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Plasticity in sunflower leaf and cell growth under high salinity

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Keywords

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ABSTRACT

A group of sunflower lines that exhibit a range of leaf Na^+ concentrations under high salinity was used to explore whether the responses to the osmotic and ionic components of salinity can be distinguished in leaf expansion kinetics analysis. It was expected that at the initial stages of the salt treatment, leaf expansion kinetics changes would be dominated by responses to the osmotic component of salinity, and that later on, ion inclusion would impose further kinetics changes. It was also expected that differential leaf Na^+ accumulation would be reflected in specific changes in cell division and expansion rates. Plants of four sunflower lines were gradually treated with a relatively high (130 mM NaCl) salt treatment. Leaf expansion kinetics curves were compared in leaves that were formed before, during and after the initiation of the salt treatment. Leaf areas were smaller in salt-treated plants, but the analysis of growth curves did not reveal differences that could be attributed to differential Na^+ accumulation, since similar changes in leaf expansion kinetics were observed in lines with different magnitudes of salt accumulation. Nevertheless, in a high leaf Na^+-including line, cell divisions were affected earlier, resulting in leaves with proportionally fewer cells than in a Na^+-excluding line. A distinct change in leaf epidermal pavement shape caused by salinity is reported for the first time. Mature pavement cells in leaves of control plants exhibited typical lobed, jigsaw-puzzle shape, whereas in treated plants, they tended to retain closer-to-circular shapes and a lower number of lobes.

INTRODUCTION

Crop yield over a period of time is directly related to leaf area development, photosynthesis rate and assimilate partitioning (Hay & Porter 2006). In sunflower, events occurring before anthesis determine the capacity to develop leaf area, and consequently to intercept radiation. Leaf area development (Rawson & Turner 1983), together with radiation use efficiency, condition carbon balance and potential yield in this crop (Trápani *et al.* 2003).

Abiotic stresses invariably result in agricultural yield reduction, and among them salinity is a growing concern (Munns & Tester 2008). Leaf expansion and carbon fixation rates in sunflower are reduced by salinity (Rawson & Munns 1984), and these effects are expected to be directly related to yield constraints under saline conditions. It is generally agreed that the negative effects of salinity on plant growth and development result from three main concurrent causes: decreased water potential in the root medium, excess ions affecting nutrient uptake and balance, and high build-up of potentially toxic ions (Munns & Tester 2008). There is also consensus that early responses observed immediately after salinity imposition are mainly attributed to osmotic effects of this stress (Munns 1993), while ion-specific effects are expressed later. In this work, we explored whether responses to the osmotic and ionic components of salinity can be distinguished in leaf expansion kinetics analysis. This question was addressed by examining the effects of high salinity on leaf area expansion in sunflower lines that differ in salt tolerance and in leaf Na^+ accumulation (Céccoli *et al.* 2012), and that had previously been characterised for their response to drought (Pereyra-Irujo *et al.* 2008). In those lines, overall salt tolerance, estimated from vegetative growth responses to short and long salt treatments (130 mM NaCl), was lines HAR2 > HAR1 > HA64 > HAR5 (Céccoli *et al.* 2012). Among which, lines HA64 and HAR2 exhibited osmotic tolerance, as evaluated from the initial effects of the salt treatment on plant elongation. A range in Na^+ concentration was observed in leaves from salt-treated plants of these lines (HAR1 > HA64 = HAR5 > HAR2). Tissue Na^+ tolerance was estimated by plotting the percentage of dead leaves against leaf blade Na^+ accumulation, and was higher in HAR1 than in the other lines.

It was expected that at initiation of the salt treatments, leaf expansion kinetics changes in response to stress would be dominated by responses to the osmotic component of salinity, and would thus be similar to those resulting from drought, as described in Pereyra-Irujo *et al.* (2008). It was also expected that as the salt treatment progressed, ion inclusion would impose further changes in leaf expansion kinetics. To this end, in the above-mentioned lines we compared leaf expansion

kinetics in leaves that were formed before, during and after the initial exposure of plants to a relatively high (but gradual) salt treatment.

Sunflower leaf growth kinetics have been described in detail in Dosio *et al.* (2003). These authors analysed leaf growth from different phytomers in several hybrids grown at various times in the field or in greenhouses. They observed that if leaf relative expansion rates (LRER) were expressed per unit thermal time, the resulting plot had a general shape conserved across years, locations, genotypes and phytomers. Leaf growth expressed in relation to thermal time was suggested as a suitable tool for interpreting leaf growth in sunflower (Granier & Tardieu 1998a). Thermal time, expressed in °Cd (degree days), is the integral over time of environmental temperature above a base temperature at which the process falls to zero (Hay & Porter 2006). Dosio *et al.* (2003) reported that for phytomers above 2, LRER were high during the first 100 °Cd following initiation, rapidly decreased before 150 °Cd, stabilised for a certain time, and subsequently continuously decreased to reach zero at the end of leaf expansion. Therefore, three consecutive stages were described in sunflower leaf expansion: an initial stage with high and stable LRER, a second phase with lower but also stable rates, and a third phase characterised by decreasing LRER (Dosio *et al.* 2003).

Cell divisions and expansion contribute to final leaf size. In sunflower, cell divisions can continue until the leaf reaches 80% of its final size (Granier & Tardieu 1999) and thus, apoplastic Na^+ supply through the transpiration stream is expected to exert an effect on both cell division and expansion. Ion balance alterations at or close to the apical meristem can exert a signalling effect on the developing leaves (Lazof & Läuchli 1991; Munns & Rawson 1999), as reviewed for grass leaves in Hu *et al.* (2005). The effects of salinity on cell division and expansion were studied in leaf 8, initiated at the onset of the salt treatment, in two of the aforementioned sunflower lines with contrasting leaf blade Na^+ accumulation.

The leaf epidermis is an important architectural control element that influences the growth properties of underlying tissues and the overall form of the organ (Zhang *et al.* 2011), therefore, the morphogenesis of epidermal pavement cells is of particular interest. In dicots, pavement cell expansion in the lateral dimension often occurs in a sinusoidal pattern, generating highly interdigitated cells (Esau 1977) and a characteristic jigsaw-puzzle pattern. Pavement cell waviness, wherever present, can vary significantly between different sites on leaves and petals and the degree of waviness is affected by environmental conditions (Watson 1942; Panteris & Galatis 2005). During this work, we observed that mature pavement cells were highly lobed in leaves from non-salinised plants, and were less lobed and more round in salinised plants. Despite genetic and ultrastructural descriptions of pavement cell growth, there is still very little information about pavement cell geometry dynamics in response to stress and its relation to cell and leaf expansion. This paper includes a detailed description of the changes in pavement cell shape induced by high salinity in sunflower. The significance of these changes and the underlying mechanisms that may determine them are discussed.

