*Journal of Experimental Botany, Vol. 59, No. 8, pp. 2221–2232, 2008*
doi:10.1093/jxb/ern087 Advance Access publication 29 April, 2008

Journal of Experimental Botany
www.jxb.oxfordjournals.org

**RESEARCH PAPER**

# Genetic variability for leaf growth rate and duration under water deficit in sunflower: analysis of responses at cell, organ, and plant level

**Gustavo A. Pereyra-Irujo, Luciano Velázquez, Leandra Lechner and Luis A. N. Aguirrezábal***

*Unidad Integrada Balcarce, Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata, Instituto Nacional de Tecnología Agropecuaria, CC 276, 7620 Balcarce, Argentina*

Received 6 December 2007; Revised 3 March 2008; Accepted 4 March 2008

## Abstract

**Plants under water deficit reduce leaf growth, thereby reducing transpiration rate at the expense of reduced photosynthesis. The objective of this work was to analyse the response of leaf growth to water deficit in several sunflower genotypes in order to identify and quantitatively describe sources of genetic variability for this trait that could be used to develop crop varieties adapted to specific scenarios. The genetic variability of the response of leaf growth to water deficit was assessed among 18 sunflower (*Helianthus annuus* L.) inbred lines representing a broad range of genetic diversity. Plants were subjected to long-term, constant-level, water-deficit treatments, and the response to water deficit quantified by means of growth models at cell-, leaf-, and plant-scale. Significant variation among lines was found for the response of leaf expansion rate and of leaf growth duration, with an equal contribution of these responses to the variability in the reduction of leaf area. Increased leaf growth duration under water deficit is usually suggested to be caused by changes in the activity of cell-wall enzymes, but the present results suggest that the duration of epidermal cell division plays a key role in this response. Intrinsic genotypic responses of rate and duration at a cellular scale were linked to genotypic differences in whole-plant leaf area profile to water deficit. The results suggest that rate and duration responses are the result of different physiological mechanisms, and therefore capable of being combined to increase the variability in leaf area response to water deficit.**

Key words: Cell division, leaf expansion, leaf growth duration, leaf growth rate, sunflower (*Helianthus annuus* L.), water deficit.

## Introduction

Plants generally respond to water deficits by reducing their transpiration rate. Depending on the species and genotype, this can be achieved by stomatal closure (Tardieu and Davies, 1993), wilting, or leaf rolling (O'Toole and Cruz, 1980). However, one of the earliest water-saving mechanisms, present in a great majority of plants, is reduced leaf growth. Changes in leaf area can be a consequence of reduced leaf number (although it is not affected to a great extent; Aguirrezábal *et al*., 2006) and/or reduced area of individual leaves. Reduced leaf expansion rates under water deficit have been found to be related to different mechanisms, such as decreased cell division rate (Schuppler *et al*., 1998; Granier *et al*., 2000), to cell wall hardening (Matthews *et al*., 1984; Neumann, 1995), or to decreased turgor (Hsiao *et al*., 1998; Bouchabké *et al*., 2006).

The extent of leaf growth reduction caused by water deficit is very important in determining the adaptation of a certain crop variety to a climate scenario. In a scenario where long-term droughts are expected, a genotype which reduces its leaf growth is more likely to reach maturity with a certain amount of available water. On the other hand, in a scenario where short-term water deficits are expected, a genotype which maintains leaf growth is likely to have higher yields (Reymond *et al*., 2003). It has been shown that a genotype can maintain its leaf area by maintaining growth rate (Reymond *et al*., 2003) or by increasing the duration of leaf

* To whom correspondence should be addressed. E-mail: laguirre@mdp.edu.ar

growth (Aguirrezábal *et al*., 2006). Moreover, an increased duration of growth could have the benefit of increasing the opportunity for recovery after rainfall (Alves and Setter, 2004). The natural genetic variability for these traits could be used to develop crop varieties adapted to specific scenarios. Despite this, breeding for these traits is not a common approach for obtaining drought resistance in crop species, probably because of a lack of well-characterized sources of genetic variability.

The genetic variability of leaf growth response to water deficit has not been studied in many crop species. In maize, the response of leaf extension rate to water deficit has been genetically analysed (Reymond *et al*., 2003). This analysis, however, disregards the effect of water deficit on the duration of leaf growth, which has been shown to be significant in the model species *Arabidopsis thaliana* (Aguirrezábal *et al*., 2006). In sunflower, reports about the response of leaf growth duration to water deficit have been contradictory (Takami *et al*., 1981; Granier and Tardieu, 1999). The analysis of responses to water deficit should also take into account how processes at cell- and leaf-level affect the leaf area of the whole plant, since radiation interception, and hence photosynthesis and transpiration, are largely determined at this scale. In sunflower, a whole-plant model of leaf growth has been developed (Dosio *et al*., 2003), which uses a set of parameters to describe the co-ordinated growth of different leaves of the plant, and could be used as a framework for quantitatively analysing the response of leaf area to water deficit.

The objective of this work was to analyse the response of leaf growth to water deficit in several sunflower genotypes in order to identify and quantitatively describe sources of genetic variability for this trait. Plants were subjected to stable, long-term, mild water deficits, and leaf growth kinetics was analysed. Genotypes with contrasting response in terms of leaf growth rate and leaf growth duration were identified. The response of these genotypes was further characterized at cell-level (cell division and cell growth kinetics), and at plant-level (using the whole-plant model of Dosio *et al*., 2003). The response of each genotype to water deficit was quantified in terms of the changes in the parameters of this model. The relationships between responses at cell, organ, and plant level were analysed.

## Materials and methods

Eighteen non-branching sunflower inbred lines were selected, based on pedigree data (Korell *et al*., 1992), so as to comprise most of the genetic variability of cultivated sunflower. This group included two lines showing high and low osmotic adjustment (lines 'OA+' and 'OA–'; Chimenti *et al*., 2002). Four experiments were carried out to investigate the response of leaf growth to a water stress in these genotypes. The first experiment was conducted with the complete set of genotypes. Based on the results from this experiment, four genotypes were selected for the second experiment. Experiments 3 and 4 were conducted with the two most contrasting genotypes in terms of the response of leaf growth rate and duration.

### *Culture methods and growth conditions*

Seeds were sown in 35-cm-deep, 10-cm-wide containers made of PVC pipe, filled with soil (Typic Argiudoll, horizon A). Soil water content was measured initially by oven-drying soil samples at 105 °C for 48 h, and then monitored daily by weighing the container. Plants were grown without water limitations (–0.05 MPa) until ~100 degree-days (base temperature = 4.8 °C; Granier and Tardieu, 1998) after leaf 8 initiation in each genotype. At this moment, soil water content was decreased until –0.65 MPa was reached, using the method described in Pereyra-Irujo *et al.* (2007). The method consists of sowing maize (*Zea mays* L.) plants earlier, in the same pots as sunflower plants. This method allows the desired soil water content to be reached quickly, the resulting soil water content in the pot is uniform, the rate of soil water depletion is independent of the sunflower genotype being evaluated (Pereyra-Irujo *et al*., 2007). Maize plants did not affect the growth of the sunflower plant (Pereyra-Irujo *et al*., 2007). The specified water content in each treatment was maintained by daily irrigation as in Granier *et al.* (2005).

Experiments were carried out under controlled (growth chamber; Refrimax SRL, Mar del Plata, Argentina) or semi-controlled (heated greenhouse) environmental conditions. Air temperature, relative humidity, incident radiation, and leaf temperature were measured every 15 min, and averaged and recorded every 1 h, with dataloggers (Cavadevices, Buenos Aires, Argentina). Thermal time was calculated as the daily integral of the difference between temperature and base temperature (4.8 °C; Granier and Tardieu, 1998). The base temperature for leaf initiation and leaf expansion did not differ significantly among the evaluated inbred lines in a preliminary experiment (not shown).

### *Leaf growth measurements*

Non-destructive measurements of length and width (in the greenhouse experiments) or area (by means of a digital photograph, in the growth chamber experiment) of the 8th leaf were made at least three times a week until the end of its expansion, in four (experiment 1) or five (experiments 2, 3, and 4) plants per genotype and treatment. A highly significant linear relationship ($R^2 > 0.99$, $n$=1755, $P < 0.0001$) was established between length×width and leaf area, which was applicable to any leaf regardless of leaf number, leaf age, genotype, or treatment. In addition, all leaves of the plant were measured every 7 d.

