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Spatial and Temporal Quantitative Analysis of Cell Division and Elongation Rate in Growing Wheat Leaves under Saline Conditions

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Abstract

Leaf growth in grasses is determined by the cell division and elongation rates, with the duration of cell elongation being one of the processes that is the most sensitive to salinity. Our objective was to investigate the distribution profiles of cell production, cell length and the duration of cell elongation in the growing zone of the wheat leaf during the steady growth phase. Plants were grown in loamy soil with or without 120 mmol/L NaCl in a growth chamber, and harvested at day 3 after leaf 4 emerged. Results show that the elongation rate of leaf 4 was reduced by 120 mmol/L NaCl during the steady growth phase. The distribution profile of the lengths of abaxial epidermal cells of leaf 4 during the steady growth stage shows a sigmoidal pattern along the leaf axis for both treatments. Although salinity did not affect or even increased the length of the epidermal cells in some locations in the growth zone compared to the control treatment, the final length of the epidermal cells was reduced by 14% at 120 mmol/L NaCl. Thus, we concluded that the observed reduction in the leaf elongation rate derived in part from the reduced cell division rate and either the shortened cell elongation zone or shortened duration of cell elongation. This suggests that more attention should be paid to the effects of salinity on those properties of cell production and the period of cell maturation that are related to the properties of cell wall.

Key words: cell production; epidermal cell length; growth zone; relative elemental growth rate; salinity; wheat.

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Leaf growth in grasses plays a central role in the development of plants. Not only is the expansion of the leaves crucial to early seedling establishment, but it is also responsible for the development of other organs such as tillers, ears and grains. The growth of wheat leaves, like other grass leaves, is limited to a small region near the leaf base that is enclosed in the older leaf sheath (i.e. the leaf growth zone), which is highly distinct and relatively simply organized (Hu et al. 2005). Thus, the grass leaf presents a good opportunity to study general leaf growth processes in plants.

Leaf growth in wheat, as for other plants like rice, barley, maize and sorghum, is one of the processes that are the most sensitive to salinity (Munns and Termaat 1986; Bernstein et al. 1993a, b; Hu et al. 2000). To fully the effect of salinity on leaf growth processes, there is a need to analyse the distribution profile of relative elemental growth rates along the growing grass leaves, which can be obtained by either: (i) an anatomical method that measures the lengths of cells or, (ii) marking methods such as making pinholes in the growing leaf with a fine needle and making a mark with ink (Davidson and Milthorpe 1966; Schnyder et al. 1987). However, in the latter case, it has been shown that making the pinholes themselves causes a reduction in the leaf elongation rate of grasses by 30%–50% (Bernstein et al. 1993a, b; Ben-Haj-Salah and Tardieu 1995; Palmer and Davies 1996; Fricke et al. 1997; Hu et al. 2000). Therefore, in studies of stressed plants, the reduction in leaf elongation rate caused by pricking will confound drawing accurate conclusions of the effects of stresses (e.g. drought, salinity, temperature) on the spatial distribution of leaf elongation. Moreover, the leaf elongation rate is influenced by the cell division and elongation rates and by the duration of cell elongation. Thus, by studying the effects of salinity (or any other stress) on the distribution profiles of cell length, the cell division rate can also be obtained in contrast to pinhole or other marking methods. Although analyses of the relative elemental growth rate under salinity have been undertaken for wheat (Hu et al.

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2000) and sorghum (Berstein et al. 1993a, b), there is still a general lack of information on the effect of salinity on the profile of the sizes of cells along the growing leaf in grasses.