MATERIAL AND METHODS

Plant culture and growth conditions

Four non-branching sunflower inbred lines were used: HAR1, HAR2, HAR5 and HA64. In this group, leaf Na^+ concentrations were HAR1 > HA64 = HAR5 > HAR2 measured 22 days after plants had been exposed to 130 mM NaCl (Céccoli *et al.* 2012). These lines also express different responses to the initial stage of salinisation, dominated by responses to the osmotic component of salinity: lines HAR1 and HAR5 were more affected by this component than HA64 and HAR2 (Céccoli *et al.* 2012).

Seeds were soaked for 20 min in a 30% commercial bleach solution, thoroughly rinsed and placed on moist tissue paper at 28 °C in the dark. Germinated seeds were transferred to 7-l pots (22-cm high × 20-cm diameter) with a mixture of sand and perlite (3:1, v:v). The trials were carried out in a growth chamber adjusted to 26/18 °C day/night temperatures, with a 16-h photoperiod providing 15.47 ± 0.55 mol·m^{-2}·day^{-1}. Plant emergence was around 100 °Cd from sowing. Thermal time was calculated from the sum of daily average environmental temperatures minus the base temperature of 4.8 °Cd (Granier & Tardieu 1998a). Upon emergence, seedlings were irrigated with half-strength Hoagland solution (Hoagland & Arnon 1950). Salinisation was initiated when the first pair of leaves was 1-cm long, at 200–240 °Cd from sowing. Gradual salinisation was provided by adding NaCl to the nutrient solution until a final concentration of 130 mM was reached (within 4 days), and subsequently maintained by periodically irrigating pots with a sufficient volume of salinised solution so as to ensure complete replacement of the previous solution to prevent salt build-up. Salinity levels were monitored throughout the trials by checking the conductivity of the drainage solutions.

Leaf growth analysis

Width and length of emerged leaves was measured every 2–3 days with digital calipers and leaf areas (LA) were calculated as 0.65 (width × length), according to Dosio *et al.* (2003). Final LA and leaf expansion duration were recorded. Leaf relative expansion rates (LRER) were calculated as the ratios between the differences in logarithms of leaf area and the thermal time interval between two successive harvests (h_{n-1} and h_n), where LRER = (ln LAh_n – lnLAh_{n-1})/(°Cdh_n – °Cd h_{n-1}). The quantification of the relative contributions of the changes in growth rate and duration to the final area of leaves from stressed plants was performed as described in Pereyra-Irujo *et al.* (2008). It was based on the growth achieved by leaves from stressed plants after those from the control treatment reached 95% of their final area,.

Epidermal cell areas measurement and estimation of number of cells per leaf

These measurements were performed in leaf 8 of genotypes HAR1 and HAR2, which have high and low leaf Na^+ concentrations under salinity, respectively (Céccoli *et al.* 2012). Plants for these experiments were grown in a separate trial, carried

out in the same growth chamber and environmental parameters. To determine leaf initiation, the shoot apical bud was dissected and observed under a stereoscopic microscope (Nikon Ltd., Tokyo, Japan). Leaves were harvested at five times during development. The first three harvests were before leaf emergence, at 275, 296 and 336 °Cd from sowing, the first post-emergence harvest was at 530 °Cd and the last one, after final area was attained, at 905 and 925 °Cd for control and salinised HAR1 plants, and 1081 and 1162 °Cd for control and salinised HAR2 plants.

In harvests before emergence, the complete apex was excised, fixed in FAA (formaldehyde:acetic acid:ethyl alcohol, 2:1:10) for 48 h, and then transferred to 70% alcohol until processed. Apices were dissected under a stereoscopic microscope, the selected leaves were photographed and leaf areas were measured with ImageJ (http://rsbweb.nih.gov/ij).

For cell area (CA) measurements, all leaf samples were rehydrated in distilled water, mounted in 50% (v/v) glycerine and observed with an inverted Nikon Eclipse Cs1 spectral confocal microscope. Samples were illuminated with a 405 nm diode laser beam and cell wall autofluorescence observed through 475/25 BP and 515/30 BP filters (blue and green channels, respectively). Images were acquired using 20 ms pixel dwelling time and 1024 × 1024 dpi resolution. At least three different images were obtained from each sample. Twenty to 100 pavement and guard cell areas in each image were measured using ImageJ.

In emerged expanding sunflower leaves, cells at the base were still growing and were therefore smaller than those at the tip, which had finished growing. Thus, to estimate average pavement cell size in emerged leaves, sections of approximately 1 cm^2 were obtained from basal, middle and tip locations and cell areas measured. Average pavement CAs in these samples were, in turn, averaged to obtain mean leaf pavement CA. Cell relative expansion rates (CRER) were calculated as described for LRER, replacing LA with pavement CA.

Stomata were evident in emerged leaf samples, and guard cells were measured in them. Stomatal indices (SI) were calculated as the number of stomata divided by the sum of stomata plus pavement cells per surface area. These indices were used to calculate the proportion of pavement to the sum of all epidermal cells (proportion of pavement cells = $2*SI(1 - SI)^{-1}$ to obtain a weighted average of epidermal CA, which was used to calculate cell number per leaf, estimated as the ratio of LA to average epidermal CA.

Pavement cell shape measurements

Pavement cell shape was measured with the circularity shape descriptor in ImageJ. Circularity is defined as 4π*area/perimeter^2. A value of 1.0 indicates a perfect circle; as values approach 0.0 they indicate increasingly elongated shapes. To quantify lobe number, cell outlines were skeletonised (Zhang *et al.* 2008) with ImageJ and the end-point pixels were counted with the 'Analyze Skeleton' function to estimate branch ends.

Statistical analysis

Statistical analyses were performed with InfoStat (2009). Details for each analysis are mentioned in the Results section. Fisher's least significant difference (LSD) test was run for comparisons of means after ANOVA.

RESULTS

Alteration of leaf growth kinetics by salinity in exposed leaves at different stages of development

Leaf 8 initiation in the sunflower lines used in this study occurs at about 200 °Cd from sowing (Pereyra-Irujo *et al.* 2008). This was confirmed in two of the genotypes: leaf 8 initiated at 235 and 254 °Cd in HAR1 and HAR2, respectively. Salt treatments were started at 200–240 °Cd from sowing, and thus the effects of this stress could be compared on leaves 4, 8 and 12, which were initiated before, during and after the onset of the salt treatment, respectively. Calendar days may be easily estimated from °Cd information through dividing by 20, which is the average °Cd accumulated per day for this work.

Final areas in these leaves were smaller in salt-treated plants than in controls (Fig. 1), and emergence was significantly delayed in leaf 12 of salt-treated HAR1 and HAR2. An emergence delay was also observed in the other two lines but differences did not reach statistically significant levels. Leaf growth duration was prolonged in leaves 4 and 8 of salt-treated HAR5 and HAR2 (Fig. 1). Leaf 12 was still growing in HAR2 and HAR5 when plants were harvested (Fig. 2), so growth duration for that leaf is shown up to the harvest time.