In experiments 3 and 4, destructive measurements of leaf area were made in leaves larger than 0.005 $mm^2$ (average area at initiation; Dosio *et al*., 2003) from three plants harvested every 2–4 d (experiment 3) or 6–12 d (experiment 4). Leaves were scanned or photographed under a microscope and measured with image analysis software (Matrox Inspector; Matrox Electronic Systems Ltd, Quebec, QC, Canada).

In addition, the number of initiated leaves was determined under a microscope in two plants harvested every 1–2 d, until the initiation of leaf 12 (experiments 1, 2, and 4) or leaf 20 (experiment 3).

Absolute leaf expansion rate (LER) was calculated as the slope of the relationship between leaf area ($L$) and thermal time ($t$) in two consecutive measurements, and leaf relative expansion rate (RER) as the slope of the relationship between the logarithm of leaf area and thermal time:

$$\mathrm{LER} = [\mathrm{d}(L)/\mathrm{d}t] \quad (1)$$

$$\mathrm{RER} = [\mathrm{d}(\ln L)/\mathrm{d}t] \quad (2)$$

### Cell growth measurements

At five different moments during experiment 4, leaf 8 was divided into two to four transects (depending on the size of the leaf). The area of each transect was measured, and imprints (obtained after evaporation of a varnish spread on the leaf) of the adaxial epidermis were taken halfway between the mid-rib and the leaf margin. The size of 25 cells/imprint was measured under a microscope coupled to an image analyser. Mean cell area of the leaf was calculated as the sum of the products of average cell area and transect area for each transect, divided by the sum of all transect areas. Epidermal cell number was calculated as the ratio between leaf area and mean cell area. Relative cell expansion rate (RCR) was calculated as the slope of the relationship between the logarithm of cell area ($C$) and thermal time ($t$) in two consecutive measurements:

$$\mathrm{RCR} = [\mathrm{d}(\ln C)/\mathrm{d}t] \quad (3)$$

### Growth curves fit

In experiments 1 and 2, growth of leaf 8 was described by means of a sigmoidal curve as described in Aguirrezábal *et al*. (2006):

$$y = A/(1 + \exp\{-[(x - x_0)/b]\}) \quad (4)$$

Final leaf area was calculated as the upper asymptote ($A$) of the sigmoidal curve. The duration of leaf expansion was calculated as the time elapsed between leaf initiation and the moment the leaf reaches 95% of its final area as calculated from the fitted curve. The mean expansion rate was calculated as the ratio between the final leaf area and the duration of leaf expansion.

In experiments 3 and 4, growth of different leaves of the plant was described by means of the model proposed by Dosio *et al*. (2003). This model divides the growth of each leaf into three distinct phases (Fig. 1). During phase 1 growth is initially exponential, at a high constant relative rate. During phase 2, growth continues to be exponential but at a lower rate (Fig. 1B). During phase 3 most of the growth of the leaf occurs (high absolute growth rate), in an almost linear fashion (i.e. with declining relative growth rate, Fig. 1B), until the end of expansion. Leaf area in each phase is defined by these equations:

$$t_0<t<t_1 \quad \ln y = \ln A_0 + R_1(x - t_0) \quad (5)$$

$$t_1<t<t_2 \quad \ln y = \ln A_1 + R_2(x - t_1) \quad (6)$$

$$t_2<t \quad \ln y = \ln A_2 + R_3/a\{1 - \exp[-a(x - t_2)]\} \quad (7)$$

Parameters for these equations are the moments of leaf initiation ($t_0$) and end of phases 1 and 2 ($t_1$ and $t_2$), relative leaf expansion rates (RER) during phases 1 and 2 ($R_1$ and $R_2$) and at the beginning of phase 3 ($R_3$), and a parameter describing the decline in RER during phase 3 ($a$). The values used for leaf area at initiation ($A_0$), and $R_1$ in well-watered conditions were those reported by Dosio *et al*. (2003), which were confirmed to be the same in the genotypes used in this study, in a preliminary experiment (not shown). Leaf area at the beginning of phases 2 and 3 ($A_1$ and $A_2$) are calculated from equations 5 and 6. The duration of each phase was determined as in Dosio *et al*. (2003). The total duration of leaf expansion was calculated as in experiments 1 and 2.

A log-normal three-parameter curve was fitted to describe the time-course of relative cell expansion rate (RCR) (Cookson and Granier, 2006):

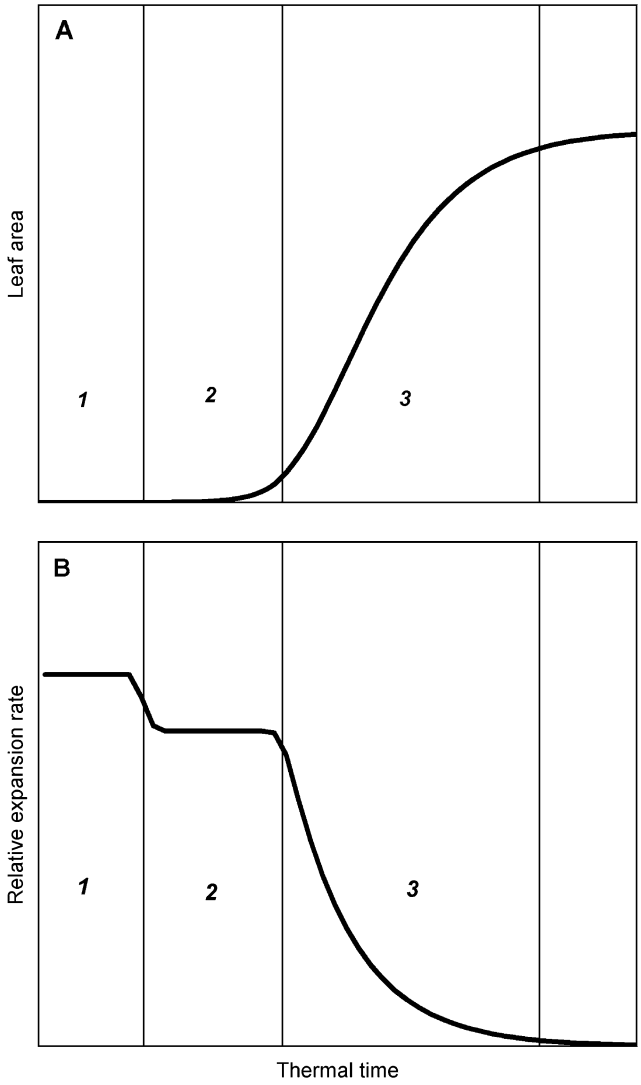


**Fig. 1.** Dynamics of leaf area (A) and relative expansion rate (B) of a leaf, according to the model of Dosio *et al*. (2003). Growth of a leaf is divided into two successive phases of exponential growth at constant relative rate (phases 1 and 2), followed by a phase of growth at declining relative rate but high absolute rate (phase 3).

$$y = a\exp\Big(-0.5\{\ln[(x/x_0)/b]\}\Big)^2 \quad (8)$$

Relative cell division rate (RDR) was estimated by subtracting RCR from RER, as in Cookson and Granier (2006).

All curves were fitted using least-squares (Marquardt–Levenberg algorithm; Sigma-Plot software version 8.0, SPSS Science, Chicago, IL, USA). Analysis of variance was carried out using SAS (SAS Institute Inc., Cary, NC, USA)

### Relative contributions of rate and duration to final leaf area

In experiments 1 and 2, the relative contributions of the changes in expansion rate and duration of growth to the changes in final leaf area under water deficit were quantified for each genotype, in order to select the most contrasting genotypes for further analyses. The impact of a reduced expansion rate was quantified, independently of changes in duration, as the reduction in leaf area observed at the

end of leaf growth in the control treatment ('rate effect'). This measure equals the reduction in final leaf area in the case of no increase in the duration of growth (Fig. 2A). The impact of an increased duration of growth was quantified by the compensatory expansion that takes place between the end of leaf growth in the control treatment and the end of leaf growth in the water-deficit treatment ('duration effect'). In the case that duration is increased, the sum of both indicators equals the reduction in final leaf area (Fig. 2B).