Kinematic methods can be used to measure rates of cell division in grass leaves because of the simple organization and distinct growth zone of the latter. More importantly, this approach can measure not only local rates of cell division, but also local rates of expansion by balancing the rates at which cells enter and exit a particular region. These latter relations are formalized with the equation of continuity from hydrodynamics (Gandar 1980; Silk 1984). Altogether, spatial analysis of leaf elongation and the use of the continuity equation (Gandar 1980; Silk 1984) provide a useful tool for analysing the cellular growth process. Studies on the effects of diverse environmental changes on the cellular processes underlying leaf expansion rates have been conducted in different crop species. Examples included the effects of temperature on maize (Ben-Haj-Salah and Tardieu 1995); of soil resistance (Beemster et al. 1996) and elevated CO_2 (Masle 2000) on wheat; of nitrogen on barley (Fricke et al. 1997) and on tall fescue (MacAdam et al. 1989; Rademacher and Nelson 2001); of temperature and drought on maize (Granier et al. 2000); and of different genotypes including *Festuca* species (Volenec and Nelson 1981), *Poa* species (Fiorani et al. 2000) and *Aegilops* species (Bultynck et al. 2003).

The objectives of this study were to investigate leaf growth at the cellular level in the growing leaves of the mainstem in wheat plants and to estimate the cellular basis (i.e. changes in any of cell division rate, cell elongation rate, or cell growth duration) for the reduction in the leaf growth rate under saline conditions using a one-dimensional continuity equation together with other numeric analysis. To our knowledge, this is the first report about the effect of salinity on the profile of the epidermal cell length along the growing leaf of wheat that was used to estimate the relative elemental growth rate, velocity displacement, cell division rate and duration of cell elongation.

Results and Discussion

The final length of the fully developed leaf 4 was about 32 cm for the control treatment and 25 cm for the saline treatment. The elongation rate of leaf 4 was determined to be 2.73 mm h^{-1} for the control treatment on the third day after the leaf emerged, compared to about 2.25 mm h^{-1} for the saline treatment. Thus, salinity reduced the leaf elongation rate by about 18%, which is consistent with the results of Hu et al. (2000), who examined the effect of salinity on the wheat leaf growth.

Spatial distribution of epidermal cell length

The distribution profile of the lengths of the abaxial epidermal cells of leaf 4 during the steady elongation phase shows a sigmoidal distribution pattern along the leaf axis for both

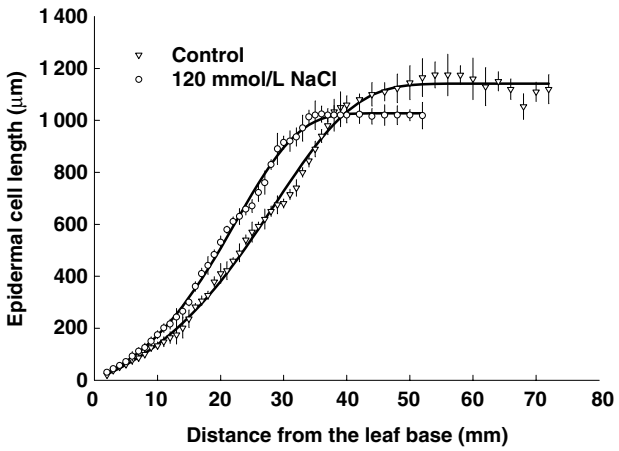

Figure 1. Distribution profiles of the abaxial epidermal cell length of the growing leaf 4 on the mainstem of wheat during the steady elongation phase. Smoothed line curves of cell length were obtained by fitting five-parameter Weibull. Plants were grown in soil with or without 120 mmol/L NaCl. Data are (means ± *SE*) of six replicates per treatments.