To compare growth in leaves from control and salt-treated plants on a uniform basis, leaf areas were plotted as a function of leaf emergence time. In general, growth kinetics of leaves 4, 8 and 12 responded very similarly to stress (Fig. 2), even though they had initiated at different stages of the salt treatment. Leaf relative expansion rates (LRER) declined from emergence onwards (Fig. 3), which is typical of the third stage of sunflower leaf expansion (Dosio *et al.* 2003). Significant differences in LRER between control and salt-treated plants (*t*-test, $P < 0.05$) were detected immediately after emergence in leaf 4 from HA64, in leaf 8 from HAR2 (also in HA64, but at $P < 0.1$) and in leaf 12 from HAR2, HAR1 and HA64. In some leaves, significant differences were also detected towards the end of expansion, and reflected the prolongation of leaf expansion in salt-treated plants. In leaf 8, prolonged leaf expansion contributed to buffer the negative effects of reduced expansion rates on final leaf areas. The increase in areas achieved by prolonging expansion was 10% of final leaf areas, on average (not shown). Changes in leaf expansion kinetics curves resulting from the salt treatment were similar in lines with different leaf Na^+ accumulation, so no association could be derived between Na^+ accumulation and leaf kinetics changes.

Effects of salinity on cell expansion and cell number per leaf

It was expected that leaf Na^+ accumulation would influence cell division and expansion. These parameters were measured in leaf 8 of lines HAR1 and HAR2, which have contrasting leaf Na^+ concentration under high salinity and was known to be significantly higher in HAR1 (Céccoli *et al.* 2012). In the first two harvests, areas of leaf 8 were similar in both treatments, but by the third harvest, leaves in salinised HAR1 plants were smaller than their controls, and thereafter also in HAR2 (Table S1). Pavement CAs were similar in control and salinised plants

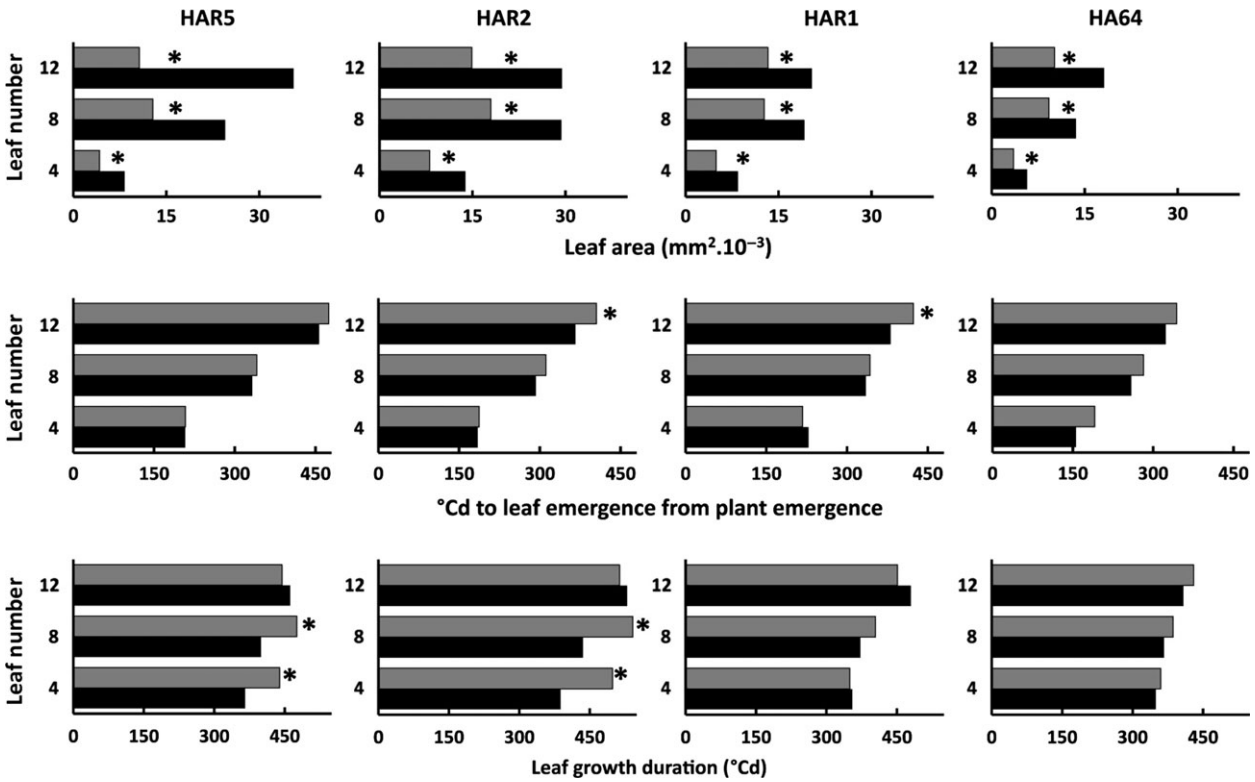

Fig. 1. Effects of salinity on area, emergence time and growth duration of leaves 4, 8 and 12 from plants of four sunflower lines grown under control (dark bars) or salinised (130 mM NaCl, light grey bars) conditions. Asterisks denote significant differences between control and salinised plants ($P < 0.05$) after ANOVA; letters are not shown for the sake of clarity. Plants were harvested a few days after leaf 8 reached the final size, which varied among lines and treatments, and the latest harvest was 53 days from sowing.

in the first three harvests (Table 1); cells then expanded and CRER were lower in salinised plants, except in the last period.

In expanded leaves of both lines there were fewer cells in salinised plants than in controls (Fig. 4). However, the number of cells per leaf was affected earlier in HAR1, resulting in fully expanded leaves with proportionally fewer cells than in HAR2. Nevertheless, in salinised plants of HAR2, the slope of cell number as a function of thermal time was steeper than in control plants in the last period, indicating the cells were still dividing and suggesting that salinity prolonged cell division in this line.

Stomata were evident in images from emerged leaves, with an average stomatal index of about 0.25, and tended to be higher at the base of the leaves of non-salinised plants (results not shown), suggesting that meristemoid cells continued to divide after pavement cells had stopped dividing. However, salinity did not induce consistent effects on the stomatal index and no differential effects could be detected between both lines.

Pavement cell shape alteration in salinised plants

When leaves were initiated, pavement cells tended to be round. In emerged leaves from control plants, average cell circularity tended to decrease, but not in leaves from salt-treated plants (Fig. 5A). Average cell circularity in emerged leaves, plotted in Fig. 5A, included cells from the base, middle and apical zones of the leaf. As leaves develop, newer growing cells tend to concentrate at the base, and a growth gradient is established from base to tip (Granier & Tardieu 1998b), the presence of cells at various stages of development contributed to the decrease in circularity observed in emerged leaves from control plants. In those leaves (control treatment), circularity decreased from base to tip (Fig. 5B), coincident with the developmental gradient. Likewise, circularity also decreased as leaves matured and stopped growing: those harvested at 530 °Cd were still growing (h4) while leaves harvested later (h5) had stopped growing, again reflecting the effects of development. However, in all emerged leaves, cells in salinised plants tended to retain closer-to-circular shapes (Fig. 5B); this was observed particularly in HAR1 (Fig. 5A).