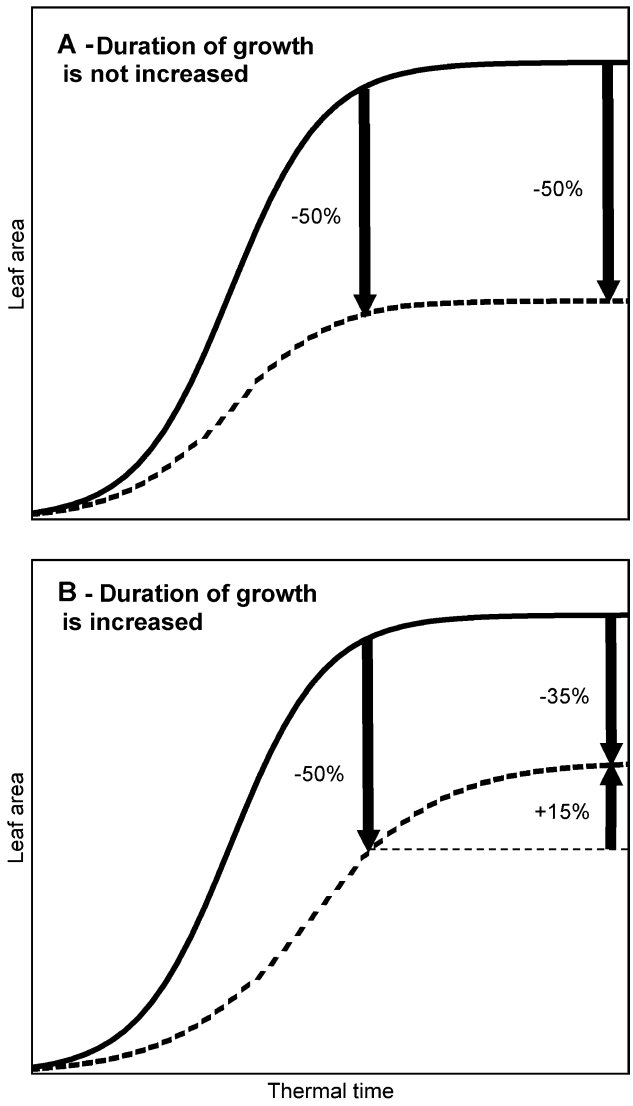


**Fig. 2.** Examples of quantification of the relative contributions of the changes in rate and duration on final leaf area. (A) If duration is not increased under water deficit, the effect of rate (a 50% reduction in area at the moment the leaf reaches 95% of its final area in the control treatment) accounts completely for the reduction in final leaf area. (B) If duration is increased under water deficit, its effect on final leaf area is quantified as the growth that takes place after the leaf reaches 95% of its final area in the control treatment. The 35% reduction in final leaf area results from the sum of the (negative) effect of reduced rate and the (positive) effect of increased duration.

## Results

### *Water deficit reduced leaf area to a different extent depending on the genotype*

In the group of 18 genotypes evaluated in experiment 1, total leaf area after 28 d of the beginning of the treatments ranged between 716 $cm^2$ and 1245 $cm^2$ in the control plants (Fig. 3A), and between 312 $cm^2$ and 569 $cm^2$ in the water-deficit treatment. This represents a reduction between 49% and 62% (Fig. 3B). The response of total leaf area is a complex combination of the responses of different leaves of the plant, which were at different developmental stages at the onset of water deficit. The response of leaf 8 was chosen for comparison between genotypes, since the initiation of this leaf was taken as reference for the imposition of treatments. The final area of leaf 8 ranged between 50 $cm^2$ and 156 $cm^2$ in control conditions (Fig. 3C), and between 30 $cm^2$ and 86 $cm^2$ under water deficit (reduction between 38% and 57%; Fig. 3D).

A two-factor (genotype and treatment) analysis of variance was performed, showing significant genetic variability for the area of leaf 8 ($P < 0.0001$) as well as a significant response to water deficit ($P < 0.0001$). The interaction between both factors, which represents the genetic variability for the response of leaf area to water deficit, was also highly significant ($P < 0.0001$). The response of leaf 8 area to water deficit was not related to the total leaf area of each genotype (compare D and A in Fig. 3).

### *Responses of leaf expansion rate and duration were variable between genotypes*

The growth of leaf 8 under well-watered conditions followed the typical sigmoidal shape, with rates and durations that differed between genotypes (Fig. 4). Water deficit altered the kinetics of leaf growth by reducing expansion rate and, in most cases, increasing the duration of leaf growth. The reduction in expansion rate in the water deficit ranged between 44% and 67%. The duration of expansion was increased between 2% and 26%.

The relative contributions of the change in leaf expansion rate and the change in the duration of growth to the final reduction in the area of leaf 8 are plotted in Fig. 5 for each genotype. As explained in Materials and methods, the contribution of the reduction in expansion rate was quantified as the reduction of leaf area observed at the end of leaf growth in the control treatment ('rate effect', Fig. 2). The effect caused by an increased duration of growth was quantified by the compensatory expansion that takes place between the end of leaf growth in the control treatment and the end of leaf growth in the water-deficit treatment ('duration effect', Fig. 2). The sum of both indicators equals the reduction in final leaf area (i.e. the reduction in final leaf area increases downward and to the left of the graph).

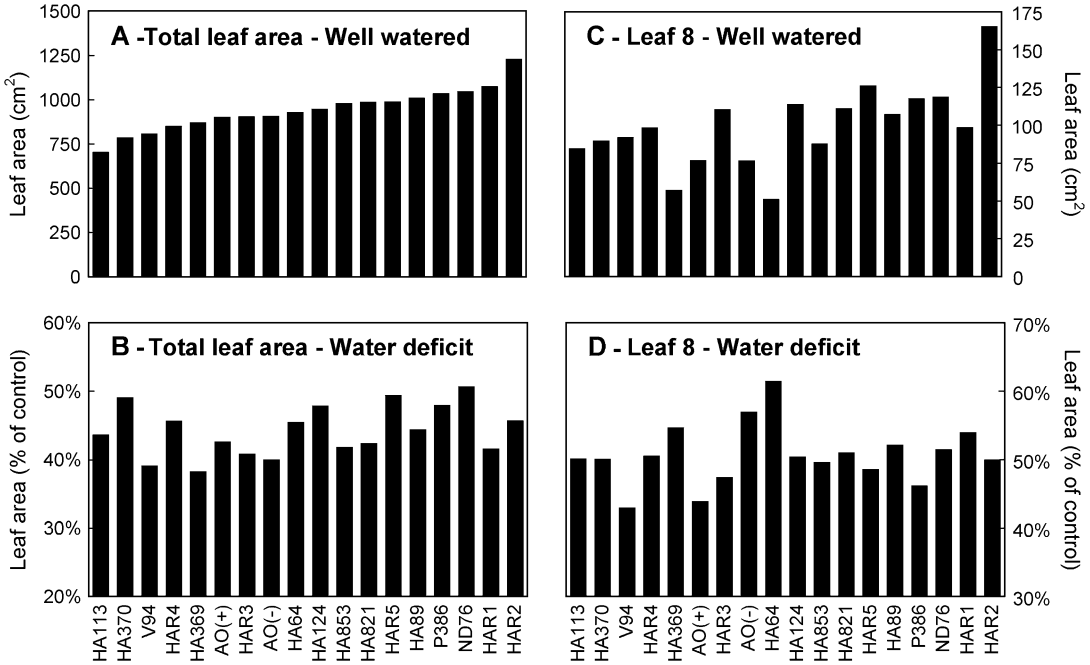


**Fig. 3.** Total plant leaf area for each genotype, in the well-watered treatment (A) and in the water-deficit treatment (B, expressed as a % of control), 28 d after the beginning of treatments. Final leaf area of leaf 8 for each genotype, in the well-watered treatment (C) and in the water-deficit treatment (D, expressed as a % of control). Data are from experiment 1.

Differences between genotypes in the reduction of final leaf area were caused either by differences in the reduction of expansion rate ('HA64' versus 'OA+') or differences in the compensatory growth during the increase in the duration of growth ('HAR2' versus 'OA+'). The genotypes showing high and low osmotic adjustment ('OA–' and 'OA+') differed mainly in the response of their expansion rate to water deficit. Relative responses of the four genotypes analysed in both experiments were conserved.