treatments (Figure 1). The cell lengths were the smallest at the leaf base regardless of the treatment and increased up to a maximum of about 1 140 μm and 1 000 μm at the distal parts of the leaf for the control and saline treatments, respectively. As such, the final cell lengths were reduced by 120 mmol/L NaCl (about 14%), which may in turn have contributed to the smaller final size of the leaf. However, cell lengths did not differ between the two treatments up to 10 mm above the leaf base, and was even higher in the saline plants between 10 and 40 mm above the leaf base than for the control plants. This might indicate that salinity does not affect or might even enhance cell elongation in some locations, which may explain the observation that turgor in the growing tissues is not decreased by an increase in salinity (Thiel et al. 1988; Yeo et al. 1991; Fricke and Peters 2002). Normally, under saline conditions, low osmotic potentials of the soil solution induce water deficit in plant tissues and, as a consequence, cell turgor pressure decreases. However, the lack of a negative effect in some locations of the growing zone may derive from a sufficient compensatory osmotic adjustment, where elongating cells adjusted to changes in external water potential by accumulating more solutes and by reducing the volume expansion (Hu and Schmidhalter 1998; Fricke and Peters 2002). Similarly, several other published reports also demonstrate that leaf growth is reduced without an associated effect on the elongation of individual cells for tall fescue under N deficiency (Volenec and Nelson 1983; MacAdam et al. 1989; Gastal and Nelson 1994), and for *Zea mays* L. under P deficiency (Assuero et al. 2004). Thus, the reduction in leaf elongation under saline conditions observed here might be due to any of a decrease in the rate of cell division, a shorter cell elongation zone, or faster cell maturation.

Velocity of displacement, relative elemental growth rate and size of the growth zone

The spatial distribution of leaf elongation was characterized by the rate of displacement of a particle from the leaf base resulting from the relative elemental growth rate (Figures 2 and 3), which was estimated in turn based on the cell length and the leaf elongation rate. The spatial distribution of the velocity of displacement along the leaf axis was similar to that of the cell length for both treatments. The maximum velocity of displacement is equal to the leaf elongation rate (Hu et al. 2000). The reduction in the leaf elongation rate was greater than that in the final cell length under saline conditions (Figures 1 and 2),

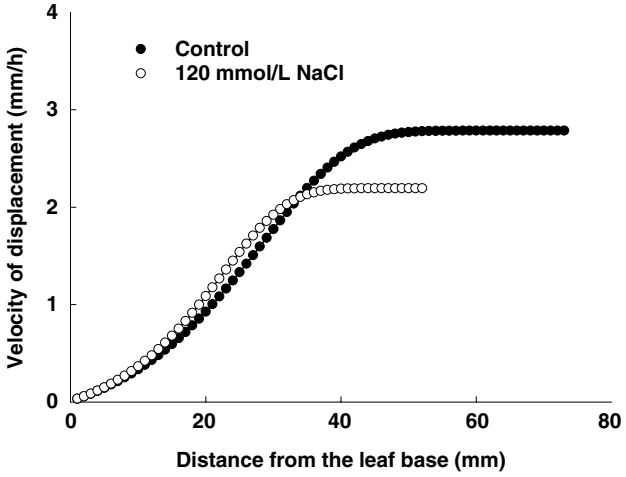

Figure 2. Spatial distribution of the displacement of velocity in the growing leaf 4 on the mainstem of wheat during the steady elongation phase. Plants were grown in soil with or without 120 mmol/L NaCl.

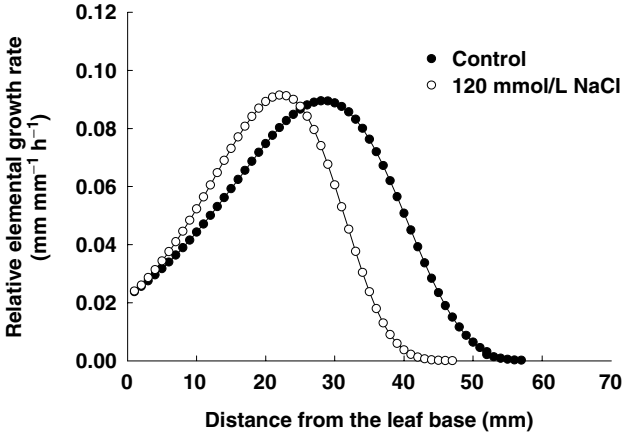

Figure 3. Spatial distribution of the relative elemental growth rate (mm per mm per hour) in the growing leaf 4 on the mainstem of wheat during the steady elongation phase. Plants were grown in soil with or without 120 mmol/L NaCl.

indicating that other parameters, such as cell production, that contribute to the leaf elongation rate may be reduced by salinity in addition to cell elongation.

