristematic cells might be regulated spatially by a gradient of some (unknown) growth regulator (*cf.* Li *et al.* 2005; Fleming 2006). Alternatively, cells may count the number of division cycles using some (unknown) internal clock. The mechanism defining the number of meristematic cells clearly merits further study.

The length of the division zone in primary roots and grass leaves has been reported to vary within two orders of magnitude (0.4 to 40 mm, Fig. V.1A). The range of the number of division cycles of the progeny of the initial cell is, however, much narrower (Fig. V.1) Calculations based on published data show that this number ranges from four to eleven. This suggests that the number of division cycles does not differ greatly between plant species, nor between different organs growing predominantly along the longitudinal axis, even though primary root meristems have an indeterminate, and grass leaf meristems a determinate growth.

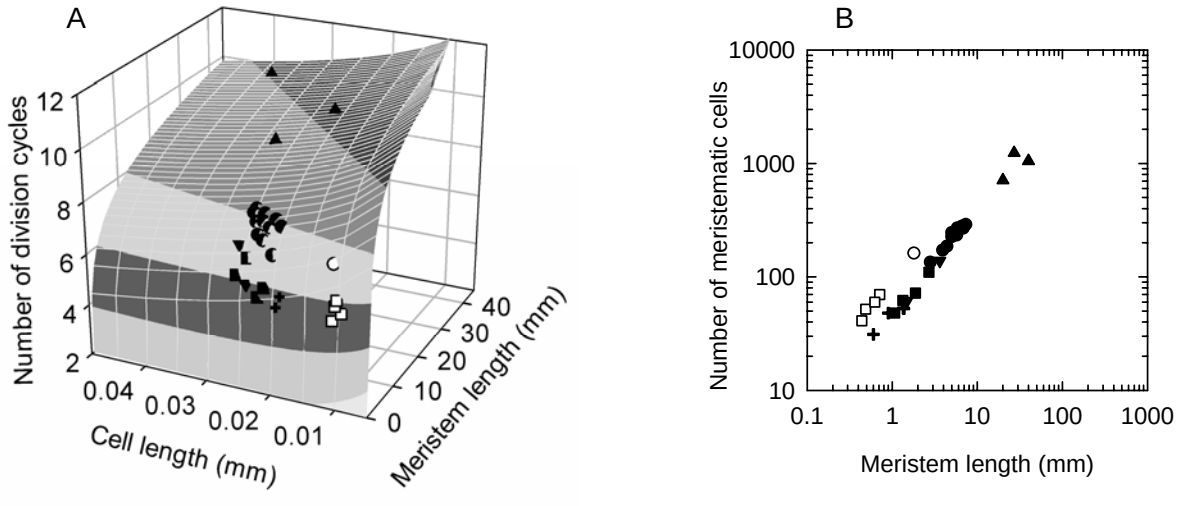

Figure V.1 (A) The number of division cycles of the progeny of the cell formed by the division of the initial cell at the base of the meristem. The plotted function represents $\log_2$(meristem length/average meristematic cell length) (see Eq. III.10). (B) Relationship between the meristem length and the number of meristematic cells. Data from leaf meristems of *Lolium perenne* (+, from the present studies), *Triticum aestivum* cultivars (● Beemster *et al.* 1996; Masle 2000), four *Poa* species (■ Fiorani *et al.* 2000), two *Aegilops* species (▼ Bultynck *et al.* 2003), and *Zea mays* (▲ Ben-Haj-Salah & Tardieu 1995; Tardieu *et al.* 2000; Assuero *et al.* 2004), and from root meristems of *Arabidopsis thaliana* (□ Beemster & Baskin 1998; West, Inzé & Beemster 2004) and *Zea mays* (○ Sacks, Silk & Burman 1997).

