

Commentary

Kinematic Analysis of Leaf Growth in Grasses: A Comment on *Spatial and Temporal Quantitative Analysis of Cell Division and Elongation Rate in Growing Wheat Leaves under Saline Conditions*

Hu and Schmidhalter (2008) conducted a study with wheat seedlings growing in saline and non-stressed (control) conditions with the aim of identifying and quantifying the cellular basis for the reduction in leaf growth. We applaud their goals as salinity is an important issue for plant ecology and food production; however, we have concerns about the methodology used and the subsequent conclusions that are drawn.

Hu and Schmidhalter measured independently the leaf elongation rate and the epidermal cell length distribution in the growth zone of leaf 4, and used these data to investigate the spatial distribution of cell division rates and the kinetics of cell elongation in the abaxial epidermis. They reported that the saline treatment significantly reduced leaf elongation rate by about 18% and final epidermal cell length by about 12%. Based on these data, and using established methods, we calculate a 6% reduction in the cell production rate due to salinity (see below). In contrast, they reported that the saline treatment significantly reduced the cell division rate by approximately 30% in the zone of most active meristematic activity and that overall cell production (the integral of cell division rate throughout the meristem) was reduced by approximately 24% (see maximum cell flux in their figure 6). This implied that cell production was impaired more by saline conditions than cell elongation. We have looked carefully at the methodology used and the data pre-

sented and find that their conclusion is in error.

An assessment of their *reported measurements* actually implies that cell production was reduced only marginally. This is demonstrated by the (measured) effects of salinity on mature epidermal cell lengths and leaf elongation rates, which are reported in the paper. In the steady state, which is a condition for their analysis, total cell production (F , cells per cell file per h, which is termed “cell flux in the mature zone” in the paper) is related to mature epidermal cell length (L_{\max} , mm/cell) and leaf elongation rate (V_{\max} , mm/h) as follows (Silk 1992):

$$F = V_{\max}/L_{\max} \quad (1)$$

Hu and Schmidhalter found that leaf elongation rate was reduced by 18% and final cell length by 12% (not a reduction of 14% as they stated) under saline conditions, meaning that cell production required to support the growth rate was reduced by only 6%. Although Equation 1 (above) is given in the paper (their equation 5), the results of this accepted calculation are not presented. Instead, the authors reported cell production rate as the summation of cell division rates throughout the meristem. This method is valid only if the parameters required for the calculation are obtained in the correct way. This is the major concern of this comment.

Local cell division rate (D_i , cells per mm cell file per h) along the length of the meristem can be estimated by applying the one-dimensional version of the continuity equation to growth velocity and cell length distribution in a contiguous file of cells along the meristem (Gandar

1980; Silk 1992). Accordingly, local cell division rate (D_i) is defined as:

$$D_i = \partial P_i / \partial t + (V_i \times \partial P_i / \partial x_i) + (\partial V_i / \partial x_i \times P_i) \quad (2)$$

where P_i is the (one-dimensional) cell number density (number of cells per unit length of a contiguous cell file, cells/mm) at location i , x_i is the distance from the leaf base (mm) at location i , V_i (mm/h) is growth velocity, that is, velocity of displacement relative to the leaf base (mm/h) at i , and $\partial V_i / \partial x_i$ is the relative elemental growth rate (denoted R_e in their paper) at i . Cell number density (cells per mm in a cell file) equals the inverse of cell length ($P = L^{-1}$). $\partial P_i / \partial t$ denotes the change of cell number density per unit time at position i and is zero in the steady-state ($\partial P_i / \partial t = 0$).

Equation 2 shows that the calculation of the cell division rate distribution requires knowledge of the cell length and velocity of displacement distributions within the “cell division” region. Hu and Schmidhalter did not measure the velocity of displacement distribution in this region, but estimated it from the cell length data (their equation 1: $V_i = V_{\max} \times L_i / L_{\max}$). This approach is valid for the “elongation-only” region of the leaf growth zone, where velocity of displacement is proportional to cell length. But it is invalid in the cell division zone, where cell length depends on two processes, cell elongation and cell division (Green 1976; Silk 1992). Cell division rates cannot be estimated with the continuity equation if independent measurements of displacement velocity and cell length distribution are not available.