Epidermal pavement cells in expanded leaves of plants grown under control condition had typical lobed, jigsaw-puzzle shapes (Fig. 6A). These shapes were only observed in emerged leaves, *i.e.* leaves in harvests h4 and h5. However, in salinised plants of those harvests, epidermal pavement cells had an almost smooth perimeter (Fig. 6B). An average of six to eight lobes (shown as end-point pixels in Fig. 6) could be distinguished in such cells located in the apical zone of leaves from control plants, while in salinised plants, generally only the two ends of a single skeleton line could be detected in this analysis (Fig. 6C, D). A gradient in cell area and in the number of lobes was also observed from base to tip in leaves from control plants. In summary, in emerged leaves from control plants, cells at the base of leaves were generally smaller, had fewer lobes and were more round than leaves in the middle and tip;

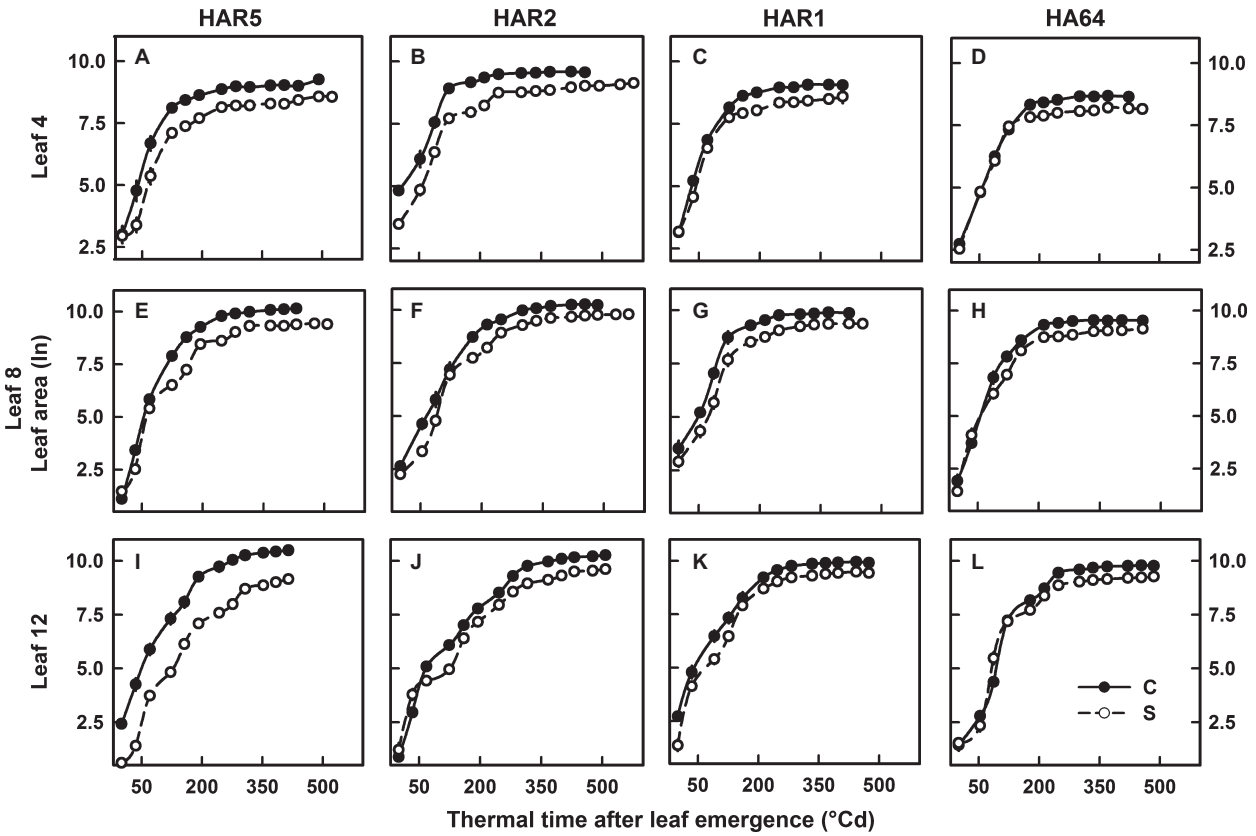

Fig. 2. Leaf areas in four sunflower lines grown under control or salinised (130 mM NaCl) conditions as a function of thermal time after leaf emergence. A–D: leaf 4; G–H: leaf 8; I–L: leaf 12. Results are from five leaves per line and treatment. Error bars are SE, those not shown are smaller than the symbol. Plants were harvested a few days after leaf 8 reached the final size, which varied among lines and treatments, and the latest harvest was 53 days from sowing.

cells in leaves from salinised plants tended to be rounder and had fewer lobes.

Pavement cells in leaves from salt-treated plants were smaller than those of non-salinised controls. We then asked whether the observed effects of salinity on circularity and end-point pixels was due exclusively to size reductions. To this end, we plotted circularity and end-point pixels as a function of cell size (Fig. 7A, B) in leaves from harvests h4 and h5. It is clear that as cell area increased, circularity decreased and end-point pixels increased. Moreover, average cell area in salinised plants did not exceed 1130 μm^2. When circularity was compared in cells of similar size from control and salt-treated plants (only cells $<1130\ \mu m^2$ were included in this analysis), significantly higher circularity and lower end-point values were observed in salt-treated plants (Table 2B). Yet the effects of salinity were similar in cells from all regions of the leaf (Table 2A, non-significant Z*T interaction). Both lines showed similar tendencies and therefore were not discriminated.

DISCUSSION

Effect of salinity on the kinetics of leaf growth expansion

Plant growth responses observed immediately after salinity imposition are considered to be caused mainly by decreased water potential in the substrate, *i.e.* by the osmotic component of this stress (Munns 1993). This initial effect is considered to be non-specific to salinity, in the sense that it is attributable to the decrease in water availability, while ion-specific effects are expressed later, after measurable ion build-up in the plant. Restrictions in leaf growth are among the earliest effects of many stress conditions, including salinity (Munns & Termaat 1986). The first question addressed in this work was whether the responses to the osmotic and ionic components of salinity could be distinguished in leaf expansion kinetics analysis. Osmotic effects of salinity were expected to be reflected in the expansion of leaves that were growing during the time the salt treatments were supplied, and we first attempted to evaluate the initial effects of salinity in the growth of leaves 1 and 2, which were expanding when the salt treatment was initiated. However, there was high variability in the data obtained from those leaves, which discouraged their use as an experimental system. Growth kinetics were then compared in leaves 4, 8 and 12. Leaf 4 was preformed before initiation of the salt treatment, and emerged and expanded during the salinisation process, while leaves 8 and 12, were formed during and after initiation of the salt treatment, respectively, and therefore expanded once the plants were salinised. The final areas of all three leaf positions were affected to a similar degree by salinity in the four sunflower lines included in this study. As expected, leaf expansion rates were lower in salt-treated plants. However, despite the differences in growth stage at the time salinity was applied, the effects of salinity in the shape of growth curves (Figs 2 and 3) and calculated growth parameters (Fig. 1) were very similar