The relative elemental growth rate for both treatments was bell-shaped (Figure 3). It increased from the leaf base, reached a maximum, and then decreased to close to zero at the leaf tip. The relative elemental growth rate under salinity was higher up to about 25 mm above the leaf base than that for the control plants, whereas it was smaller under salinity beyond this point. The maximum of the relative elemental growth rate was slightly higher under salinity than it was for the control treatment.

The size of the growth zone of grass leaves is defined as the distance between the leaf base and the position where the relative elemental growth rate first approaches zero. In this study, the growth zone was about 42 mm long under salinity compared to about 54 mm for the control treatment (Figure 3), a reduction of about 22%. These results differed from those in our previous study (Hu et al. 2000), which showed the length of the growth zone of wheat leaf 4 during the steady elongation phase was about 30 mm and was not affected by adding 120 mmol/L NaCl. These conflicting results may derive from the different approaches used in both studies, where the previous measurements were determined using the pinning method, which reduced the leaf elongation rate by about 40% to 50%. Thus, this study suggests that the pinning method reduces the leaf elongation rate due to both the reduced relative elemental growth rate and the reduced length of the growth zone, because the leaf elongation rate is a function of both these variables (Hu et al. 2000). The differential effects of salinity on growth in the leaf elongation zone between these two studies also suggest that caution must be used in determining the relative elemental growth rate and the length of the leaf growth zone when the pinning method is applied. This caution is reinforced in noting that other pinning studies of the effect of salinity on the relative elemental growth rate and the size of the leaf growth zone produced conflicting results to those noted above. For example, Bernstein et al. (1993a, 1993b) reported that salinity inhibited leaf growth in sorghum by shortening the leaf growth zone and by reducing the relative elemental growth rate. Furthermore, the pinning method results in a smaller size of the growth zone in wheat plants regardless of the treatment type, a finding that is also true for the leaves of tall fescue (R. Schäufele, personal communication).

The relative elemental growth rate is a parameter that allows comparisons of the efficiency of growth for different leaves under different conditions, where the more efficient leaves are those that are elongating more per unit of growing tissue (Kemp 1980). As mentioned, the relative elemental growth rate in some locations of the growth zone is greater under saline conditions than for the control plants, indicating a more efficient leaf growth. However, the size of the growth zone was markedly reduced, suggesting the shorter period of cell growth.

Duration of cell elongation

In addition to its spatial aspect, the leaf axis also constitutes a time gradient representing the relative age of cells as they are moving away from the leaf base. Hence the time to cross the growth zone of the leaves represents the duration of the corresponding developmental stage for the constituent cells. During the steady elongation phase of the leaves, the duration of cell elongation was about 135 h versus 122 h for the control and saline treatments, respectively (Figure 4). Thus, the residence times for each cell in the growth zone was shorter under salinity than under control conditions, which may result in the shortened growth zone observed under saline conditions. Because the elongation time is also reduced by salinity, the cells at the end of the growth zone of salt stressed leaves were also smaller. The increase in cell length occurred largely between 100 and 130 hours for both treatments (Figure 4 Inset).