Elongation-only zone

The studies summarized in this thesis showed that the length of the leaf growth zone was not related to the nutrient status. The mechanisms how the length of the leaf growth zone is determined are so far unknown. Possible determinants include both positional and temporal controls of tissue expansion. The possibility that cells require a fixed time interval to complete expansion was evaluated. However, time trajectories showed that such a temporal control was unlikely, because phosphorus or nitrogen deficiency led to significantly longer residence times of cells in the growth zone (Fig. III.6 & IV.4). Several studies (Kemp 1980a; Casey *et al.* 1999; Arredondo & Schnyder 2003), and also the present one (Fig. II.5), showed that growth zone length is proportional to the sheath length of the youngest expanded leaf. Further, Casey *et al.* (1999) showed that artificial shortening of sheaths reduced leaf elongation rate by decreasing the leaf growth zone length. Taken together, this suggests that leaf growth zone length is regulated spatially. A coordination between leaf growth zone length and enclosing sheath length mediated by morphogenic effects of light quality has been proposed several times (*cf.* Begg & Wright 1962; Skinner & Simmons 1993; Gautier & Varlet-Grancher 1996), but direct experimental evidence is still missing.

Cell division and cell growth rates response to the nutrient status

Interestingly, from all the cytological processes determining leaf elongation rate, only the cell division rate and the rates of mitotic and postmitotic cell elongation were directly affected by the nutrient status in *L. perenne* leaves. It is also intriguing that the relative rates of elongation were reduced to a similar extent during the mitotic and postmitotic growth phase. During the mitotic phase, cell growth consists of an increase in the volume of the cytoplasm and organelles. This requires large amounts of nitrogen and phosphorus for the biosynthetic processes. During the postmitotic phase, cell growth is largely based on an increase in vacuolar volume, accompanied by uptake of water and osmotica along with the deposition of new cell wall material. The growth rate of a cell depends on cell wall extensibility, tissue hydraulic conductance and turgor pressure in excess of the yield threshold of the cell wall (Fricke 2002a). Only limited information exists about the role of these parameters in nutrient-deficient leaves. Changes in turgor have only a minor role in leaf growth responses to nitrogen and carbon stress (Fricke 2002a, and references therein). Thus, it is more likely that nutrient status induces changes in cell wall extensibility (possibly through expansins) or in tissue hydraulic conductivity (possibly mediated by aquaporins that are highly expressed in dividing and elongating cells; Chaumont *et al.* 1998). Whether the reduction in the growth rate during the mitotic and postmitotic growth phase was a direct result of the low availability of phosphorus and nitrogen that limited biosynthetic processes, or whether the reduction was mediated by some signalling pathway, also remains to be elucidated.

Particularly revealing is the finding that the division and growth rate of meristematic cells was highly coordinated under nutrient stress. It is not clear whether one of these two processes was limiting the other one, or whether both responded co-ordinately to the same signal. The rates of cell production and average cell division were positively correlated in the present studies, because the number of meristematic cells did not change under nutrient stress. Whether this also occurred in other studies reporting a decrease in cell production rate under unfavourable nutrient conditions (Volenec & Nelson 1983; Gastal & Nelson 1994; Fricke *et al.* 1997; Rademacher & Nelson 2001; Assuero *et al.* 2004) can not be conclusively established. However, it is necessary to stress that a change in cell production rate associated to a change in cell division rate is not necessarily a result of cell cycle regulation, because the causality between changes in cell division rate and mitotic growth rate is not clear.

The control of meristematic and final cell size

Meristematic cell length

The maintenance of a constant size in nutrient-deficient and sufficient meristematic cells, which differed in their growth and division rate, illustrates the tight control of cell size. The relatively small size of meristematic cells (20 and 25 μm, Table III.2 & IV.1) is probably dictated by the need for a high rate of intracellular transport in proliferating cells (Silk 1992). A high rate of protein synthesis in meristematic cells demands a short distance between the nucleus and the cytoplasm. This is manifested by the strict allometric relationship that exists between the nuclear and cell volume in

meristematic cells (Price, Sparrow & Nauman 1973). Similar relationship exists between meristem length and the number of meristematic cells: bigger meristems have more cells, but these cells are not bigger (Fig. V.1B).