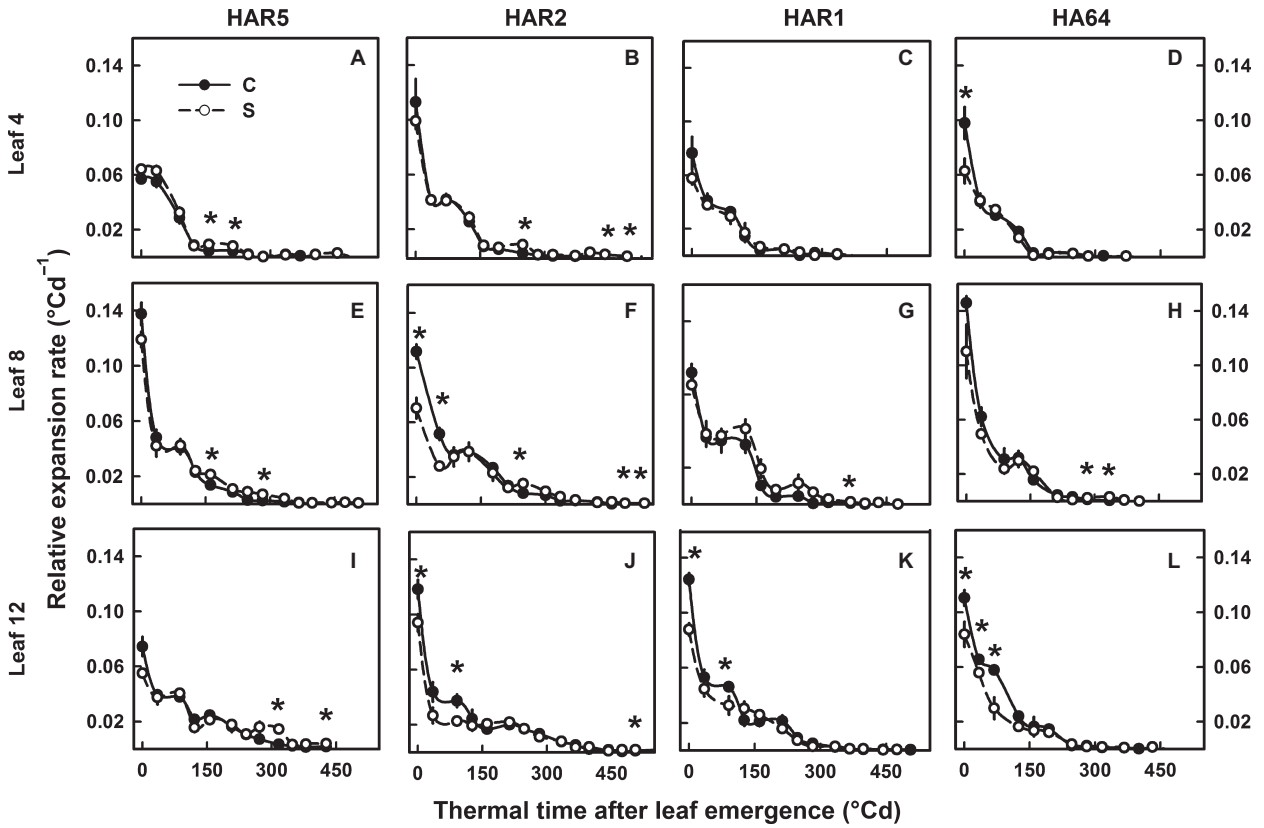

Fig. 3. Leaf relative expansion rates (LRER) in four sunflower lines grown under control or saline (130 mM NaCl) conditions as a function of thermal time after leaf emergence. A–D: leaf 4; G–H: leaf 8; I–L: leaf 12. Results from five leaves per line and treatment. Error bars are SE, those not shown are smaller than the symbol. Asterisks denote significant differences between control and salinised plants (*t*-test, $P < 0.05$) at a specific sampling time. Plants were harvested a few days after leaf 8 reached the final size, which varied among lines and treatments, and the latest harvest was 53 days from sowing.

Table 1. Epidermal pavement cell area (μm^2) and relative cell expansion rates (CRER) $°Cd^{-1}$ at various points along leaf 8 development, in plants of two sunflower lines grown under control (C) or salinised (S, 130 mM NaCl) conditions. CRERs are average expansion rates in the period between two successive harvests. The final harvest was made after leaves stopped expanding. In the first column, in the last two rows, the two figures separated by a slash indicate harvest times in control/salinised plants. For cell areas, different letters indicate significant ($P < 0.05$) differences among all samples, after ANOVA.

	HAR 1				HAR2			
	cell area (μm^2)		CRER ($°Cd^{-1}$)		cell area (μm^2)		CRER ($°Cd^{-1}$)	
°Cd from sowing	C	S	C	S	C	S	C	S
275	53.03a	49.47a			56.78a	53.02a		
296	58.32a	53.58a	0.0045	0.0038	61.64a	53.70a	0.0039	0.0006
336	77.45a	56.59a	0.0071	0.0014	95.78a	59.86a	0.0110	0.0027
530	484.33c	212.45b	0.0096	0.0068	252.33b	76.83a	0.0044	0.0013
905/925	1343.33g	792.52e	0.0029	0.0033				
1081/1162					1169.50f	630.63d	0.0030	0.0033

in all three leaf positions. Thus, the osmotic effects of salinity on leaf expansion could not be distinguished from ion-specific effects; it was expected that the latter would be clearly expressed in leaf 12, which was initiated once the plant had been exposed to salinity for a longer period. In fact, an inspection of Fig. 2 indicates that genotype-related differences in leaf expansion were more remarkable than differences among leaf positions of a given genotype. The lines in this study differ in leaf Na^+ accumulation in response to salt treatment, however, no association could be drawn between the leaf Na^+ concentration and expansion kinetics In a previous work (Céccoli *et al.* 2012): HAR1 and HAR5 were found to be more sensitive to the osmotic component of salinity than HAR2 and HA64, measured as effects on plant height. However, relative sensitivity to this aspect of the stress was also not reflected in the response of leaf expansion kinetics to the salt treatment. Thus, leaf growth kinetics alterations by salinity cannot simply be ascribed to either ion-specific or osmotic effects of this stress, but undoubtedly result from complex signalling events triggered by salinity (Huang *et al.* 2012).