Cell division in a file of cells begins when an initial divides to form two daughter cells. The initial in tall fescue (*Festuca arundinacea* Schreb.) is often about 20 μm long and expands up to about 50 μm long to divide and form two cells each about 25 μm long, one of which remains as the initial. Both increase in length, again to about 50 μm then divide with the proximal one staying at the base as the initial where it continues to enlarge, divide and enlarge again. The daughter cells that are produced also enlarge, divide and so on, such that there are several cells in the file that are dividing, enlarging and dividing again (MacAdam et al. 1989; Skinner and Nelson 1994). At the distal end of the cell division region the cell enlarges, but does not divide and enters the cell elongation region. Thus, cell lengths in the cell division region do not increase in a permanent manner and the cell length stays within the range given by the minimal cell length (recently divided cell) and maximal cell length (mitotic cell). So, while active expansion growth occurs inside the meristem, this is not mirrored in the cell length distribution in this region.

Hu and Schmidhalter did not address the condition, that independent measurements of displacement velocity and cell length in the meristem are required for estimation of cell division rates. But they recognized that the equation relating velocity to cell length (their equation 1) "might underestimate the velocity of displacement in the cell division zone" and generated "adjusted curves of V_{di} between the leaf base and about 5 mm above the leaf base". This adjustment, "obtained by fitting five-parameter Weibull curves", seems an arbitrary procedure and effectively produced the erroneous rate and distribution of cell division. Without the adjustment, the velocity of displacement data (calculated with their equation 1) would have yielded cell division rates equal to zero everywhere in the meristem. Furthermore, the magnitude and distribution of cell division rates obtained by the adjustment

were also unrealistic. In addition, and most importantly, the curve adjustment procedure invalidated the subsequent analyses.

As a result, it remains unclear if the slight decrease in the cell production rate was due to a decrease in the cell division rate or to a reduction in the number of meristematic cells in the division zone. Other parameters calculated with the erroneous adjusted velocity data (Figure 2) were also faulty and include the relative elemental growth rate (Figure 3), duration of cell growth (Figure 4), cell division rate (Figure 5), and cell flux (Figure 6). Accordingly, the conclusions drawn from the calculations are also incorrect and some of the data are contradictory. For example, the cell production rate in the control treatment (see maximum cell flux in Figure 6) can not under any circumstance be 30 cells/h, since the final cell length was 1.14 mm and leaf elongation rate was 2.73 mm/h. If it was real, then the leaf elongation rate must have been 34.2 mm/h, more than 12 times the measured rate.

Moreover, their analysis suggests that epidermal cell division continued to the end of the growth zone (cell flux increased until the end of the growth zone in Figure 6). This contrasts with all other studies in leaves of Poaceae (including studies of wheat and its ancestors), which show that epidermal cell division is restricted to the basal 5% to 25% of the leaf growth zone (MacAdam et al. 1989; Beemster et al. 1996; Masle 2000; Fiorani et al. 2000; Bultynck et al. 2003; Kavanova et al. 2006, 2008).

Clearly, a different approach is needed to estimate the velocity and elemental growth rate distribution in the cell division-region of the leaf growth zone. In roots or hypocotyls, on which marks can be observed directly and continuously, velocity data have been derived from observational records (e.g. streak photographs, image sequence analysis) of displacement of natural or artificial marks on the surface of the tissue in the meristematic regions (Erickson and Sax 1956; Walter et al. 2002; van der Weele

et al. 2003). But, such measurements cannot be made in a non-destructive way in the leaf growth zone of Poaceae, since it is enclosed by the sheaths of older leaves and is therefore not directly accessible.

At present, aside from cell-length profiles in the elongation-only region, the only available alternative technique for the derivation of growth velocities in the leaf growth zone of grasses is the marking of the growth zone by pinning through the surrounding sheaths and measuring the pin-hole displacement after a short time interval (Schnyder et al. 1987; Bernstein et al. 1993; Ben-Haj-Salah and Tardieu 1995) or by following ink marks placed on the growth zone after removal of the surrounding sheaths (Volencic and Nelson 1981; Schnyder et al. 1987). In principle, the pinning method can also be used to determine the velocity distribution in the cell division zone of the leaf, which is necessary for calculation of cell division distribution using the continuity equation. But since the velocity is slow the time interval for accuracy becomes a challenge. In contrast with the anatomical method, however, the marking methods do not depend on the steady-state condition. This also means that the anatomical and marking methods will give different growth rate distributions, if the steady-state condition is not satisfied.