A comparable range of values of the meristematic cell length has been reported in other C3 grasses (20-26 μm; Beemster *et al.* 1996; Fiorani *et al.* 2000; Masle 2000; Bultynck *et al.* 2003). In maize, a C4 species, the average meristematic cell length was slightly higher (22-38 μm; Ben-Haj-Salah & Tardieu 1995; Tardieu *et al.* 2000; Assuero *et al.* 2004). In contrast, root meristematic cells are shorter in arabidopsis (9-11 μm; Beemster & Baskin 1998; West *et al.* 2004) and maize (11 μm; Sacks *et al.* 1997). The difference in size between root and leaf meristematic cells might be related to a higher need for mechanical resistance in root meristematic cells. Smaller highly curved cells need relatively thinner cell walls to withstand a certain external or internal pressure (Silk 1992).

Although a coordination between cell growth and division rates in proliferating cells seems logical, conclusive proves and mechanisms explaining this phenomenon in plants are largely unknown (Ingram & Waites 2006). Indeed, little is known about the maintenance of optimum cell size in higher plants. In the unicellular *Chlamydomonas*, the size homeostasis is achieved by the need to exceed a critical minimum cell size for the progression from G1 to the subsequent phases of the cell cycle (John *et al.* 1993). In yeast, proteins involved in the translation affect both the cell cycle progression and cell growth (Jorgensen & Tyers 2004). First mechanistic links between the regulation of cell growth and cell division in plants have been reported recently (Li *et al.* 2005). The transcription factor encoded by arabidopsis *TCP20* binds specifically to the cyclin B1;1 promoter, and to several ribosomal protein promoters. A co-regulation of cell cycle and translation machinery may thus play a role in coordinating cell division and cell growth in plants as well. Cytokinins are another putative candidate, because their levels are regulated by phosphorus and nitrogen availability, and they affect both the cell cycle progression at the G1-to-S and G2-to-M transition (del Pozo *et al.* 2005), and have an effect on the expression of expansins and thereby on cell wall expansibility (Downes *et al.* 2001).

Final cell length

On the other hand, final cell length does not seem to be tightly regulated. The final length of a cell depends on several factors: the length of the cell leaving the division zone, the duration of the elongation phase, and the relative rate of elongation. Thus, not surprisingly, diverse results of nutrient deficiency have been reported, ranging from no effect on the final cell length (Volenec & Nelson 1983; Assuero *et al.* 2004), to slightly (Fricke *et al.* 1997), and substantially decreased cell length (Kavanová *et al.* 2006b). As discussed previously, when plants differing both in tiller size and nutrient status are compared, the decrease in the growth zone length can be counterbalanced by a slower displacement velocity in nutrient deficient cells, leading thus to a similar or longer duration of the elongation phase, which may eventually compensate for the lower relative elongation rate. Thus, no straightforward conclusion can be drawn from mature cell size regarding changes in elongation rates.

Conclusion

Under phosphorus and nitrogen deficiency, meristematic cells maintained proliferative activity for a substantially longer period of time, and the duration of the postmitotic elongation phase was also significantly increased. Thus, cell differentiation and maturation were delayed in nutrient deficient cells. The prolongation of the proliferative period was due to the fact that whereas the number of division cycles before a meristematic cells started elongating without further division was not affected by nutrient status, the time necessary to complete one cell cycle was substantially increased. Similarly, whereas the length of the elongation-only zone was not affected by nutrient deficiency, nutrient deficient cells were being displaced more slowly over its length due to the lower elongation rate of cells situated in the zone. In summary, nutrient deficiency did not affect the putative controls of the cell morphogenetic program, but, by slowing down the rates of cell division and growth and increasing thus the residence time in the growth zone, it slowed down the pace at which it was carried out.

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