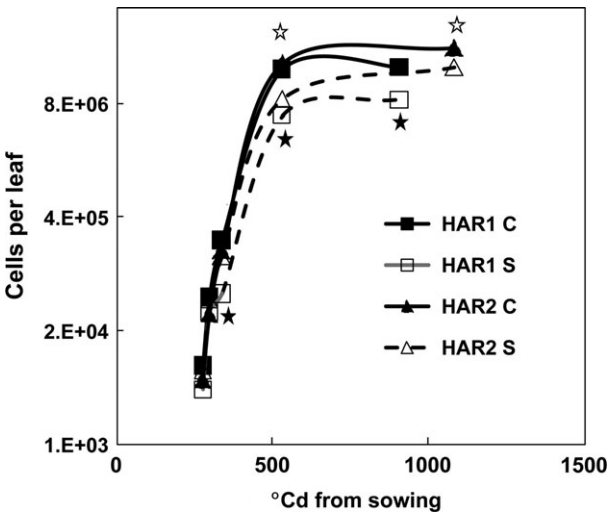

Fig. 4. Estimated cell number in the abaxial leaf epidermis at various points during leaf 8 development in plants of two sunflower lines grown under control (C) or salinised (S, 130 mM NaCl) conditions. In the first three harvests, before leaf emergence, average cell number was calculated from the ratio between leaf size and average cell area. For the last three samples, cell number estimates took into account a weighted estimate of guard and average pavement cell size. In the last harvest, samples were taken after leaves stopped expanding, which occurred earlier in HAR1. In each harvest, filled and empty asterisks indicate significant (*t*-test, $P < 0.05$) differences between control and salt-treated plants in HAR1 and HAR2, respectively.

Analysis of sunflower LRER reveals three consecutive phases in leaf growth: an initial stage with high and stable LRER, a second phase with lower but also stable rates, and a third phase characterised by decreasing LRER (Dosio *et al.* 2003). The leaves shown in Figs 1–3 were in the third phase, which spans the emerged stage. Dosio *et al.* (2003) showed that in sunflower plants growing either isolated or within a canopy, relative leaf expansion rates in the third phase were similar despite observed differences in leaf area resulting from differences in irradiation. The rate and duration of the second growth phase, but not the third, reflected the effects of growth conditions, supporting results of Granier & Tardieu (1999) who pointed out that events occurring in young expanding leaves, smaller than 40% of their final area, determine final leaf size in sunflower. Thus, if the events that determined final leaf area occurred largely before leaf emergence, relative growth rates in the third growth phase would not be related to final leaf size attainment. Therefore, it was not unexpected that we detected relatively few differences in LRER between leaves grown in control or Na^+-stressed conditions.

Alteration of leaf growth duration under stress

Effects of stress on leaf expansion prolongation have often been reported in the literature. It was reported that leaf expansion was prolonged by salinity in *Chloris gayana* (Ortega & Taleisnik 2003), by soil impedance in wheat (Beemster & Masle 1996) and by nitrogen deficiency in *Lolium perenne* (Kavanová *et al.* 2008), and by moderate water stress (Aguirrezábal *et al.* 2006)

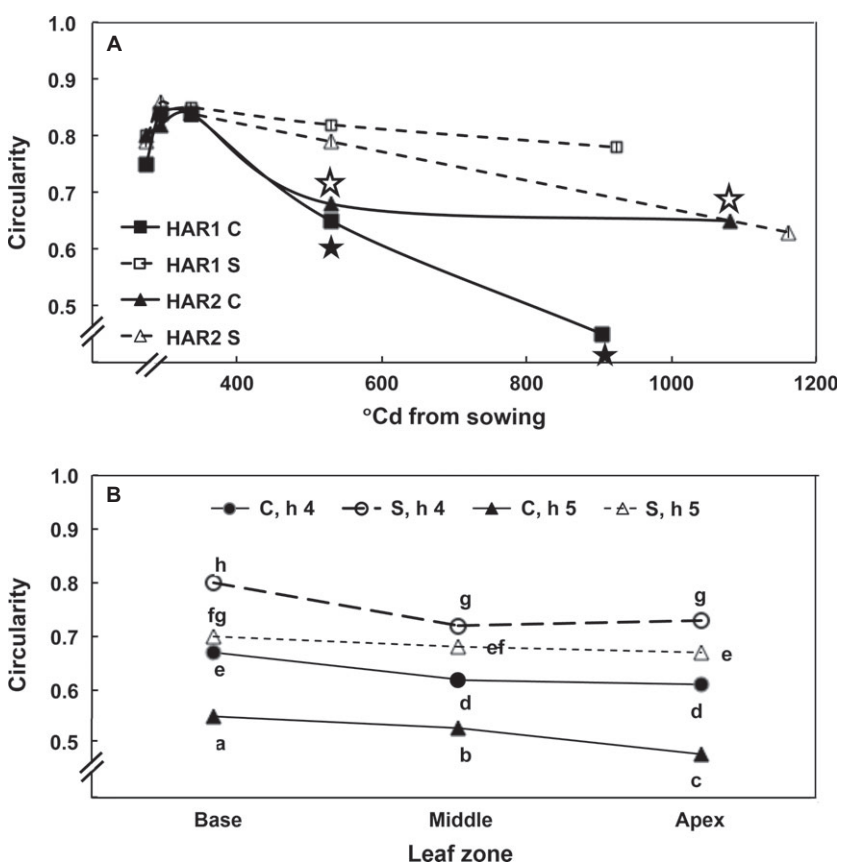

Fig. 5. Epidermal pavement cell circularity in leaf 8 of two sunflower lines grown under control (C) and salinised (S, 130 mM NaCl) conditions. A: Average circularity at successive thermal time points. In each harvest, filled and empty asterisks indicate significant ($P < 0.05$) differences between control and salt-treated plants of HAR1 and HAR2, respectively. B: Circularity in different zones (base, middle and tip) of emerged leaf 8 of the two cultivars harvested after 500 °Cd. Leaves harvested at 530 °Cd (h4) were still expanding, while those in the final harvest (h5) were fully expanded. Different letters indicate significant differences at $P < 0.05$. Averages are from 15 to 20 cells measured in each of five to ten leaves per line, harvest and treatment.