Hu and Schmidhalter disregarded the pinning method. They argued that it causes artefacts in the growth rate distribution, stating "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" and more particularly they write that "the pinning method results in a smaller size of the growth zone ...". However, Hu and Schmidhalter did not present direct evidence for their statement. Also, their concern is not new and has been addressed in several studies. Schnyder et al. (1987) demonstrated that although leaf

elongation rate was substantially reduced for several hours after pinning the relative distribution of growth was not affected. Moreover, in leaves of *Lolium perenne* L. during the phase of near-constant leaf elongation, the spatial distribution of relative elemental growth rates obtained from pinning corresponded well with that derived from the anatomical record (Schnyder et al. 1990). These studies indicated that the relative effect of pinning on local growth rate was uniform over the growth zone and could be therefore corrected by applying a correction coefficient (Schnyder et al. 1987; Ben-Haj-Salah and Tardieu 1995).

Using a two-pinhole method, Hu and Schmidhalter (2000) confirmed that the *relative* reduction in tissue expansion due to the pinning did not differ along the whole growth zone. Of interest is whether there is an interaction between the effect of saline conditions and pinning, but Hu and Schmidhalter did not examine this. Nevertheless, they noted that the growth rate distributions obtained in their study (which used the anatomical method) differed from that obtained in their former investigations (which used the pinning method) and suggested that artefacts by the pinning method were responsible for the difference. In view of the above, we deem it more likely that the differences were associated with other factors, including the described errors in the derivation of the growth rate distribution from the anatomical data, size effects on growth zone dimensions (Kavanova et al. 2005), or experimental variation.

A disadvantage of the pinning method is its limited spatial resolution, which reduces its usefulness in studies of cell division in short meristems, such as that of wheat leaves. However, the method was successfully used with maize leaves (Granier et al. 2000; Assuero et al. 2004) (the work by Granier et al. 2000 also demonstrated a close correspondence between the activity of p34^{cdc2} kinase, involved in the progression of the cell cycle, and the cell division rate

assessed by the continuity equation that was based on pinning and cell number data). Unfortunately, at present there seems to be no better alternative method to assess directly the distributions of growth rates in the cell division region of grass leaves. Therefore, studies of cell division in leaves of Poaceae have generally used different approaches. Since the information on growth rates in the meristem is so difficult to obtain, these alternative approaches all depended on the measurement of some cell division marker (see below). These studies can be grouped in two categories: (i) those that assessed *average* division rates of cells in the meristem (e.g. Fiorani et al. 2000; Kavanova et al. 2006) and (ii) the ones that also resolved the *spatial distribution* of cell division rates in the meristem (Beemster et al. 1996).

Average cell division rate (D_{avg} , cells per cell per h) is defined as the number of cells produced per meristematic cell per unit of time, and is calculated by dividing total cell production in a cell file (F) by the number of meristematic cells in that cell file (N_{div} , cells per meristematic cell file):

$$D_{\text{avg}} = F/N_{\text{div}} \quad (3)$$

The number of meristematic cells is obtained by counting the number of cells in a contiguous cell file between the basal and distal limit of the meristem (i.e. division zone). Several markers have been used as an indicator for the boundary between the cell division zone and the elongation-only zone (that is the distal limit of the division zone): an increase in the average cell length (Skinner and Nelson 1994), observation of the last mitosis in stained preparations (Fiorani et al. 2000) or determinations of the last freshly deposited transverse cell wall (indicative of a recent division) in cleared leaves (Beemster et al. 1996; Masle 2000) or on replicas of the abaxial epidermis (Kavanova et al. 2006). Under steady state conditions, this approach to cell division rate only requires measurements of leaf elongation rate and

final cell size, in addition to the number of meristematic cells per cell file (see equations 1 and 3). Additional functional parameters of meristematic activity can be derived from cell production rate and cell number in the meristem, including: average cell cycle duration, the average residence time for a cell in the meristem, and the average number of division cycles of the initial's cell progeny (e.g. Skinner and Nelson 1994; Kavanova et al. 2006).

The only way by which the spatial distribution of cell division rates can be determined without knowledge of cell length and growth rate distribution is by the use of a direct quantitative indicator of cell division. Thus, Beemster et al. (1996) analyzed the spatial distribution of freshly deposited cell walls in the meristem of wheat leaves and estimated the spatial distribution of cell division rate by dividing the number of freshly deposited cell walls by the number of cells in certain intervals along the division zone. In view of the difficulties associated with the assessment of growth rates in the meristems of grass leaves (which are, as discussed above, necessary for quantifying the spatial distribution of cell division rate from cell length profiles), the development of improved and alternative methodologies for the direct quantification of cell division activity in grass leaf meristems is a commendable goal. Such advances may come from the use of alternative microscopic markers (e.g. phragmoplast as an unambiguous marker for cytokinesis) or from work with reporter lines for cell cycle activity, which has already been useful in developmental and growth studies in dicots (Donnelly et al. 1999, West et al. 2004).

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