The duration of individual cell elongation is also important because any reduction might reduce the leaf elongation rate without concomitant changes in the cell division and elongation rates (MacAdam et al. 1989). The duration of cell elongation can be determined by the chemical composition and properties of the cell wall. A modified capacitance of cell walls to irreversibly yield was suggested to be the major factor limiting growth under salt stress (Cramer and Bowman 1991; Neumann et al. 1994). The deposition of the secondary cell walls follows cell elongation, which might prevent further cell expansion (MacAdam et al. 1989). Other factors that might contribute to the cessation of cell elongation have also been investigated. Extensibility of the cell wall is thought to be reduced by the formation of covalent bonds between phenolic residues of pectins, hemicelluose, and structural proteins of the cell wall (Fry 1986). Proxidase promotes this bonding by catalyzing the formation of free radicals of the residues (de Souza and McAdam 2001). Recent reports have highlighted the biochemical regulation such as expansion of cell wall extensibility as a key process in controlling growth in plants and have led to the identification of several proteins that are potentially involved in this process (Cosgrove 1999). For instance, the XET-related gene, *Fp*XET1, is a potential marker for leaf elongation in the growth zone of grasses (Reidy et al. 2001). Apoplastic pH is also considered to play an important role in cell wall loosening and tissue growth, with several environmental conditions that affect growth having been shown to alter apoplast acidification. For example, growth inhibition by water stress is accompanied by an increase in apoplastic pH and a decrease in the acidification rate (Van Volkenburgh and Boyer 1985; Hartung et al. 1988). However, Neves-Piestun and Bernstein (2001) reported that the salinity-induced inhibition of leaf elongation in maize is not mediated by changes in the acidification capacity of the cell wall in growing tissues of the leaves.

Cell division rate and cell flux

The cell division rate for both treatments decreased markedly within the first 5 mm above the leaf base, with a further, albeit slower decline up to 30 mm above the leaf base (Figure 5), implying that the cell division zone must be restricted to the initial 5 mm above the leaf base for both treatments. The cell division rate at the leaf base was about 8 and 5 cells mm^{-1} h^{-1} for the control and saline treatments, respectively. As such, salinity significantly reduced the cell division rate by about 30% within the initial 1–2 mm above the base. In tall fescue, epidermal

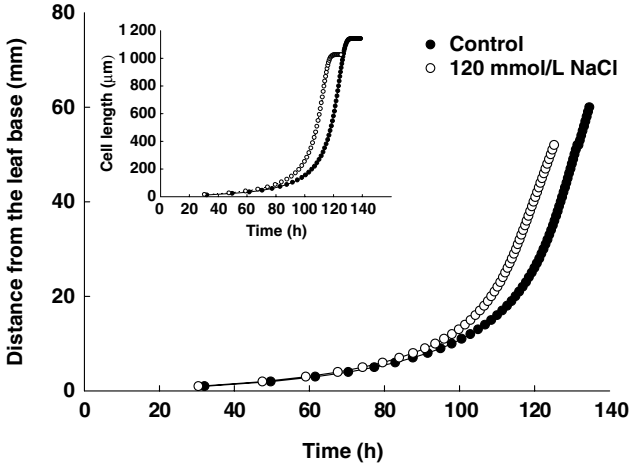

Figure 4. Time of cell elongation needed to reach the distal limit of the elongation zone in the growing leaf 4 on the mainstem of wheat during the steady elongation phase. Plants were grown in soil with or without 120 mmol/L NaCl.

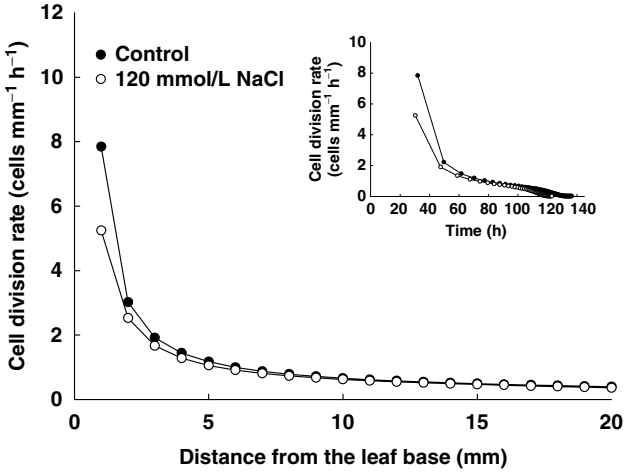

Figure 5. Spatial distribution of the cell division rate in the growing leaf 4 on the mainstem of wheat during the steady elongation phase. Plants were grown in soil with or without 120 mmol/L NaCl.