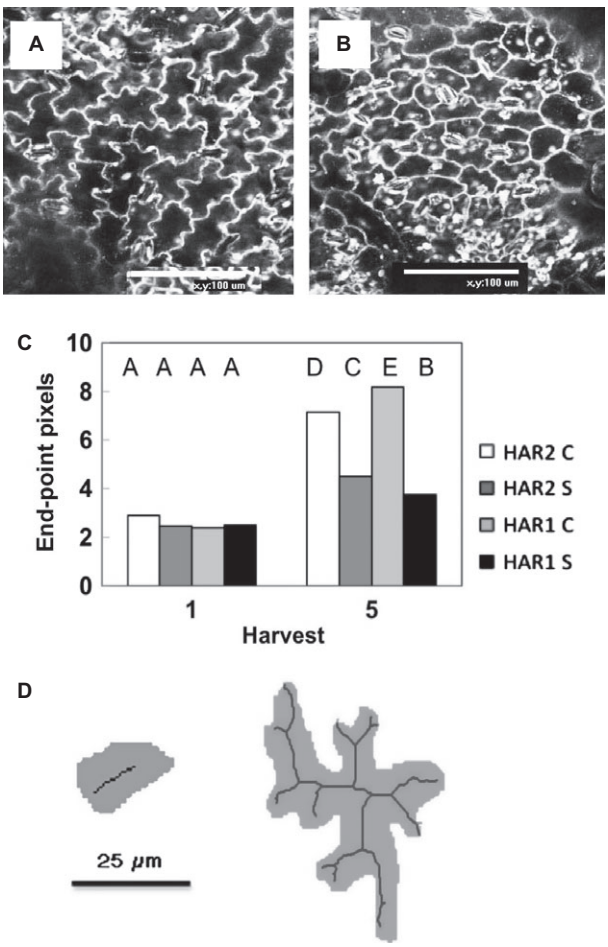

Fig. 6. Epidermal pavement cell shape analysis in cells from leaf 8 of two sunflower lines grown under control (C) and salinised (S, 130 mM NaCl) conditions. A: and B: Confocal images of the apical zone of leaf 8 epidermis in HAR2 leaves obtained from fully expanded leaves; A: control, B: salt-treated plants. C: Number of end-point pixels in cells from harvests 1 and 5 (see Table 1 for details of harvest times). Results are average of 35–115 cells, different letters above bars indicate significant differences at $P < 0.05$. D: Skeletons of an epidermal pavement cell from harvests 1 (left) and 5 (right), taken from HAR2 control plants. Digital images were analysed with the 'Analyze Skeleton' function of ImageJ.

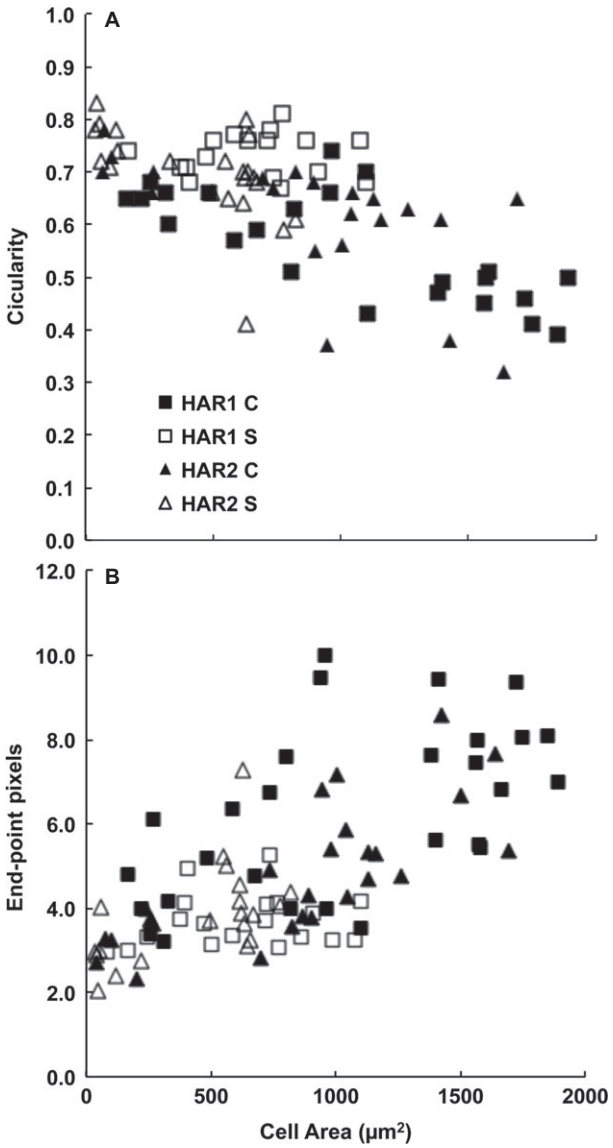

Fig. 7. Effects of salinity on leaf pavement cell shape. A: Circularity as a function of cell area. B: End-point pixels as a function of cell area. Average circularity values obtained from the basal, middle or tip zones of individual emerged leaves were plotted against the average cell size in the same sample. Each point is the average of cell measurements in a single sample. SE not shown for clarity.

and decreasing day length (Cookson *et al.* 2007) in *Arabidopsis*. Increased leaf expansion duration has been observed in water-stressed sunflower (Takami *et al.* 1981; Pereyra-Irujo *et al.* 2008), but not in response to nitrogen availability (Trápani *et al.* 1999). In water-stressed plants, an increase in leaf expansion duration can partially buffer the negative effects of stress on leaf area. Leaf growth duration tended to increase in salt-treated plants in our trials, and, in general, the effect was more marked in lines HAR5 and HAR2. The contribution of prolonged leaf expansion duration to final leaf area in salt-treated plants was estimated as approximately 10% in HAR2 and HAR5, half as much in HAR1, and even less in HA64. The range of these values is lower than those estimated under water stress (Pereyra-Irujo *et al.* 2008), however, as also observed by those authors, the relative magnitude of the effect was always lower in HA64 than in the other sunflower lines, independently of the trial, highlighting that the effect was genotype-specific.

Pereyra-Irujo *et al.* (2008) associated prolonged leaf expansion in leaf 8 with prolonged duration of cell divisions, as in salinised plants of HAR2. Since HAR2 contains very low leaf Na^+ concentrations, the effect may be related to the osmotic component of salinity. It is likely that a common response to various stresses may have a common physiological basis, and it could be speculated that the observed intraspecific variability for leaf growth prolongation under stress in sunflower must have a genetic basis.

Effects of salinity on cell expansion and cell number per leaf

Final leaf size depends on cell number and size, together with the size of intercellular spaces (Marcotrigiano 2010). In terms

Table 2. Salinity effects on sunflower leaf pavement cell shape. (A) ANOVA results for the effects of salinity on end-point pixels and circularity in cells obtained from the basal, middle and apical zones of leaf 8 harvested at two time points (harvests h4 and h5, see Fig. 5 for details). *P*-values < 0.05 indicate significant differences for variables or interactions. (B) Average values for end-point pixels and circularity in these cells. For each variable, figures followed by different letters are significantly different ($P < 0.05$).