The genetic variability for both effects was quantitatively similar. Average (experiments 1 and 2) differences between genotypes for the relative contribution of reduced rate were 15.6 percentage points, while differences for the relative contribution of increased duration were 15.3 percentage points.

### *Water deficit affected the rate and duration of different phases of leaf growth*

Two genotypes, 'HAR2' and 'HA64', were selected for further analysis based on their contrasting response to water deficit, both in terms of rate and duration of growth (Fig. 5). In experiments 3 and 4, leaf growth dynamics showed the same three-phase pattern described by Dosio *et al.* (2003), consisting of two phases of exponential growth followed by a phase of quasi-linear growth. Water deficit altered the duration of the phases and the relative expansion rates in each of them, as can be observed for leaf 8 in Fig. 6.

In leaf 8, growth rates were reduced to a greater extent in genotype 'HAR2' than in 'HA64', both during phase 2 (30% versus 12%, average of experiments 3 and 4) and phase 3 (57% versus 38%). The increase in the duration of growth was greater for 'HAR2' than 'HA64', both for phase 2 (40% versus 6%) and for phase 3 (48% versus 8%). The effect of water deficit on phase 1 could not be analysed in leaf 8 since the treatments began approximately at the end of this phase, but in upper leaves it was found that its response was similar in both exponential phases (phases 1 and 2; not shown).

The whole-plant framework of analysis described by Dosio *et al.* (2003) was applied to leaf growth data from experiment 3. Figure 7A and E shows the beginning of the water-deficit treatment relative to the leaf development in each genotype. These figures also show that durations of exponential growth and total growth were increased in genotype 'HAR2' and were similar to the controls in 'HA64'. Leaves of genotype 'HAR2' that were already at phase 3 at the onset of water deficit (up to leaf 4) showed less response than leaves that were at phase 1 or 2 (e.g. leaves 8 and 10). In both genotypes, the rates of expansion during the exponential phases of growth (Fig. 7B, F) were increasingly affected from leaf 6 onwards, while the expansion rates during phase 3 were affected in all the measured leaves. Water deficit reduced the final area of leaves 8 onwards in genotype 'HAR2' (Fig. 7D), while in 'HA64' leaves 4 onwards were affected (Fig. 7H).

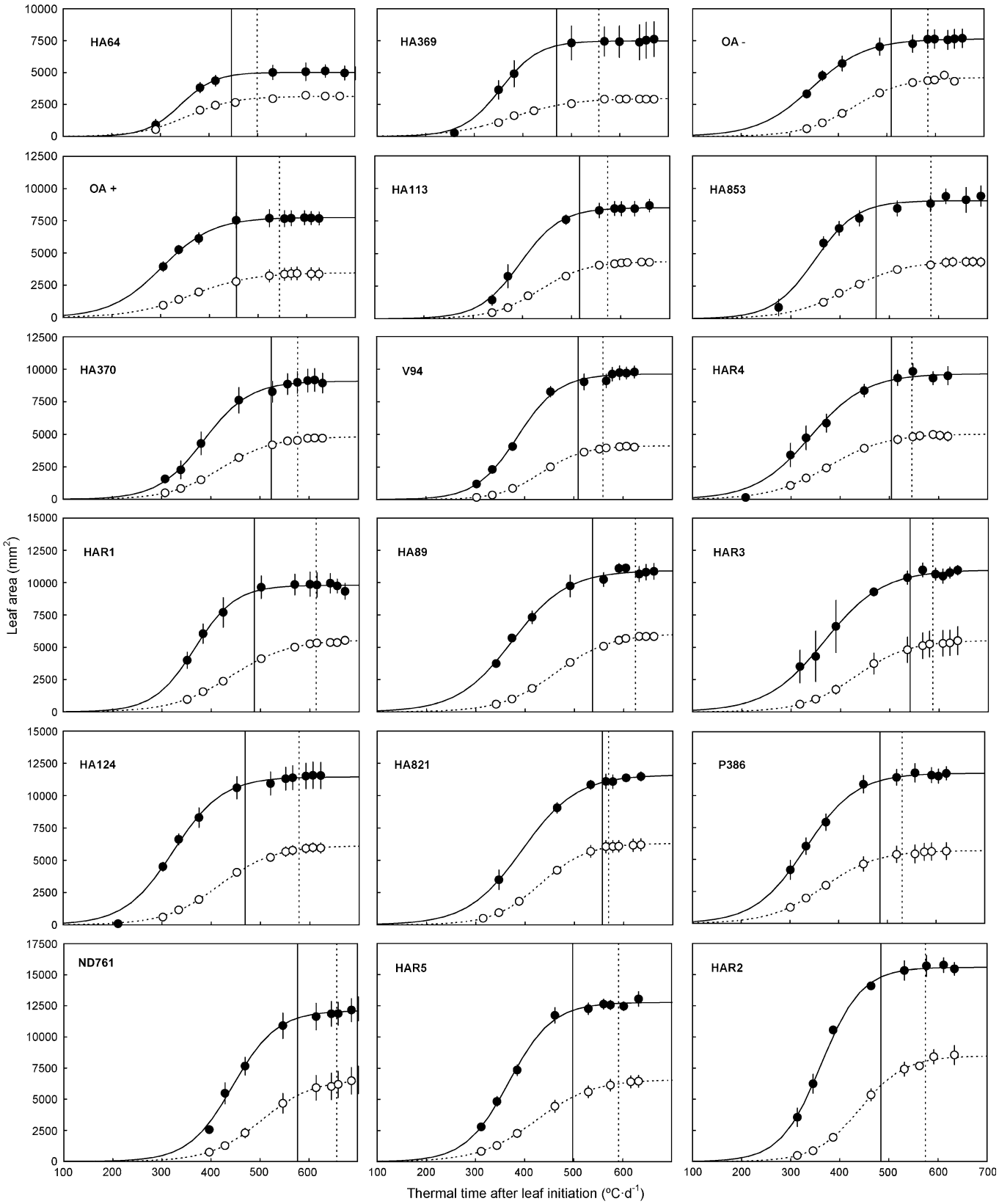


**Fig. 4.** Growth of leaf 8 of each genotype, in well-watered (black circles) and water-deficit treatments (white circles). Fitted lines are sigmoidal growth curves (equation 4) for well-watered (continuous line) and water-deficit (dotted line) treatments. Vertical lines indicate the moment at which 95% of final leaf area is reached, in well-watered (continuous line) and water-deficit (dotted line) treatments. Vertical bars represent ±SE. Data are from experiment 1.

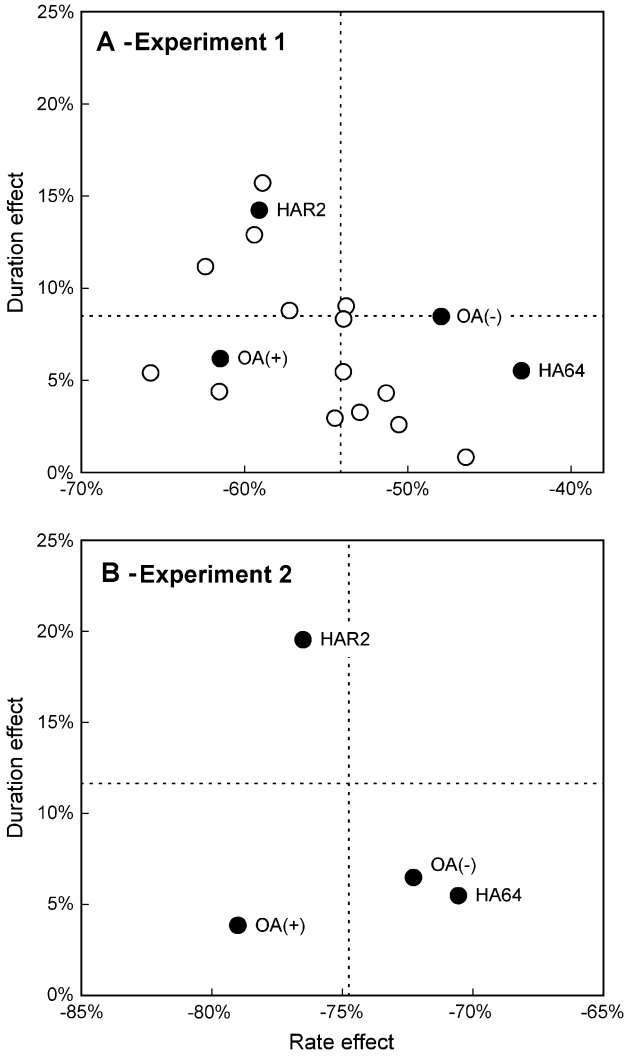


**Fig. 5.** The effect of increased duration on final leaf area, plotted against the effect of reduced rate, for each genotype in experiment 1 (A) and in experiment 2 (B). The four genotypes used in both experiments are shown as black circles. Dotted lines indicate the midpoint of the variation range of each variable, in each experiment.