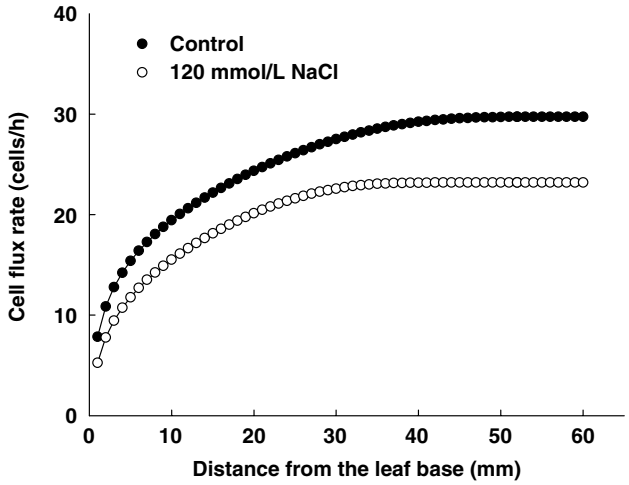

Figure 6. Spatial distribution of the cell flux rate in the growing leaf 4 on the mainstem of wheat during the steady elongation phase. Plants were grown in soil with or without 120 mmol/L NaCl.

cell division is restricted to the basal 1.5–2.0 mm of the leaf, with the division zone of mesophyll cells being extended to 5–10 mm above the leaf base (MacAdam et al. 1989; Skinner and Nelson 1995). During the 122 hours of cell growth for the saline treatment and the 135 hours for the control treatment, the most rapid cell division occurred in the first 60 hours (Figure 5 Inset). This observation, together with the effect of salinity on the cell elongation, indicates that salt stress decreased the rate of leaf elongation by reducing the mature cell size and lowering the number of dividing cells. A recent study of the effect of salinity on cell production in the roots of *Arabidopsis* (West et al. 2004) also reported similar findings in that the elongation rate of the roots was reduced by salinity due to decreases in the cell division rate and final cell length. However, the response of the leaf expansion rate to salinity was analysed mainly in terms of changes in cell turgor and cell wall properties.

Because the time required for a cell to leave the growth zone was higher under control conditions than under salinity, cell flux in the mature zone (i.e. outside the growth zone) essentially reflected the higher cell production associated with a longer cell division zone and with higher cell production rate under control conditions (Figure 6).

Cell division is probably controlled by signalling and candidate genes. However, the connection between stress signalling and the control of cell division still needs to be better understood (Zhu 2001). A potentially important link between stress and cell division was revealed by induction of ICK1 in *Arabidopsis* by ABA (Wang et al. 1998). ICK1, a cyclin-dependent-protein-kinase inhibitor, might hinder cell division by reducing the activities of cyclin-dependent protein kinases that help to drive the cell cycle (Zhu 2001). Salt stress therefore might inhibit cell division by causing the accumulation of ABA, which, in turn, induces ICK1. The recent study by Cramer and Quarrie (2002) showed that ABA concentrations in the leaf growth zone of maize were highly correlated with the reduction in the leaf elongation rate for all four genotypes they examined. Their results thus suggest that ABA concentration in the growth zone of the leaf is a good predictor of the leaf elongation response to salinity. As such, it is clear that a combination of spatial analyses of the cell division and expansion rates together with the activities of candidate enzymes and of the accumulation of hormones (such as ABA) can be a powerful tool to identify the mechanisms that limit leaf expansion in grasses under saline conditions.