	P-values	
	end-point pixels	circularity
(A) ANOVA results		
model	0.0311	0.0025
zone (Z)	0.2336	0.0278
harvest (H)	0.0052	0.0004
treatment (T)	0.0001	<0.0001
Z*H	0.9365	0.0214
Z*T	0.5302	0.1749
H*T	0.2568	0.2024
Z*H*T	0.7922	0.0160
(B) average values		
harvest 4	3.90A	0.70A
harvest 5	5.10B	0.61B
control	4.86B	0.65A
salt-treated	3.75A	0.72B

of cell division and expansion, leaf growth can be described as the succession of five overlapping and interconnected stages: an initiation phase, a general cell division phase, a transition phase, a cell expansion phase, and a meristem division phase. Environmental conditions that alter final leaf size can affect all five of these activities. In this work, salt stress affected both cell size and number, in coincidence with numerous reports in the literature on the influence of stress on cell division and expansion, both in dicots (*e.g.* Lecoeur *et al.* 1995; Trápani *et al.* 1999; Alves & Setter 2004; Aguirrezábal *et al.* 2006; Pereyra-Irujo *et al.* 2008) and monocots (*e.g.* MacAdam *et al.* 1989; Gastal & Nelson 1994; Assuero *et al.* 2004; Hu *et al.* 2005; Taleisnik *et al.* 2009). Furthermore, stress may exert differential effects on cell division and expansion, as observed in water-stressed *Arabidopsis* (Aguirrezábal *et al.* 2006) and nitrogen-deficient *Lolium perenne* (Kavanová *et al.* 2008).

Cell expansion rates are determined by relative water uptake capacity as well as wall yielding properties (Lockhart 1965; Cosgrove 1997). If dominated by the former, leaf expansion rates may not reflect ion-specific effects that are expected to influence wall-yielding properties (Cosgrove 1997), yet walls obviously change in stressed plants even in the absence of direct ion effects (Neumann 2008). Lechner *et al.* (2008) suggested that processes linked to cell wall tightening seem to be delayed in water-stressed sunflower plants. However, Palmer *et al.* (1996) observed reduced cell expansion in sunflower in response to decreased nitrate availability without concurrent reduction in cell turgor pressure and concluded that reduced cell expansion was due to changes in cell wall properties that were associated with increases in abscisic acid following the reduction in nitrate availability. Our data on the effects of salinity on cell expansion, however, do not allow us to distinguish the effects of this stress on cell wall properties.

The cell division rate is an important contributing factor to the final organ size (Gonzalez *et al.* 2012). While analyses of the effects of salinity on cell size are numerous in the literature (Taleisnik *et al.* 2009), there is much less information on the effects of salinity on cell division. In our trials we observed that the number of cells in expanded leaves was reduced in salt-treated plants. In dicot leaves, cell division and expansion overlap, both in space and time, and, in sunflower, cell divisions can continue until the leaf reaches 80% of its final size (Granier & Tardieu 1998b). Accordingly, cell numbers continued to increase at least up to the fourth harvest (h4) in our trials, and in salinised HAR2, up to the final harvest. Thus, both cell division and expansion were likely to be influenced by the various apoplastic supplies of Na^+ from the xylem stream in lines that differ in leaf Na^+ concentration, such as HAR1 and HAR2. It may be tempting to assume that the earlier effect on cell division observed in HAR1 and the prolongation of cell divisions observed in HAR2 could be associated with differences in apoplastic Na^+ in these lines, however, we have no evidence for this. There are many regulators involved in the control of the cell division. In *Arabidopsis*, upon NaCl treatment, the promoter activities and transcript levels of all cell cycle genes initially fell in the shoot apex and were subsequently induced during salt-stress adaptation (Burssens *et al.* 2000). However, in *Arabidopsis* root tips, decreased root length under salinity was due to a smaller number of dividing cells, and was suggested to be mediated by post-translational control of cyclin-dependent kinase activity (West *et al.* 2004). In leaves of water-stressed *Arabidopsis* plants, ethylene plays a role in cell cycle arrest *via* inhibition of cyclin-dependent kinase A activity, leading to leaves with fewer and smaller cells (Skirycz *et al.* 2011). Verelst *et al.* (2010) found that ethylene and gibberellic acid affect cell division in leaf cells of plants under water stress, while abscisic acid affects cell expansion. Research on the link between changes in hormonal balance under salt stress and sunflower leaf expansion would provide insight into the effects of stress on cell divisions and expansion.

Alteration in pavement cell shape in salinised plants

Pavement cells are the most abundant cell type in the epidermal layers of all plant organs (Glover 2000), where their function is to protect the underlying cell layers. In dicot leaves they often show jigsaw-puzzle shapes, which are assumed to provide mechanical strength necessary to support the large air spaces within the mesophyll (Glover 2000). In cotyledons, analysis of epidermal pavement cell populations indicates circularity decreases and the number of lobes per cell increases as cells grow (Zhang *et al.* 2011). Similar results were observed in the current work with sunflower. Size and shape of pavement cells have also been suggested to be correlated indicators of the developmental stage of the leaf (Andriankaja *et al.* 2012). In agreement, we observed that circularity decreased as a function of leaf development in non-salinised sunflower plants (Fig. 5), where fully expanded cells had a higher number of lobes (Fig. 6). However, these changes were less pronounced in cells from salinised plants, which tended to remain more circular and with a smoother perimeter. Growth analysis of individual cells has shown that lobe initiation takes place in waves; an initial stage is completed at an early stage of cell growth, followed by a subsequent period of diffuse growth, which is in turn

followed by additional lobe initiation events later during cotyledon development (Zhang *et al.* 2011). It is impossible to discern from our measurements whether lobe initiation was continuous or in waves, and at what stage differences in cell shape between salinised and control plants became established.

Complex underlying processes determine how plant cell shapes are built. These include cell wall synthesis, regulation of activity of the actin and microtubule cytoskeletons, the involvement of plant hormone signalling and vesicular membrane trafficking (Ivakov & Persson 2013). Recently, Xia *et al.* (2012) demonstrated that extracellular hydrogen peroxide directs ingrowth wall formation in epidermal transfer cells of *Vicia faba* cotyledons. Salt stress has been shown to reduce production of reactive oxygen species (ROS) in maize leaves (Rodríguez *et al.* 2004) through effects on NADPH oxidase activity (Rodríguez *et al.* 2007). It is interesting to note that changes in lobe number and circularity resulting from the salt treatment were more evident in HAR1, the genotype having a higher Na^+ concentration in leaves. Whether salt-related effects on ROS production and signalling may influence pavement cell shape remains to be investigated. Regardless of origin, the change in epidermal pavement cell shape must have a bearing on palisade cell packing, which in turn would influence photosynthetic activity on a unit leaf basis. This has not yet been explored and would provide clues to the physiological consequences of pavement cell shape alterations.

In short, our results suggest that leaf growth alterations in sunflower plants exposed to salinity are not dominated by responses to the ionic component of salinity, since similar alterations in growth kinetics were observed in leaves that were initiated before, during and after initial exposure of plants to salinity. Moreover, our results do not allow us to infer whether effects on cell division and expansion observed in lines with different magnitudes of salt accumulation could be attributed to this feature. Effects of salinity on pavement cell shape changes had not previously been reported; it would be interesting to analyse whether similar changes result under other environmental stresses and to investigate their physiological causes and consequences.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Leaf area (mm^2) and leaf relative expansion rates LRER ($°Cd^{-1}$) at various points along leaf 8 development, in plants of two sunflower lines grown under control (C) or salinized (S, 130 mM NaCl) conditions.

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