### *Water deficit affected cell division and expansion*

In experiment 4, mean adaxial epidermal cell area and cell number were measured at different moments during growth of leaf 8. Water deficit reduced the relative rates of leaf expansion (Fig. 8A, D), cell expansion (Fig. 8B, E), and cell division (Fig. 8C, F), in both genotypes. In genotype 'HAR2' the period of cell division was extended by 80 degree-days (Fig. 8C), and the end of cell (and leaf) expansion was delayed by an additional 70 degree-days (Fig. 8B). In this genotype, the increase in the duration of cell division under water deficit is similar to the increase in the duration of exponential leaf growth (Fig. 8A). In genotype 'HA64', the duration of the phases of exponential and linear growth were only slightly modified (Fig. 8B). The duration of cell division and expansion also remained almost unchanged (Fig. 8E, F).

## Discussion

A water-deficit treatment was imposed on 18 sunflower genotypes by maintaining a reduced soil water content for a long period. A method specially designed for sunflower (Pereyra-Irujo *et al*., 2007) was used to apply this treatment independently of the leaf area of the sunflower plant, thus allowing a fair comparison between genotypes. This kind of water-deficit (constant intensity) allowed intrinsic genotypic differences in leaf growth responses to be identified independently of differences in total plant leaf area (Fig. 3) or water depletion rate (not shown).

It has been generally assumed that the effect of water deficit on leaf growth consists solely of a reduction in expansion rate, without any change in the duration of growth, as evidenced by the assumptions included in crop models, both in specific (e.g. for sunflower; Villalobos *et al*., 1996) and general (Connor and Fereres, 1999) crop models. In the case of sunflower, this assumption was later supported by experimental evidence that showed that, in one sunflower hybrid, water deficit did not increase leaf growth duration (Granier and Tardieu, 1999). On the other hand, it was recently reported that several *A. thaliana* genotypes showed increased leaf growth duration under water deficit, with significant genetic variability (Aguirrezábal *et al*., 2006). The present results showed that there are sunflower genotypes that respond to water deficit by considerably increasing leaf growth duration. This variability in the responses of leaf growth to water deficit could be incorporated into existing sunflower simulation models, in order to improve the estimations of the performance of different genotypes under water stress.

### *Changes in rate and duration contributed in a similar amount to the variability in the response of final leaf area*

The analysis of the growth dynamics of individual leaves permitted the dissection of the response of each genotype into its components, leaf growth rate and duration. A novel approach that quantifies the relative contributions of the changes in rate and duration on final leaf area was used to select genotypes of contrasting response to water deficit. The effect of an increased duration on final leaf area was smaller than the effect of a reduced growth rate, since expansion rate is usually low during the final stage of leaf expansion. Nevertheless, the average (experiments 1 and 2) range of variation between genotypes in the effect of increased duration (15.3%) was similar to that of reduced rate (15.6%), rendering both components of the response equally interesting from a breeding point of view.

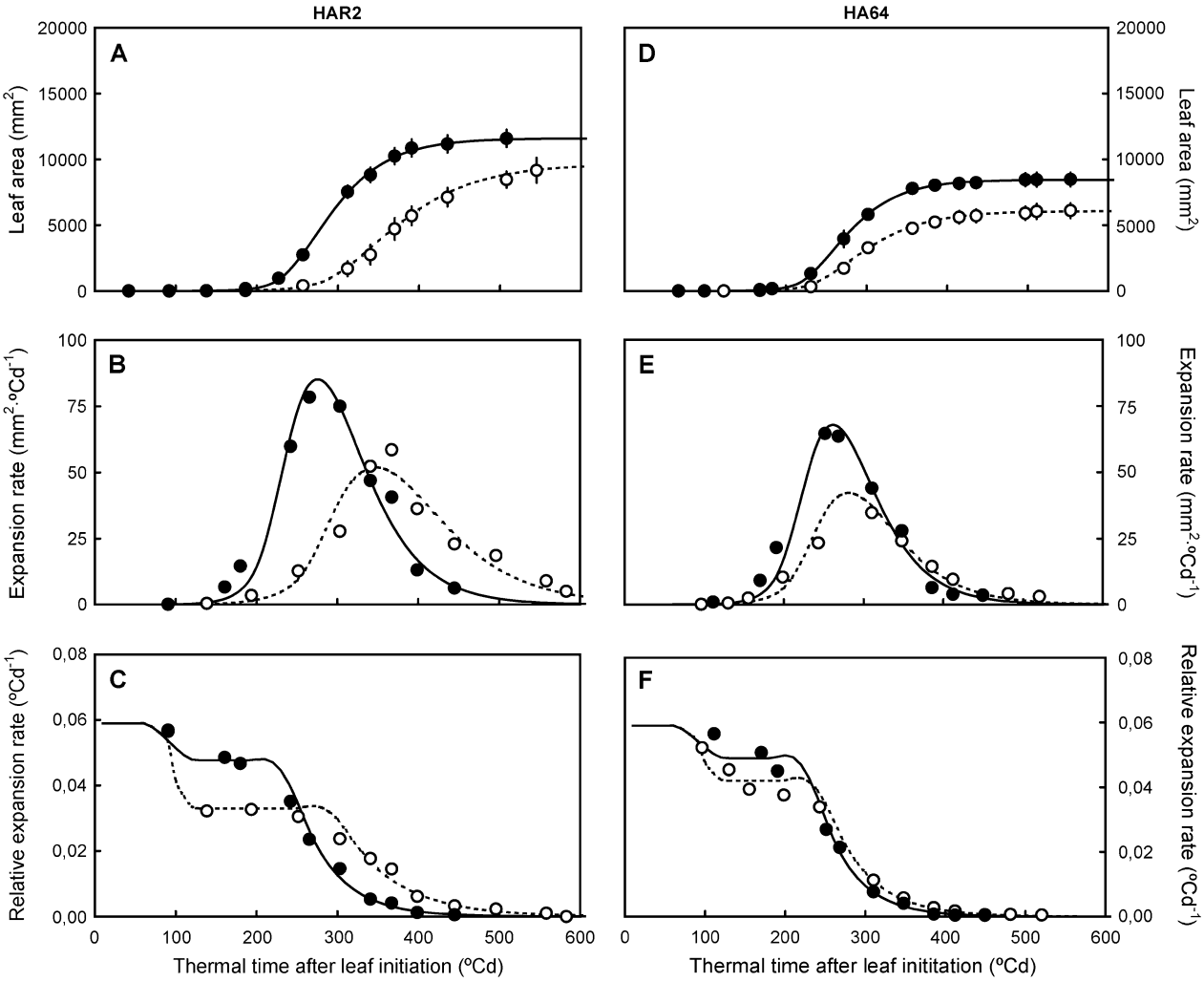


**Fig. 6.** Time-course of leaf area (A, D), leaf expansion rates (B, E), and relative leaf expansion rates (C, F) in well-watered (black circles) and water-deficit treatments (white circles), for genotypes 'HAR2' (left) and 'HA64' (right). Fitted lines in (A) and (D) are leaf growth curves obtained by the model of Dosio *et al*. (2003) using equations 5–7. Fitted lines of leaf expansion rate (B, E), and relative leaf expansion rates (C, F) were calculated using equation 1 and equation 2, respectively. Continuous lines correspond to the well-watered treatment, and dotted lines to the water-deficit treatment. Vertical bars represent ±SE. Data are from experiment 3.