In conclusion, our results show that the lower leaf elongation rate of wheat leaf 4 on the mainstem during the steady growth phase under saline conditions was associated with a reduced cell division rate and either, a shortened cell elongation zone, or duration of cell elongation rather than a reduction in the amount of cell elongation itself. Therefore, our study suggests that more attention should be focussed on investigating the effect of salinity on the cell production and period of cell maturation that is related to the properties of the cell wall.

Materials and Methods

Growth conditions

Six seeds of spring wheat (*Triticum aestivum* L. cv. Thasos) that were pre-germinated for two days on filter paper wetted by tap water at 20 °C were sown in 1.5-L pots (10 cm in diameter and 20 cm high) containing loamy soil. The soil was initially watered to 0.25 g H_2O g^{-1} dry soil (which allowed for the optimum aeration) with 0.2 g NH_4NO_3 per kg dry soil also being added. Plants in the experimental saline treatment were subjected to a salt level of 120 mmol/L NaCl, which was obtained by adding NaCl to the nutrient solution. The soil was thoroughly mixed and kept in tightly closed plastic boxes for one week to facilitate equilibration. Thereafter, the soil was sieved and put into the pots. The soil moisture content was maintained at the initial level by watering with tap water as needed. To minimize water loss by evaporation, the pots were covered with a perforated plastic film, where plants could grow through the small holes. One week after sowing, the seedlings were thinned to four plants per pot.

The experiment was conducted in a growth chamber with a 16-h photoperiod. The light intensity was approximately 550 μmol photon^{-2} s^{-1} (PPFD). The air temperature was 20 °C (day/night) and the relative humidity was maintained at 55%–65%.

Determination of the leaf elongation rate and cell lengths in the growing leaf 4

Because salinity delays the development of grass leaves (Hu et al. 2000), all measurements in each treatment (i.e. control

and saline) were taken at the same development stage. The leaf elongation rate during the light period was determined by using a ruler to measure the increase in the length of leaf 4 during 10 hours on day 3 after its emergence. There were six replicates. These plants were grown until leaf 4 was fully developed at which point their final leaf blade length was determined. On the same day as the measurement of the elongation rate of leaf 4, the remaining plants were used to determine the cell lengths. The sampling started at 5-h into the 16-h photoperiod. Six replicates were harvested successively; all sampling was finished within 1–2 h. To ensure that sampling occurred in the steady growth phase, the leaf blades 14–16 cm in length were selected for sampling according to Hu et al. (2000). The basal parts of the leaves (0–10 cm above the leaf base) were placed on a glass plate. A 4% solution (w/w) of polyvinylformaldehyde (Formvar 1595E, Merck, Darmstadt, Germany) in chloroform was carefully painted on the leaf surface using a fine paintbrush (Rademacher and Nelson 2001). After evaporation of the chloroform, the Formvar formed a transparent negative film of the leaf abaxial epidermis. This film was removed by using transparent adhesive tape, and was then transferred to a microscope slide and stored for later use. Cell lengths in the replicates were measured using a Zeiss microscope (Zeiss axioscope, Germany) at a magnification of 2.5–20.0 times, with the objectives being connected to a PC-based image processing system. Images were captured using a Zeiss Axio Vision system mounted on top of the microscope. An image analyzer, SigmaScan Pro 5 (SYSTAT Software Inc., Point Richmond, CA, USA), was used to measure the cell lengths. Starting from the point of attachment of the leaf to a point 20 mm above the leaf base, the lengths of at least 10 cells were measured at 1 mm increments, and then 5–10 cells at 2 mm increments from 20 to 80 mm at control and to 60 mm at 120 mmol/L NaCl above the leaf base. According to the cell types in abaxial epidermal cells of wheat leaves as classified by Beemster and Masle (1996), the elongated cells along the same cell files were determined.

The lengths of the epidermal cells in the growth zone are assumed to increase smoothly and continuously with distance (Fiorani et al. 2000; Bultynck et al. 2003). Thus, adjusted curves of cell length were obtained by fitting five-parameter Weibull curves using the TableCurve 2D® (Systat Software Inc., CA, USA). The smoothed data were used for the calculations described in the following sections.