Correlation between the response of rate and duration was weak, as was previously found in *A. thaliana* (Aguirezábal *et al*., 2006). However, of the four possible combinations of high or low rate response and high or low duration response, only three of them were identified (i.e. responses of 'HAR2', 'HA64', and 'OA+'). No genotype was found combining a relatively low reduction in rate and a relatively large increase in duration. In *A. thaliana*, genotype 'An-1' showed this kind of response, reaching a similar leaf area under well-watered or water-deficit conditions (Aguirrezábal *et al*., 2006). Under the hypothesis that rate and duration are governed by different physiological mechanisms, a cross between two genotypes showing a differential response in terms of rate and duration (e.g. 'HAR2' and 'HA64') could theoretically produce an offspring with a response to water deficit ranging from that of 'AO+' (high rate response, low duration response) to a final leaf area response smaller than that of the parents (low rate response, high duration response). This hypothesis is supported by the low correlation between both components of the response. This is currently being tested by means of a genetic analysis of a segregating population derived from the cross between 'HAR2' and 'HA64'.

The rate of tissue expansion is proportional to cell wall extensibility and turgor pressure above a yield threshold (Lockhart, 1965). The genotypes differing in osmotic adjustment capacity differed mainly in the response of their leaf expansion rate (Fig. 4), with the genotype with high osmotic adjustment showing the greatest reduction in expansion rate. This is in agreement with previous findings which showed that sunflower genotypes having a high degree of osmotic adjustment showed relatively lower leaf expansion and tissue elasticity (increased bulk

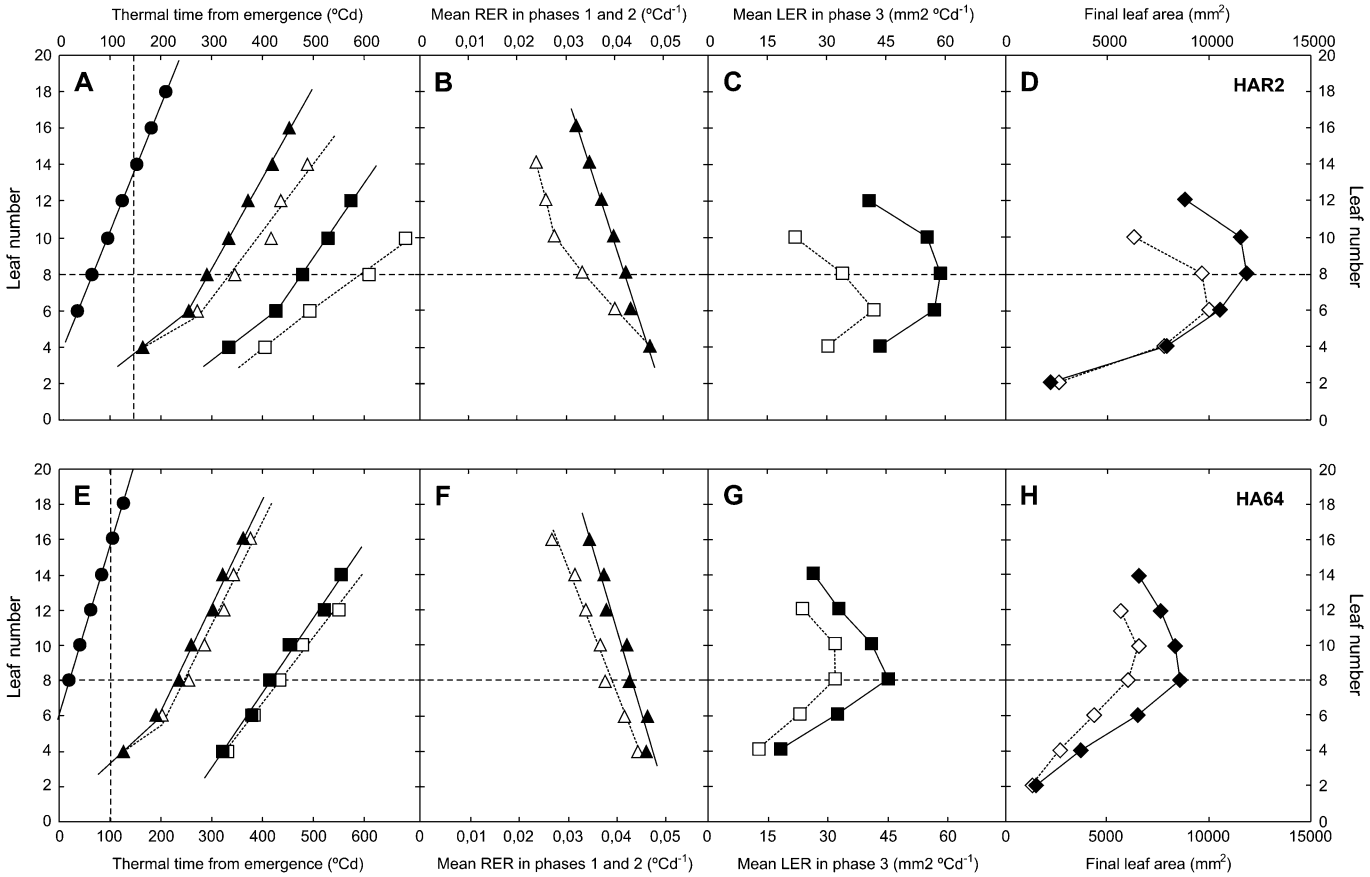


**Fig. 7.** Whole-plant representation of leaf growth responses of genotypes 'HAR2' (top) and 'HA64' (bottom) to water deficit, using the framework proposed by Dosio *et al*. (2003). (A, E) Number of leaves which are initiated (circles), which have ended the exponential growth (triangles), and which have ceased to expand (squares). (B, F) Mean RER of each leaf during phases 1 and 2 (exponential growth). (C, G) Mean LER of each leaf during phase 3 (quasi-linear growth). (D, H) Final leaf area of each leaf. In all cases, black symbols correspond to the well-watered treatment, and white symbols to the water-deficit treatment. The vertical dotted line in (A) and (E) indicates the beginning of the water-deficit treatment. The horizontal dotted lines indicate data from leaf 8. Data are from experiment 3.

modulus of elasticity) under water stress (Chimenti and Hall, 1994). These results suggest that, in this case, differences in growth rate could be mainly determined by cell wall characteristics, with osmotic adjustment being a consequence of reduced growth decreasing the rate of solute dilution, as has been previously suggested (Van Volkenburgh and Boyer, 1985; Munns, 1988).

### *Using a whole-plant framework to describe the response of the leaf area profile to water deficit*

Granier and Tardieu (1999) showed that the effect of water deficit depended largely on the timing of the treatment, relative to the development of the leaf. A similar behaviour was observed in the present experiments, where young leaves generally showed larger reductions in their area under water deficit. In spite of this, the whole-plant framework used allowed the genetic differences in the response of leaf growth to water deficit to be highlighted, and changes in the leaf area profile to be understood.

Irrespective of leaf position, genotype 'HAR2' showed a higher sensitivity to water deficit, both in terms of expansion rate and growth duration. In 'HAR2', the increase in duration of leaves stressed at phase 3 (e.g. leaf 4) was able to compensate the reduction in relative growth rate, allowing them to reach the same area as in the control treatment. In 'HA64', these leaves showed a similar reduction in relative growth rate but no increased duration, leading to a reduced final area. In younger leaves (e.g. leaf 10), the more marked reduction in relative growth rate shown by 'HAR2' prevails over the compensatory effect of increased duration, and the reductions in final leaf area become larger than in 'HA64'. These differences in the response to water deficit result in the plant's leaf area profile being altered in a different fashion on each genotype (Fig. 7D, H). On the other hand, the relatively high expansion rate of 'HA64' under water deficit results in a leaf area profile similar to that of control plants (Fig. 7H).

To allow a fair comparison of genotypes in the experiments where only leaf 8 was analysed (experiments 1 and

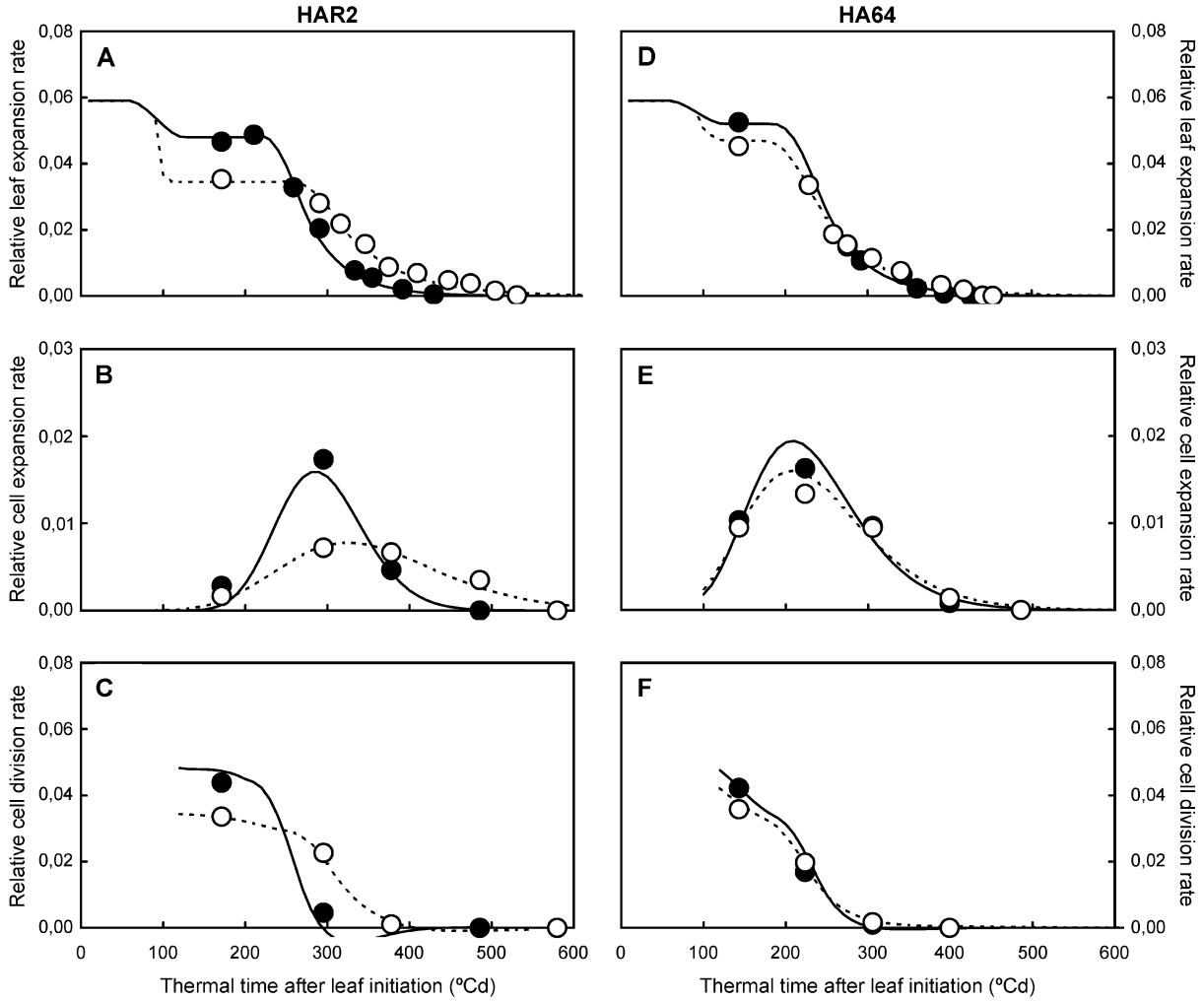


**Fig. 8.** Time-course of relative leaf expansion rates (A, D), relative cell expansion rates (B, E), and relative cell division rates (C, F) in well-watered (black circles) and water-deficit treatments (white circles), for genotypes 'HAR2' (left) and 'HA64' (right). Fitted curves of relative leaf expansion rates (A, D) were calculated using equation 2, from the leaf growth curve fitted using equations 5–7. Fitted curves of relative cell expansion rate (B, E) were fitted using equation 8. The curves of relative cell division rate (C, F) were calculated by subtracting the fitted values of relative cell expansion rate from those of relative leaf expansion rate. Continuous lines correspond to the well-watered treatment, and dotted lines to the water-deficit treatment. Data are from experiment 4.

2), care was taken to start the water-deficit treatment at a fixed developmental stage of this leaf, and not at a fixed date after emergence. The whole-plant analysis of the two contrasting genotypes confirmed that their differential response was not due to differences in timing of the treatment relative to leaf age, since each genotype's specific responses could be observed in different leaves of the plant.

### *The increase in the duration of exponential growth was correlated to an extended period of epidermal cell division*

In the present work, it was found that, in one of the two genotypes analysed in detail ('HAR2'), the increase in the duration of the phase of exponential growth coincided with an increase in the duration of epidermal cell division, while in the other genotype ('HA64') neither of these durations were appreciably affected. An increase in the duration of cell division in response to water stress was also observed in wheat leaves (Schuppler *et al*., 1998). In sunflower, however, it had been reported that neither water nor light stresses caused any increases in the period of cell division in leaves (Granier *et al*., 2000). These results, obtained with a different genotype (cv. 'Albena'), support the existence of genetic variability for this trait.

Increased leaf growth duration under water deficit is usually suggested to be caused by changes in the activity of cell-wall enzymes (Aguirrezábal *et al*., 2006), such as peroxidases (which increases drastically at the end of cell expansion; MacAdam and Grabber, 2002). The present results, however, suggest that the duration of cell division in the epidermis plays a key role in this response. During

the early stages of leaf development, proliferative growth is highly energy-requiring (Fleming, 2006). Maintaining cells under a proliferative growth state in the epidermis could allow the leaf to maintain the high relative expansion rates which occur during the first two phases of leaf growth, by acting as a strong sink for carbon and nutrients (as suggested previously by Van Volkenburgh, 1999). This would be in agreement with the fact that the epidermis has been shown to be the leaf tissue which drives and restricts leaf growth (Savaldi-Goldstein *et al*., 2007). Subsequently, a delayed entry of cells into a non-proliferative, vacuole-associated growth state could lead to delayed leaf growth cessation at leaf level. A similar increase in the duration of epidermal cell division, accompanied by an increase in total leaf growth duration has been reported in *A. thaliana* plants subjected to light stress (Cookson and Granier, 2006). Based on these results, the genetic variability for the response of the duration of cell division can be considered a likely cause of the apparently contradictory responses of leaf growth duration to water deficit in sunflower (Takami *et al*., 1981; Granier and Tardieu, 1999).

In the genotypes analysed in detail, the largest increase in duration of cell division coincided with the largest decrease in cell division rate. Genetic evidence in *A. thaliana*, however, suggests that rate and duration of cell division are not necessarily linked. Inactivation or overexpression of different genes in this species were able independently to alter cell division rate (cyclin-dependent-kinase inhibitors KRP1 and KRP2: Wang *et al*., 2000; de Veylder *et al*., 2001) or the duration of the period of cell division (*AINTEGUMENTA*: Mizukami and Fischer, 2000; *STRUWWELPETER*: Autran *et al*., 2002). The genotypes of contrasting response described in the present paper could be useful for further studying the relationship between duration and rate of epidermal cell division under changing environmental conditions.

## Conclusions

Significant variation among lines was found for the response of leaf expansion rate and for leaf growth duration. The experimental and statistical methods applied allowed the identification of intrinsic genotypic differences in leaf growth responses at cell-, leaf-, and plant-level, that were not due to differences in timing or intensity of the treatment, or differences in plant leaf or water depletion rate.

One of the objectives of this work was to identify the mechanisms underlying genetic differences in the response of leaf growth to water deficit, as has been done previously for different environmental factors (Harrison *et al*., 1998; Masle, 2000; Stiles and Van Volkenburgh, 2002). The present results suggest that genetic differences in leaf growth rate under water deficit could be determined by cell wall properties, while increased duration of leaf growth is partly due to a prolonged phase of epidermal cell division. This implies that rate and duration responses could be the result of different physiological mechanisms, and are therefore capable of being combined to increase the variability in leaf area response to water deficit in sunflower.