Spatial analysis of leaf elongation and the length of the growth zone

The spatial analysis of leaf elongation is characterized by the displacement velocity (V_{di}), the rate of displacement of a particle from the leaf base, and by the relative elemental growth rate (R_{ei}). Based on the measurements of leaf elongation and cell length along the leaf axis, the velocity of displacement was calculated as:

$$V_{di} = V_{max} L_i / L_{max}, \quad (1)$$

where V_{di} is the velocity of displacement (mm h^{-1}) at the position i (i = 1, 2, . . ., n), V_{max} is leaf elongation rate (mm h^{-1}), L_{max} is the final length of the epidermal cells (mm/cell) and L_i is the length of an epidermal cell at position i (Gandar and Hall 1988; Silk et al. 1989; Bultynck et al. 2003). Equation 1 might underestimate the velocity of displacement in the cell division zone of epidermal cells in different grass leaves that is mainly located up to about 2–3 mm above the leaf base (MacAdam et al. 1989; Beemster et al. 1996). Since V_{di} like the cell length are assumed to increase smoothly and continuously with distance, adjusted curves of V_{di} between the leaf base and about 5 mm above the leaf base were obtained by fitting five-parameter Weibull curves using the TableCurve 2D® (Systat Software Inc., CA, USA).

Local relative elemental growth rate (relative elemental growth rate expressed as mm per mm per hour (mm mm^{-1} h^{-1}; R_{ei}) was obtained by differentiating local velocity (V_{di}) at position i:

$$R_{ei} = f'(x), \quad (2)$$

where x is the distance from the leaf base (Silk and Erickson 1979; Gandar 1980). The TableCurve 2D® (Systat Software Inc., CA, USA) was employed to differentiate the local velocity.

The length of the growth zone was calculated as the distance from the leaf base to the position at which R_{ei} was around 2% of the maximum relative elemental growth rate (Hu et al. 2000).

Estimation of the cell division rate, cell flux, and the duration of cell elongation

Cell division rate (D_i)

The cell division rate (cell number per mm of file length per hour) was calculated according to the one-dimensional version of the continuity equation as described by Gandar (1980) and Silk (1992):

$$D_i = (\partial P_i / \partial t) + V_{di} \cdot (\partial P_i / \partial x_i) + (R_{ei} \cdot P_i), \quad (3)$$

where P_i is the cell density per unit of leaf length at position i, and x_i is the distance from the base at position i. During the steady phase of leaf elongation, the cell density should not change with time. Thus, $\partial P_i / \partial t = 0$.

Duration of cell growth (T_i)

The time course of displacement of a given cellular particle through the elongation zone is given by the growth trajectory. The time needed for a material point to move from the proximal to the distal end of segment i (T_i) was calculated as the ratio of the length of the segment ($\triangle x_{i,0}$) to the local velocity (V_{di}):

$$T_i =_{\triangle} x_{i,0} / V_{di}. \quad (4)$$

The total time needed for a material point to move from its present position to the distal end of the elongation zone was calculated as the sum of T_i from the material point to the distal limit of segment i in the elongation zone (Schnyder et al. 1990).

Cell flux rate (f_m)

Cell flux in the mature zone (f_m, cells h^{-1}) was calculated (Fraser et al. 1990) as the ratio of V_{max} to mature cell length (L_{max}):

$$f_m = V_{max}/L_{max}. \quad (5)$$

The local cell flux in position i (f_i, cells h^{-1}) was calculated as the integral of the division rates from the ligule to the position i. This integral equals ($V_{di} \cdot P_i$) and can also be calculated with the summation formula:

$$f_i \overset{i}{=} \sum_0 D_i \Delta x_{i,0} (i = 0, 1, 2, \cdots n) \quad (6)$$

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