## Acknowledgements

We are grateful to Luis Méndez, for his technical assistance, and to Carlos Sala, José Bruniard, Claudio Chimenti, Advanta Semillas, and the North Central Regional Plant Introduction Station (USDA) for providing the seeds of the inbred lines used in this study. This research was supported by Asociación Argentina de Girasol (ASAGIR) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), grant PICT-O 13168, and by Instituto Nacional de Tecnología Agropecuaria (INTA), grant PNCER1336. Luis Aguirrezábal is a member of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Gustavo Pereyra-Irujo and Leandra Lechner hold scholarships form CONICET. This work is part of a thesis submitted by Gustavo Pereyra-Irujo in partial fulfilment for the requirements for a Doctor's degree.

## References

**Aguirrezábal L, Bouchier-Combaud S, Radziejwoski A, Dauzat M, Cookson SJ, Granier G.** 2006. Plasticity to soil water deficit in *Arabidopsis thaliana*: dissection of leaf development into underlying growth dynamic and cellular variables reveals invisible phenotypes. *Plant, Cell and Environment* **29,** 2216–2227.

**Alves AAC, Setter TL.** 2004. Response of cassava leaf area expansion to water deficit: cell proliferation, cell expansion and delayed development. *Annals of Botany* **94,** 605–613.

**Autran D, Jonak C, Belcram K, Beemster GTS, Kronenberger J, Grandjean O, Inzé D, Traas J.** 2002. Cell numbers and leaf development in arabidopsis: a functional analysis of the struwwelpeter gene. *EMBO Journal* **21,** 6036–6049.

**Bouchabké O, Tardieu F, Simonneau T.** 2006. Leaf growth and turgor in growing cells of maize (*Zea mays* L.) respond to evaporative demand under moderate irrigation but not in water-saturated soil. *Plant, Cell and Environment* **29,** 1138–1148.

**Chimenti CA, Hall AJ.** 1994. Responses to water stress of apoplastic water fraction and bulk modulus of elasticity in sunflower (*Helianthus annuus* L.) genotypes of contrasting capacity for osmotic adjustment. *Plant and Soil* **166,** 101–107.

**Chimenti CA, Pearson J, Hall AJ.** 2002. Osmotic adjustment and yield maintenance under drought in sunflower. *Field Crops Research* **75,** 235–246.

**Connor DJ, Fereres E.** 1999. A dynamic model of crop growth and partitioning of biomass. *Field Crops Research* **63,** 139–157.

**Cookson SJ, Granier C.** 2006. A dynamic analysis of the shade-induced plasticity in *Arabidopsis thaliana* rosette leaf development reveals new components of the shade-adaptive response. *Annals of Botany* **97,** 443–452.

**de Veylder L, Beeckman T, Beemster GTS, Krols L, Terras F, Landrieu I, Van Der Schueren E, Maes S, Naudts M, Inzé D.** 2001. Functional analysis of cyclin-dependent kinase inhibitors of arabidopsis. *The Plant Cell* **13,** 1653–1667.

**Dosio GAA, Rey H, Lecoeur J, Izquierdo NG, Aguirrezábal LAN, Tardieu F, Turc O.** 2003. A whole-plant analysis of the dynamics of expansion of individual leaves of two sunflower hybrids. *Journal of Experimental Botany* **54,** 2541–2552.

**Fleming AJ.** 2006. The integration of cell poliferation and growth in leaf morphogenesis. *Journal of Plant Research* **119,** 31–36.

**Granier C, Aguirrezabal L, Chenu K, *et al.*** 2005. Phenopsis, an automated platform for reproducible phenotyping of plant responses to soil water deficit in *Arabidopsis thaliana* permitted the identification of an accession with low sensitivity to soil water deficit. *New Phytologist* **169,** 623–635.

**Granier C, Tardieu F.** 1998. Is thermal time adequate for expressing the effects of temperature on sunflower leaf development? *Plant, Cell and Environment* **21,** 695–703.

**Granier C, Tardieu F.** 1999. Water deficit and spatial pattern of leaf development: variability in responses can be simulated using a simple model of leaf development. *Plant Physiology* **119,** 609–619.

**Granier C, Turc O, Tardieu F.** 2000. Co-ordination of cell division and tissue expansion in sunflower, tobacco, and pea leaves: dependence or independence of both processes? *Journal of Plant Growth Regulation* **19,** 45–54.

**Harrison J, Nicot C, Ougham H.** 1998. The effect of low temperature on patterns of cell division in developing second leaves of wild-type and *slender* mutant barley (*Hordeum vulgare* L.). *Plant, Cell and Environment* **21,** 79–86.

**Hsiao TC, Frensch J, Rojas-Lara BA.** 1998. The pressure-jump technique shows maize leaf growth to be enhanced by increases in turgor only when water status is not too high. *Plant, Cell and Environment* **21,** 33–42.

**Korell M, Mosges G, Friedt W.** 1992. Construction of a sunflower pedigree map. *Helia* **15,** 7–16.

**Lockhart JA.** 1965. An analysis of irreversible plant cell elongation. *Journal of Theoretical Biology* **8,** 264–275.

**MacAdam JW, Grabber JH.** 2002. Relationship of growth cessation with the formation of diferulate cross-links and *p*-coumaroylated lignins in tall fescue leaf blades. *Planta* **215,** 785–793.

**Masle J.** 2000. The effects of elevated $CO_2$ concentrations on cell division rates, growth patterns, and blade anatomy in young wheat plants are modulated by factors related to leaf position, vernalization, and genotype. *Plant Physiology* **122,** 1399–1415.

**Matthews MA, Van Volkenburgh E, Boyer JS.** 1984. Acclimation of leaf growth to low water potentials in sunflower. *Plant, Cell and Environment* **7,** 199–206.

**Mizukami Y, Fischer RL.** 2000. Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. *Proceedings of the National Academy of Sciences, USA* **97,** 942–947.

**Munns R.** 1998. Why measure osmotic adjustment? *Australian Journal of Plant Physiology* **15,** 717–726.

**Neumann PM.** 1995. The role of cell wall adjustments in plant resistance to water deficits. *Crop Science* **35,** 1258–1266.

**O'Toole J, Cruz R.** 1980. Response of leaf water potential, stomatal resistance, and leaf rolling to water stress. *Plant Physiology* **65,** 428–432.

**Pereyra-Irujo G, Velázquez L, Granier C, Aguirrezábal LAN.** 2007. A method for drought tolerance screening in sunflower. *Plant Breeding* **126,** 445–448.

**Reymond M, Muller B, Leonardi A, Charcosset A, Tardieu F.** 2003. Combining quantitative trait loci analysis and an ecophysiological model to analyze the genetic variability of the responses of maize leaf growth to temperature and water deficit. *Plant Physiology* **131,** 664–675.

**Savaldi-Goldstein S, Peto C, Chory J.** 2007. The epidermis both drives and restricts plant shoot growth. *Nature* **446,** 199–202.

**Schuppler U, He P, John PCL, Munns R.** 1998. Effect of water stress on cell division and cell-division-cycle 2-like cell-cycle kinase activity in wheat leaves. *Plant Physiology* **117,** 667–678.

**Stiles KA, Van Volkenburgh E.** 2002. Light-regulated leaf expansion in two *Populus* species: dependence on developmentally controlled ion transport. *Journal of Experimental Botany* **53,** 1651–1657.

**Takami S, Turner N, Rawson H.** 1981. Leaf expansion of four sunflower (*Helianthus annuus* L.) cultivars in relation to water deficits. I. Patterns during plant development. *Plant, Cell and Environment* **4,** 399–407.

**Tardieu F, Davies W.** 1993. Integration of hydraulic and chemical signalling in the control of stomatal conductance and water status of droughted plants. *Plant, Cell and Environment* **16,** 341–349.

**Van Volkenburgh E.** 1999. Leaf expansion – an integrating plant behaviour. *Plant, Cell and Environment* **22,** 1463–1473.

**Van Volkenburgh E, Boyer JS.** 1985. Inhibitory effects of water deficit in maize leaf elongation. *Plant Physiology* **77,** 190–194.

**Villalobos FJ, Hall AJ, Ritchie JT, Orgaz F.** 1996. OILCROP-SUN: a development, growth and yield model for the sunflower crop. *Agronomy Journal* **88,** 403–415.

**Wang H, Zhou Y, Gilmer S, Whitwill S, Fowke LC.** 2000. Expression of the plant cyclin-dependent kinase inhibitor ICK1 affects cell division, plant growth and morphology. *The Plant Journal* **24,** 613–623.